THE BIOLOGY OF CRAYFISH PLAGUE (APHANOMYCES ASTACI: SCHIKORA) IN GREAT BRITAIN.

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ABSTRACT

Aspects of the biology of the crayfish plague fungus (Aphanomyces astaci : Schikora) were studied and a chronology of the spread of crayfish plague in the rivers of Europe was constucted. A study of the spread of crayfish plague in the River Lea and its tributaries indicated a decline in crayfish populations throughout the system with residual populations of Austropotamobius pallipes remaining in the Mimram and in the Stort. Populations may also exist in the Ash and at two sites in the Lea. The occurrence of five Pacifastacus leniusculus introductions in the system may explain the occurrence of the disease in these waterways.

Six tested invertebrate species (including Gammarus pulex) did not appear to be suitable alternative hosts for the fungus and fish did not appear to act as transport hosts. Growth and sporulation of the fungus occurred on plant substrates but could not be demonstrated under non-sterile conditions. Putative sexual stages were noted both on sterile hemp seeds and during a laboratory infection of Astacus leptodactylus. polyplanetism was demonstrated in culture and a maximum of five generations of zoospores occurred at 15°C.

Histopathological examinations of infected animals showed the spread of the fungus within the host to be limited. Locally, cuticle, epidermis and connective tissue showed most involvement. Although nervous tissue was infected, gross involvement of the nerve chord was not seen and nervous involvement was not believed to be the major cause of death. The infection of certain tissues may explain exhibited behaviour patterns. Attempts to demonstrate an effect of infection on urine production were unsuccessful.

Infection of the proximal leg joints could be enhanced by wiping with a mixture of chloroform and methanol in Ast. leptodactylus but not in P. leniusculus. Solvent extracts of both crayfish species and of G. pulex prevented germination of Aph. astaci zoospores and affected the growth of sporelings. Calcium chloride extracts of crayfish enhanced germination and increased sporeling growth. The Ast. leptodactylus extract appeared to kill motile zoospores, whilst its effect on encysted spores could be reversed by replacing the medium. None of the effects demonstrated appeared to account for the differential susceptibility of the two species to Aph. astaci.

I hearby declare that this thesis is my own work, except where the contrary is specifically indicated. No other registration for an award of either the CNAA or any university occurred during the period of this research programme.

M.W. Rodgers

April 1988

Advanced studies undertaken in connection with this programme of work included the attendance of seminars and conferences both at the City of London Polytechnic and at other institutions.

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Matthew William Rodgers BSc. April 1988

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THE BIOLOGY OF "CRAYFISH PLAGUE" (APHANOMYCES ASTACI : SCHIKORA) IN GREAT BRITAIN.

INTRODUCTION.

"Crayfish plague" is a colloquial term used to describe a disease of crayfish caused by the fungus Aphanomyces astaci: Schikora (Oomycetes, Saprolegniaceae). The disease is fatal to species other than those from North America (Unestam 1972 and 1975) and death is rapid, occurring within a few days of infection in acute cases.

Whilst the fungus does not have any complex nutrient requirement (Unestam 1965), it appears to be more or less specific to freshwater crayfish (Unestam 1972). Whilst laboratory experiments have shown that the fungus is capable of parasitizing living flies (Schikora 1903), the disease is confined generally to the Decapod crustaceans (Unestam 1972). Within the Decapoda, the disease is largely confined to the freshwater crayfish, North American species being markedly more resistant to the disease than those of European, Asian or Australasian origin (Unestam 1969b, 1972, and 1975). However, Benisch (1940) demonstrated that the disease could infect a Chinese crab.

In infected populations, crayfish plague causes 100% mortality amongst both adult and juvenile animals. It is thus an important disease, both ecologically and

commercially.

Since the animals are both scavengers and grazers, their disappearance, particularly from slow flowing or still waters, may lead to an overgrowth of weed and eventually result in the water body becoming silted up (Abrahamsson 1966, Unestam 1974). It has also been suggested (Lowery pers. com.) that the removal of a crayfish population may lead to a dramatic increase in the population of snails, due to a decrease in predation.

In areas where crayfish are farmed or harvested commercially, the disease has had an enormous impact on the industry, especially in the early part of this century. For example, in Sweden, in the years prior to the arrival of crayfish plague in 1907, the estimated annual catch, was in the order of 10 million animals, and it is estimated that 4 million were exported from the Hjalmaren alone each year, mainly to Germany. In the years immediately after the plague first appeared, crayfish catches were reduced to only a few thousand animals annually (Alm 1929). By 1972, it was estimated that 50-70 % of Sweden's crayfish bearing waterways had been devastated by the disease, with losses estimated at 10 million Swedish Krone annually (Hastein and Unestam 1972).

In Finland, before the disease affected the major crayfish waters, the estimated annual export of crayfish reached 15.5 million animals. Exports now total 0.7-0.8 million animals and the disease is estimated to have caused losses of around $2.3 million by 1971 (Westman

1973).

As recently as 1986 (Alderman pers. com.), Turkish crayfish have been affected by mortalities, the cause of which was then unknown. These mortalities, together with the recent ban on exports of crayfish to France, may well adversely affect the Turkish crayfish industry.

Aph. astaci has coenocytic hyphae approximately 7.5 - 9.5 um. in diameter. They are abundantly branched and ramify within the soft cuticle and underlying tissues of the host. Reproduction is thought to be mainly asexual, oospores rarely being seen (Rennerfelt 1935, Unestam 1969b, Cerenius and Soderhall 1984a). At or near the time of death of the host, hyphae project through the cuticle of the host and their contents become differentiated into a single row of up to 40 primary zoospores, which swim to the mouth of the sporangium, where they encyst, forming a characteristic tight cluster. Secondary zoospores then emerge through pores in the primary zoospore cyst wall. These secondary zoospores are laterally biflagellate and free swimming. Upon reaching a crayfish (possibly aided by a non-specific chemotactic attraction, and/or trapping of the zoospores in folds of the cuticle (Unestam 1969, Svensson 1979, Cerenius and Soderhall 1984b)), the two flagellae are cast off and the spore becomes attached to the crayfish cuticle. This is believed to be facilitated by interactions involving lipids or lipoproteins (Svennson 1978).

Upon germination, an infection peg, or germ tube,

penetrates the cuticle of the new host, passing through the lipid layer of the epicuticle by both enzymolysis and physical force (Nyhlen and Unestam 1975), after which the hyphae ramify within the soft cuticle of the animal, which is degraded by secreted chitinase and protease (Soderhall and Unestam 1975, Unestam 1978). After growing within the cuticle the fungal hyphae may pass into, and ramify within, the tissues below.

Infected animals may exhibit altered behaviour patterns and a variety of physical symptoms, although, particularly in acute cases of the disease, the physical symptoms are not always visible to the naked eye and animals may succumb to the disease without showing any gross signs of infection.

Infected animals are often seen in the open during daylight and may exhibit uncoordinated limb movements (Reuf 1879, Alderman et al 1984) or an unsteady gait, walk on the tips of straight legs (described as "walking on stilts"), or may roll onto their backs and spasmodically twitch their limbs. Their escape responses are weak (Alderman et al 1984) and they may carry their tails curled under their abdomen (Tsukerzis 1986). When picked out of the water the animals' limbs often hang limp (Schapperclaus 1935).

The animals may also exhibit orange or brown spots or patches on the soft cuticle, particularly the arthrodial membranes of the legs, the dorsal and ventral abdomen and the anal region. The eyes are often also

infected (Schapperclaus 1928, 1935, Unestam 1973, Alderman et al 1984), and hyphae may be seen protruding from them or ramifying over the outer surface. In addition, more or less melanized fungal hyphae are often seen within the infected cuticle.

In later stages of the disease, internal tissues such as epidermis, connective tissue, muscle and nerve may become involved, and a whitening or necrosis of subcuticular tissues may be seen. The infection may become severe enough locally, to cause loss of appendages. Secondary bacterial and fungal infections may take place, particularly in more chronic cases.

The crayfish is capable of mounting a defence reaction against the invading fungus. This reaction is mediated by the haemocytes via a complex enzyme cascade, known as the phenol oxidase activating system (PAS) (Soderhall et al 1979, Hall 1983, Soderhall and Smith 1986, Soderhall et al 1986).

The haemolymph of the animal contains numbers of circulating haemocytes, some of which (the granular and semi-granular cells), contain prophenol oxidase (ProPO), the inactive form of the enzyme phenol oxidase (PO). Haemocytes accumulate at the site of invasion and encapsulate the invading organism. β-1,3 glucans, from the fungal cell wall, stimulate the release of ProPO from the semi-granular cells (Soderhall et al 1986) and also cause the PAS to be activated. The final step of the PAS is the conversion of ProPO to PO, by a serine protease.

Degranulation of the semi-granular cells releases a factor that stimulates degranulation of the granular cells, thus amplifying the production of PO (Soderhall et al 1986). PO is a sticky protein and attaches to the surface of the fungal cell, where it catalyses the conversion of phenols to quinones and thence to melanin. Melanin forms a capsule around the fungus, isolating it and preventing it's spread. Intermediates in the pathway of melanin synthesis such as 5, 6 dihydroxyindole and 1, 4 Napthoquinone, have been demonstrated to be fungistatic (Soderhall and Ajaxon 1982) and may contribute to the defence mechanism. PO activity has also been detected in the outer endocuticle (Unestam and Nyhlen 1974, Unestam 1975) and melanization of hyphae also occurs here.

Although the defence systems of both resistant and susceptible species have been studied with a view to explaining the difference in susceptibility between them, the mechanism of resistance to the disease in North American species is still poorly understood. Unestam and Wiess (1970) demonstrated that melanization of injected spores and hyphae of Aph. astaci within the haemocoel was stronger in a resistant species Pacifasticus leniusculus than in a susceptible species Astacus astacus. Melanization of hyphae in serum from the two species of crayfish in vitro gave similar results (Unestam and Nylund 1972).

In studies on the susceptibility of Australasian crayfish to the disease, Unestam (1975) showed that,

although it was possible to infect all species tested, the degree of susceptibility varied from species to species. This susceptibility was, to some extent, correlated to the degree of melanization of Aph. astaci hyphae within the cuticle. Moreover, Unestam and Ajaxon (1976) demonstrated that the activity of PO in the cuticle of P. leniusculus was greater than that in Ast. astacus. It is clear therefore, that the resistance demonstrated by some species of crayfish, to infection by Aph. astaci, can be explained to some extent by stronger melanization reactions both in the cuticle and in the haemolymph. However, Unestam and Weiss (1970) demonstrated that simply pricking the epicuticle of P. leniusculus rendered it susceptible to the disease, whilst Nyhlen and Unestam (1975) noted that the number of penetrations of the epicuticle of P. leniusculus was far less than in Ast. astacus, when exposed to a suspension of Aph. astaci zoospores. This suggests that the epicuticle plays a large role in resistance to the disease in P. leniusculus.

Whilst infection of resistant species rarely leads to the animals' death (Unestam and Weiss 1970), the disease has been demonstrated to be present, in a chronic form, in P. leniusculus from lake Tahoe, North America (Unestam 1972). This has led to the suggestion that North American species are the natural hosts of the organism, to which they have a high degree of resistance. Further, it is suggested that the appearance of the disease in Europe in the 1860's was due to the introduction of American

crayfish species, carrying the parasite, to waters where the endemic species had no innate resistance (Unestam 1972 & 1974). The discovery of the disease in a Finnish population of P. leniusculus, originating from from Lake Tahoe (Nylund and Westman 1981), and the demonstration of chronic infections in laboratory infected Orconectes limosus (Vey et al 1982), have strengthened this argument. Indeed, the appearance of the disease in England in the first part of this decade, occurred only 5 years after the first commercial introductions of P. leniusculus into British waterways (Richards 1981, Alderman et al 1984).

The earliest report of a mass crayfish mortality, which could have been attributable to crayfish plague, was made in 1860 by Professor Emilio Cornalia. He described a disease affecting large areas around Lombardy and Brescia, in northern Italy, in the summer of 1859. In the next 10 to 20 years mortalities spread across Europe to France and Germany and infected animals were reported in Finland and Russia before 1900 (Schikora 1926). The disease was then detected in Sweden in 1907 (Alm 1929), Spain in 1956 (Ceuller and Coll 1983), Norway in 1971 (Hastein and Gladhaug 1973) and was first identified in the British Isles in 1981 (Alderman et al 1984). In recent months crayfish mortalities have been reported from Turkey and Greece (Alderman Pers. com.)

Outbreaks of the disease are characterized by a rapid and widespread mortality usually occurring in the spring, summer or autumn. The disease spreads rapidly to

further populations both up and downstream, either by natural movement of crayfish or by water currents. In several instances the disease is thought to have been spread by man. For example, the disease is believed to have been introduced to Sweden in 1907 by the dumping of infected animals, from Finland, into the Malaren at Stockholm, causing an outbreak of the disease that destroyed the crayfish stocks of the most productive area of the country (Alm 1929). Aph. astaci can also be spread by the movement of infected fishing nets, which have been shown to harbour the parasite when wet, and by the movement of fish that have not previously been treated with fungicides such as malachite green (Alderman pers com).

Attempts to prevent the spread of the disease from country to country have taken the form of bans on the import of live crayfish, such as those introduced in Sweden and more recently, in France. However, in cases where waterways cross the border between countries, it is almost impossible to prevent the spread of the disease between them.

The Vrangselven waterway is such a water-course, which crosses the border between Sweden and Norway. In Autumn 1971 crayfish plague was diagnosed in Sweden, close to the border at Eda, and within 4 weeks it had crossed the border into Norway and later spread further along the waterway despite attempts to prevent it (Lund 1975).

Attempts to prevent the spread of Aph. astaci

within river systems have, however, met with some success. The techniques employed have centred on the prevention of the spread of the disease upstream by crayfish migration, since prevention of its spread downstream by zoospores would be impractical. Methods have included the addition of slaked lime to water bodies, in order to kill the crayfish present, thus creating a zone free of crayfish, as a barrier to the spread of the disease. This technique is, however, unreliable and can only be used in stationary water bodies (Svensson et al 1976, Unestam 1977).

Another method employed is the use of electrical barriers, set up across a river to prevent the movement of infected crayfish upstream. Such a method was employed in Norway, in an attempt to prevent the spread of the disease from Sweden along the Vrengselven waterway (Unestam and Hastein 1971, Lund 1975). However, this proved unsuccessful and the disease was soon found further upstream (Lund 1975). It proved more successful when used in conjunction with the creation of crayfish free zones, by the controlled use of Aph. astaci and by liming, down stream of the barriers (Sodderhall et al 1977).

Despite the limited success of these methods, devastation of crayfish stocks in infected rivers is usually complete. However, not all tributaries in a catchment area may be infected.

Attempted reintroductions of crayfish to waters previously denuded by the plague, have met with varying success. Early attempts at restocking simply involved

introducing new stocks of crayfish to denuded waterways after a few years (Franke 1894). These often met with failure, probably because residual numbers of infected crayfish still remained in the waterways, or because the disease was simply reintroduced from surrounding infected waters. Later restocking techniques have involved the eradication of remaining crayfish stocks by liming the water or use of Aph. astaci under controlled conditions.

These techniques, coupled with more stringent controls of the movement of crayfish, have lead to more successful reintroductions in Sweden (Soderhall et al 1977). In some areas, populations of native crayfish wiped out by the disease have been replaced with resistant and faster growing American species such as P. leniusculus, but the ecological effect of such introductions remains unknown. Certainly, introductions of Procambarus clarkii to areas of the Donna park in the Guadalquivir river estuary in southern Spain have had disasterous effects. Numbers of these animals have reached epidemic proportions since their introduction in the 1970's, and their burrowing habit has lead to great damage being done to rice fields (Mackenzie 1986). The possibility also exists that such introduced animals are carriers of crayfish plague, and as such pose a threat to remaining populations of native species (Unestam 1972 and 1974).

For four decades following the first reports of widespread crayfish mortalities, controversy raged as to the nature of the causative organism. The agents were

initially thought to be "Vaginicolae", common crayfish ectoparasites (Ninni 1865). It was later suspected that these were in fact Vorticella (Hofer 1900). Other parasites, such as small "worms" (Reuff 1879), the internally parasitic trematodes Distomum isostomum or D. cirrigerum and later on, bacteria, were also put forward as the agent of disease (Franke 1894).

Despite the fact that fungi of the genus Aphanomyces had been identified in diseased animals, both living and dead, and proposed as the agent of crayfish plague comparativly early on (Harz 1881 and Leuckhart 1884), it was firmly believed at the end of the 19th century that bacteria were the causal agent of the disease (Weber 1899, Hofer 1900, Zacharius 1904). It was not until 1903, when Schikora published a short report in which he suggested that a species of Aphanomyces, which he named Aph. astaci (and later Aph magnusii, although the second name was later ruled invalid) that this theory met with serious challenge, and for a short period the subject was hotly debated in the German fisheries journals (Schikora 1903, Zacharius 1904, Schikora 1904 and 1905a & b and Hofer 1906).

It was not until Nyblin (1934) succeeded in establishing a pure culture of the fungus in vitro and was subsequently able artificially to infect crayfish from these cultures, that Aph. astaci was proven to be the causative agent of crayfish plague. Further work by Nyblin (1936) and Rennerfeld (1936) elucidated some aspects of

the physiology of the fungus, but it was not until the 1960's that further studies were begun on the biology of the organism (Unestam 1965, 1966, 1969a, b & c, 1975, Unestam and Weiss 1970, Unestam and Svensson 1971, Soderhall and Unestam 1978).

Although crayfish mortalities have been reported in the British Isles in the past (Cornish 1902, Boulenger 1927 and Duffield 1933), whether or not these mortalities were due to crayfish plague is uncertain (see chapter 1). In the early part of this decade however, a number of large scale, rapid and spreading mortalities have been noted (Alderman et al 1984).

In 1981 there was a large mortality of native crayfish, Austropotamobius pallipes in the Sherston branch of the River Avon in Wiltshire and in the River Lea in Hertfordshire, although crayfish plague was not confirmed as the cause. Similarly, 1982 saw the demise of crayfish in the River Whitewater and the River Rib, a tributary of the Lea (Alderman et al 1984). However, in 1984, mortalities occurred in the Tetbury branch of the River Avon and in the River Wey. Aph. astaci was isolated from animals from both these rivers (Alderman and Polglase 1984).

The object of this research is to learn more about the biology and spread of the disease in Great Britain, the possibilities for the reintroduction of native crayfish in areas denuded by the disease, the physiological effects of the disease on the animals

themselves and the reasons for the differential susceptibility of native, European and American species. The animals chosen for this work are the Turkish crayfish Ast. leptodactylus, due to their susceptibility to the disease (Alderman pers. com.), their ready availability from local fishmongers and the conveniently regular size range of these imported animals. The British crayfish Austropotamobius pallipes was also used when necessary as was the American signal crayfish P. leniusculus.

A CHRONOLOGY OF THE SPREAD OF CRAYFISH PLAGUE.

INTRODUCTION.

Although a number of reviews have been written in the past, describing the spread of Crayfish Plague in particular regions (Schikora 1926, Alm 1929, Mansfield 1942, Vivier 1965, Westman *et al* 1972), no review to date has detailed the spread of the disease throughout Europe.

This chapter constitites a review of the literature regarding the spread of crayfish plague from the first widespread crayfish mortalities in Italy in the middle of the 19th century, to the present day. Major waterways that have been infected are shown on a series of maps (Figures 1 to 10) detailing the infected waterbodies in each area. Where reference is made to a water body being infected at a certain point, that location is given where possible. Where reports indicate that a river was infected with the disease, the whole course of the river is marked on the map. Where possible, the names of places and infected water bodies given in the text are followed by the present day names in parenthesis; on the maps the names used in the original reports are used.

This review is by no means exhaustive, data on certain areas being difficult to obtain (e.g. The USSR and other Eastern Bloc countries). Readers wishing to investigate the history of the spread of crayfish plague further are refered to Hart and Clark (1987), a detailed bibliography on the subject of crayfish, published after

the completion of this study.

CHRONOLOGY.

PRE1859

A disease of crayfish was reported to have been known for some years previous to 1859, by an Italian worker, Fedrighini (in Cornalia 1860), who reported that all crayfish around Sarnico, on the shores of lake Iseo and at the mouth of the River Oglio, were dead.

The symptoms of the disease described by Cornalia (1860) were somewhat different to those now classically asociated with Crayfish Plague. A similar description was given by Martinati (1862) in a later paper based on the work of Cornalia. The living animals were described as having yellow and then red blotches, and after death, becoming reddened all over. It was also noted that the animals were sluggish and that the legs easily became detached. Later Hofer (1900) noted similar symptoms in animals said to have died of Crayfish Plague.

Such red and yellow colouration is not a symptom of aphanomycosis which I have observed. Crayfish that have been dead for some time often take on a red colour as they decompose, alhough I have not observed them to ever turn red all over.

1859

A severe crayfish mortality occurred in Lombardy,

Italy, in Summer and spread towards Veneto. From midsummer to September mortalities occurred in Gambara, Isorella, and Verolanova, villages in the Bresica region, and in December in the Trevignano area NW of Treviso. The disease was also present in the Cremona, Lodi and Como districts (Cornalia 1860) (Figure 1.).

1860

Crayfish mortalities were reported in the province of Milan, with widespread mortalities occurring around Melegnano. In Bresica too mortalities were widespread (Cornalia 1860).

1861

In lake Garda (lake Benaco), crayfish began to die in the spring. East of the River Adige in the water ways around Zevio, Persacco, Raldon, San Giovanni Lupatoto, Buttapietra, Oppeano, in the Upper Menago, the Bra Channel the Bongiovanni and the Crea Vales, and around Palu and Vallese, crayfish populations were also affected. The disease spread to Belfiore di Porcile, Bionde and other places on the west bank of the Adige, and appeared in the Upper Fibbio, the Antanello, the Fossa Balbi and spread down stream from the Ferrazze di San Martino. It also appeared in the Dugale Fontane, the Sarega, in the lower Tartoro, and in the Cerea valley, and the Casaleone valley, between the Adige and the Po Rivers (Figure 1).

In the vicinity of Albaredo (Albareto) there was

widespread crayfish mortality between autumn and the following spring (Martinati 1862).

1862

After widespread mortalities in 1861 crayfish were again caught in lake Garda.

1864

The disease reached the source of the Sile in December at Casa Corba (Ninni 1865)(Figure1)

In this year too, a crayfish mortality was recorded in Germany, in the Spree at Beeskow. However, it seems unlikely that this was due to crayfish plague, since it was a short-lived, isolated case (Seligo 1865)(Figure 1).

1865

In February the Italian crayfish mortalities spread up the Sile and Botteniga and then to the Rivers Storga, Melma, Limbraga and Magnagola. By April they had spread to the Rivers Musestre, Musestrelle, Pero, Valilo and other lesser waterways (Ninni 1865)(Figure 1).

1876

In France (Figure 2.); the disease appeared for the first time in 1876. The first areas infected were those in the north east. From this plateau area arise many rivers and other waterways, and via these the disease spread rapidly, within seven years, to infect some 39 of the 95

French departements. In 1876 the river basins of the Meuse and its tributary the Mouzon, the Madon (a tributary of the Moselle) the Seine, the Saone, the Ornain and the Yonne were infected (Vivier 1965).

In Yugoslavia, the River Raduljabach became infected, the disease spread as far as Klingenfels, and upstream 16 Km from the mouth of the river (Franke 1894).

1877

This year the disease continued to spread in northern France, attacking the departments of Vosges (the Vair), Aisne, Hte-Marne and the Cote d'Or (Vivier 1965)(Figure 2).

Crayfish mortalities were also noted in the vicinity of Frankfurt-on-Maine (Tsukerzis 1964)(Figure 3).

1878

The spread of the disease continued in France, with the rivers of the Meuse region becoming infected (except the Vinte and the Loivon), whilst in the Seine basin the rivers of the Marne district (the Saulx, the Marne, the Chee, the Viere, the Bruxenelle, the Germenelle, and the Marne Canal in the Rhin region), the Aube district (the Seine and all its tributaries in the district; except the Amance, the Vanne the Landeon and the Brevennes(Figure 2). tributaries of the Aube), the Seine-et-Marne district (the Rivers Loing, Fusin and Lunain), the River Bresle in the Manche (Channel) district and the Andelle (a tributary of the Seine) in the Seine Maritime district were infected.

In the Rhone basin the picture was not so bleak, two rivers were reported infected, the Veyle, a tributary of the Soane, and the Seran, a tributary of the Rhone (Anon 1879, Vivier 1965).

In Germany (Figure 3.) the disease had reached Strassburg and Alsace by March, whilst in July and August, crayfish brought from the Rivers Hunsbruck and Eifel to Mainz all perished, suggesting these rivers were infected. At the same time, crayfish in Hessen-Darmstadt (Hesse) and Baden became infected (Hofer 1906).

This year the disease was also reported to have reached Luxemberg and Belgium (Vivier 1965).

1879

In January Crayfish Plague appeared in Munich. In the River March too, all the crayfish perished in 1879 (Seligo 1865)(Figure 2).

In Austria the disease was also reported in the Klambach at Grein, in lake Traun at Gmunden and the Krems. At this time crayfish mortalities were also reported to be spreading east along the Danube (Figure 3.)(Anon 1879).

In France the plague continued to spread rapidly. It was identified in the Coney, a river of the Vosges region. In the Seine basin nearly all the crayfish of the Aisne district had died by the end of the year. In the River Therain (in the Oise region) the spread was equally rapid, however the Divette and the Vondy were spared.

In the Rhone basin the Rivers Loue and Lizon,

tributaries of the Doubs, were infected, followed a few months later by most of the other rivers in the Doubs district. The disease then proceeded to spread to all the waterways of the Loue, to the Ain and the Bienne, in the Jura district and to the Furan (a tributary of the Rhone) in the Ain district(Figure 2).

By 1879 the disease had also spread to the Loire basin and was reported in the Cher, Creuse and Vienne regions. In the Deux-Sevres region the Bretonne inferieure and the Boutonne were infected (Vivier 1965)(Figure 2).

1880

This year crayfish died out in certain stretches of the Altmuhl and its tributaries eg the Wieseth and the Sulz. Crayfish Plague also appeared in Lorraine (Lotheringia) in the Rivers Moselle, Saar, Orne, Seille, Nied (Seilgo 1895) and by December, Rohrsee, Thuringia, Mecklenburg, and Saxony were suffering from the disease (Figure 3) (Hofer 1906).

During this period the disease also spread to the Brandonbergh March (Schapperclaus 1927) and began to spread from the Oder up the Meitzel (Van dem Borne 1883).

This year saw the first reports of the disease in Slavonia, Northern Yugoslavia (Figure 3.). It was detected in the Krain (now the Carniola) region, in the River Krain (Franke 1894), and in the Drava the Mura, and the Savinja, rivers in the Stajerska district. Soon after it was detected in the Dolenjska and Bela-Krajina regions in the

Krka a rich and very productive crayfish river, and in the Kolpa, spreading both up and down stream (Herfort-Michieli 1973).

In France, the disease continued to spread in the Seine basin. Here, crayfish in the Blaize, the Blaiseron (a tributary of the Marne) and the Aube and its tributaries, flowing through the Hte-Marne district, died out. In the Loire basin the disease was reported in the Loiret region and also in the Botonne, a river in the Sevres Niortaise district, whilst in the Saone district it was reported in the Vingeanne(Figure 2).

An outbreak of crayfish mortalities occurred in the Echez, in the Htes-Pyrennes, but was never confirmed as Crayfish Plague (Vivier 1965)(Figure 2).

1881

During this year crayfish deaths occured in the Rhine province of Switzerland at Berne and in Silesia, in a side stream of the River Bober (now the Bobrawa or Bobr in Poland : Figure 3.). An outbreak also occured at Angermunde in Brandonburgh (Seligo 1895).

Deaths were also reported at Wels and in the Eiterbach in upper Austria during October and November (Roch 1881). Mortalities also occured in the Tauber, tributaries of the Mainz, in the Kuddow river in the Oder region and in the Ferse (now the Wierzyca in Poland) and Schwarzwasser in the Weichsel region of Western Prussia (Figures 3 and 4.) (Seligo 1895).

In France the disease continued to spread among the rivers of the Rhone basin, infecting the Serine, the Cotey, the Gland, tributaries of the Rhone and several rivers in the Belley area. It had, by now, completely over run the Loire basin (Figure 2).

In the Manche district, several rivers in the Mortain area were attacked, whilst in the Maine-et-Loire district only the Couesnon, downstream of Authion was affected (Vivier 1965) (Figure 2).

1882

This year the disease spread further in Yugoslavia, advancing from the Kolpa to the streams of Tschernembl (Cernemble), whose stocks of crayfish were destroyed within the next two years (Franke 1894). Mortalities were also reported in the Gorenjska district and around Ljubljana and in the rivers and streams of Notranjska (Herfort-Michieli 1973) (Figure 6)

In France the Plague continued its spread through the Rhone basin, attacking nearly all the waterways around the town of Nantua in the district of Ain.

In the Cher district of the Loire basin the disease was still widespread, infecting the Rivers Arcueil, Auron, Yevre (between Bourges and Mehun) and the Arnon and its tributaries. In the Loiret district, the disease also continued its destructive course (Vivier 1965) (Figure 2)

1883

Crayfish deaths also occured in lake Boethin this year at Deutch Krone (now Walcz in Poland : Figure 4) (Hofer 1906), and it was reported that there were no longer any crayfish left in the Altmuhl (Anon 1893).

Spread of the disease continued in Slavonia, where the tributaries of the River Sava were infected (Figure 3.) (Herfort-Michieli 1973).

1884

In 1884 the Crayfish Plague was first observed in the Brahe (now the Brda in Poland) in Western Prussia, it also attacked the fast flowing waters of the Kamionka and Zemplona and was observed in the Wildgartenfleiss. In this year too, the crayfish of the Weichsel and Mischkerfleiss were destroyed by the disease. Between the years 1884 and 1890 the Rivers Drewenz (or Drweca), Ossa and Liebe in Western Prussia were all affected by crayfish plague (Figure 4.) (Seligo 1895).

The upland areas of the Glatz province of Silesia (now the Klodzko district of Poland) were also invaded, via the slow flowing streams of the eastern (Glatzer) Neisse (Figure 3.) (Schikora 1906).

In Yugoslavia, the head waters of a major crayfish river, the Gurk, became infected, along with the River Temenica, also in Yugoslavia. Restocking of this River in later years failed because crayfish in the upper waters were still falling victim to the disease as late as 1893

(Franke 1894).

This year signaled the end of the period of rapid spread of the disease in French waterways. In the Rhone basin, Crayfish Plague had, by now, affected most of the rivers of the Ain district, except the Albarine, the Chalarone and several small rivers of the Trevoux and Belley areas which were not conected to the main course of the infeced rivers (Figure 2)

Developement of the disease in the rivers of the Cote d'Or region continued, not only downstream but also upstream towards their sources. The crayfish finally disapeared from the Aube, the Aubette (except upstream of Lesgarde) and the Ource (except upstream of Voulaines). The topmost sections of the Seine and some of its tributaries (the Brevon, the Coquille and the Revinson) remained uninfected as did rivers such as the Laigne, without comunications with the main watercourse. Rivers in the Dijon area were also attacked, as were the Sorgues in the Vaucluse district (Vivier 1965) (Figure 2).

In England (Figure 5) crayfish began to die out in stretches of the River Ock, a tributary of the Thames. The cause was unknown, but the mortalities spread throughout the river until 1887 when no animals were caught (Duffield 1933).

1885

After the rapid spread of the disease in the French waterways between 1876 and 1884, crayfish began to

reappear in the Vienne district, which had been infected in 1879 (Vivier 1965).

1886

This year saw the demise of crayfish stocks in lake Doubuzhis in the Rokishksk district of N.E. Latvia (Tsukerzis 1964), and also of those in the Wiexelbach in Yugoslavia (Figures 3 and 4). Attempts at repopulating the Yugoslavian river several years later, failed (Franke 1894).

1888

Crayfish populations of the Nievre district of France, upstream of Chateau-Chinon, which were destroyed in 1876, began to re-establish themselves (Vivier 1965). (Figure 2)

1889

An outbreak of "Crayfish plague" was reported in the Oeuf, a tributary of the Essonne, which in turn flows into the Seine. Reports of the disease in France were, by now, few (Vivier 1965) (Figure 2).

In England, crayfish died out in the River Thame, from it confluence with the Thames upwards (Figure 5). Side streams were also affected. The cause was never ascertained (Duffield 1933).

1890

This year the Plague spread to East Prussia having

taken 20 years to cross Germany (Schikora 1926a) and Lake Drewenz and Lake Ewing in the Passar region of Western Prussia were affected (Seligo 1895) (Figures 2 & 3)

1891

Between 1891 and 1892 the Plague spread to the River Luga (Leningrad district) and the lake Onega basin in Russia (Figure 6) (Arnold 1900). The waters of the Baltic lake area then conducted it to the River Volga by 1892 (Arnold 1900, Schikora 1926).

During this period crayfish in the River Clery, a tributary of the Loing in the Seine basin, fell victim to the disease also (Vivier 1965).

1892

This year the disease appeared in Masuren in Western Prussia, an area of many lakes including lake Sniardy and lake Manry (Seligo 1895), and in waters around Shialiai in central Latvia (Tsukerzis 1964) (Figure 3).

The disease now appeared in the high valleys of the Karst region of Yugoslavia, in the River Rinnse (Franke 1984) (Figure 3)

Crayfish reintroduced into the Main recently were reported to be thriving (Scherpf 1892) (Figures 2 & 3)

During the period 1892-1894, the disease appeared in the Upper Stople and in the River Lyke (a tributary of the Narew) (Figure 4).

1893

In 1893, the Crayfish Plague reached Finland (Figure 7), probably as the result of the import of infected crayfish from Russia (Westman 1973). The first reports were from lake Saimaa in the Vuoksi water course, and the surrounding waters but between 1893 and 1972 the disease spread to infect 750 waters in 46 river systems (Westman et al 1972).

In Russia, the River Dniepa was affected down as far as the rapids at Ekaterinoslav (Dneipopetrovsk)(Figure 6) (Arnold 1900). This year also saw the demise of crayfish stocks in the lakes and rivers of the Niamunas (Neman) and Niaris (Vilnia) river basins, completly wiping out the animals in the waters of Shvianchensk, Trakaisk, Utiansk, Birzhaisk (E and central Latvia), Varniaisk (W. Latvia) (Figure 6). The rapid spread in Latvia was said to have been due to the import of infected crayfishing gear from Germany (Tsukerzis 1964).

1894

In late autumn of 1894 a further outbreak of the disease occurred in lake Boethin after the crayfish had begun to reappear. The disease also appeared in Courland (Southern Latvia), Livonia (Northern Latvia), and Estonia (Schapperclaus 1954 : Figure 4).

In Russia the River Klyazma, a tributary of the Volga, was attacked as were the River Dvina (Vitebsk district) and a few lakes of the Vladimir district (Arnold

1900 : Figure 6).

In Finland (Figure 7), the disease spread to the Kymijoki waterway, infecting the Sarkavesi, the Lahnavesti and the Tarhavesi as well as other waterbodies (Westman et al 1972).

This year saw the demise of crayfish stocks in the River Windrush in England (Figure 5). The cause of this mortality was unknown (Cornish 1902, Duffield 1933).

1895

The decimation of Russian rivers and lakes continued this year with the loss of crayfish stocks in the River Beresina, a tributary of the Dnieper, the River Moscow, the River Oka and lakes in the Suvalki district (now the Suwalki district of Poland : Figure 6) (Arnold 1900).

1896

Further crayfish mortalities occurred in Russia this year, in the River Scheksna in the Jaroslav district (now in Poland) and the River Embach in Livonia was affected (Arnold 1900) (Figures 4 and 6)

The lowland areas of the Glatz region of Silesia was reached this year, the upland areas having been affected in 1884 (Figure 3).

In France, crayfish reappeared in an affluent of Nantua lake, the River Merloz (Vivier 1965).

1897

This year Schikora (1926a) described the disease reaching the Urals in the Kama district and climbing upstream to cross the mountain barrier, from whence it spread to the Ob via the Tura and on to Siberia (Figure 6).

In this year the crayfish of the Tchernigoff (Cernigov) and Tver (Kalinin) districts of Russia fell victim to the disease. Some lakes of the Novgorod district were also affected (Arnold 1900 : Figure 6).

In Livonia the River Woo, and lake Werro through wich it flows were affected (Hofer 1900 : Figure 4).

1898

This year the rivers of the Poltava, Charkov and Pskoff (Pskov) districts of Russia were affected (Arnold 1900 : Figure 6), and a German fisherman reported the death of crayfish in a stock lake at Lubben (Schiemenz 1934).

In Livonia (Figure 4), the disease spread to the Livlandische Aa (The Gauja) and the Duma (Vivier 1965).

1902

This year the disease appeared in the Pansdorfer See in Silesia during October (Schikora 1906).

In Latvia, the spread of the disease appeared to have abated (Tsukerzis 1964).

1903

In the spring, the disease reached the Malzsee in Neumark and during July reached Lake Sawindasee (Figure 3). In the summer, mortalities were also reported in the Volzkowsee near the town of Neustettin (Szczecinek) and in the River Rohra in the region of Deutche-Krone in western Prussia (Anon 1903 : Figure 4). In late August the disease was reported in the River Aurach between Emskurchem and Munchaurach and in September, in the trout streams around Selb in the Oberfranken region of Bavaria (Surbeck 1903). Work done by Schikora (1906) identified the disease in samples from the Nariensee at Guldenboden West Germany, a lake in Mecklenburg, Volhynia in Russia and an area (unnamed) of Eastern Russia (Figures 6 & 3)

1904

This year the plague was identified in the Zanzhausener See in Neumark, Germany (an area S.E. of the Oder and S. of Pomerania, now incorporated into Poland). It was not confined to this water however, and spread from lake to lake (Schikora 1905 and 1906) (Figure 3).

1905

The disease continued to spread in Neumark this year. It was identified in the Kloppsee in April and in the Muckenburger See in May (Schikora 1905 and 1906).

1906

In England (Figure 5), the crayfish of the upper course of the River Kennet had died out by this time (Duffield 1933).

1907

In 1907 the disease reached Sweden (Figure 8), probably via Finland, when infected animals were dumped into the Malaren at Stockholm (Alm 1929). Deaths began the following year.

In Finland (Figure 7), the first appearence of the disease in the Eurajoki, Karvianjoki and Kokemaenjoki water systems was noted (Westman et al 1972).

1908

This year saw the devestation of the most productive crayfish lakes in Sweden, the Malaren and Hjalmaren. By the end of 1908 all the crayfish in the Hjalmaren were dead. The disease spread between the two lakes via a connecting river, the Eskilstunaa, in which the crayfish also perished (Alm 1929).

In Finland, the disease appeared in the Karjaanjoki water system (Westman et al 1972).

1909

Between 1909 and 1910 the last of the crayfish in the Malaren died and the disease spread to the Fyrisaan, Arbogaan, Svarta in Nerike and Talga. (Alm 1929). In

Slavonia the disease occurred in lake Cerknica (Herfort-Michieli 1973).

1910

In Finland the disease spread to further river systems including the Porvoonjoki and the Mantsalanjoki systems (Westman et al 1972).

1911

Between 1911 and 1912 the disease spread further up the Fyrisaan and its tributaries, as well as the Hagaan, Orsundaan, Eukopingsaan, Saga, the Svarta in Vastmanland, the Kolbacksa, Kopingsa, Hedstrommen, the Arbogaa and its tributaries and also a large number of smaller rivers and streams. (Alm 1929).

In 1912 a large mortality of crayfish in the Uckermark region of Germany (Figure 3) was diagnosed as being due to Bacillus pestis astaci, an agent which, at the time, was believed to cause crayfish plague (Schapperclaus 1927).

In France (Figure 2), the Gau de Champlive, a river conected to the Doubs by an underground course and thus protected from the spread of crayfish plague from this river, eventually fell victim this year. This was the last identification of the disease in France, up till 1965 (Vivier 1965).

In Finland the disease spread to the Koskenkylanjoki and Painionjoki water systems (Westman et al 1972).

1912

In 1912, the disease was detected in the Kiskonjoki river system in Finland (Westman et al 1972 : Figure 7)

1913

Between 1913 and 1916 the spread of the disease continued up the Fyrisa, Hagaa, Orsundaa, Kolbacksa, Kopingsa, Hedstrommen, Arbogaa, and the Svarta in Nerike. The advance halted in the smaller rivers before 1913, and in larger ones by 1915-1916 (Alm 1929 : Figure 8).

In 1915 another large mortality of crayfish was reported in the Uckermark region of Germany (Schapperclaus 1927).

1916

Most of the rivers and lakes of the Kolbacksa system in Sweden were devastated between 1916 and 1919. The disease front also advanced up the Kopingsa and the tributaries of the Arbogaa and Svarta in Nerike (Alm 1929).

1918

This year saw a further rapid decline in the numbers of crayfish in the River Thame in England (Figure 5). The animals had become re-established since their demise in 1889. In the River Windrush too a further decine in the animals occurred after they had become re-established. The cause of these declines was never

established. (Duffield 1933).

1919

Between 1919 and 1921, deaths began to occur in the uppermost branches of the Kolbacksa, but had subsided in the Vastmanland Svarta, the Nerike Svarta and the Arbogaa by 1921 (Alm 1929). Thus, during the period 1907-1922 the disease appeared to be confined to the Hjalmaren, the Malaren and associated rivers (Figure 8).

1920

Following a period when there was little or no spread of the diease in Latvia, new outbreaks of plague were reported in Lake Obelija and Lake Dusia (Tsukerzis 1964 : Figure 4).

New outbreaks of plague were also reported in Lithuania (Mazylis and Grigelis 1976).

1923-1925

No new outbreaks occured in Sweden during this period (Alm 1929). However, another outbreak occurred in the Uckermark region of Germany, in the same area as those in 1912 and 1915 (Schapperclaus 1927).

In Livonia (Northern Latvia : Figure 4) the disease broke out anew after a lull of some six years. It was reported in 1924 in the main branch of the Oger and in its associated rivers and lakes (Vivier 1965).

1925

Having broken out again in Livonia in 1924, the disease gained new ground this year, affecting crayfish stocks over the entire length of the Jagel and its tributaries, in the Lede (a tributary of the Ewst) and the Tirsee, an affluent of the Aa. Whilst in the neighbouring province of Courland (Southern Latvia), the epizootic invaded lake Usmaiten, the Pussenscher See, the Spahrenscher See and the Irbe and its side streams the Anger and the Stende, as well as the Sirgumbach and the Suhrsche Bach (Mannsfield 1942).

1926

New outbreaks of crayfish plague occurred in Sweden this year, in parts of the Fyrisa system which had previously been spared or restocked (Alm 1929). In Livonia the spread of the disease continued unabated, the Salin-see, the Wadersee and the Waddain-see (in the Walck district) as well as the Salis and the Burtneck see becoming infected. In the Aa river system the disease continued to work its way through the tributaries and in Courland, Lake Dulme, Lake Lohuste (in the Irbe system) and its affluents, the Moritzbach the Palse and the Racke were affected by the disease (Mannsfield 1942 : Figure 4).

1927

As the new Swedish outbreak continued, the Magelungen-Drevviken lake system in Sodertorn became

infected (Alm 1929), whilst in Livonia, Crayfish Plague spread further through the Aa river system, affecting crayfish in the Lore, the Ligat and the Merge. In Courland the epizootic appeared in the River Hasau from were it spread to its tributar ies (Mannsfield 1942).

This year there was a large scale mortality of crayfish in the River Colne, a tributary of the Thames, in England (Duffield 1933 : Figure 5).

1926-1928

During this period disease occurred in several waters in Lettgallen, namely the lakes Ekscho, Jescho, Gelenowo, Kategrader, Rasno, Solwa, Ruschon, Bolta, Swojatschij, Schugari, Jerscha, Bischa and Baldosch, whilst in Livonia the disease was recorded in the Behrse, a river confluent with the Duna near the island of Dahlen (Mansfield 1942).

1928

In Sweden, Nyblin (1931) reported deaths in Lake Addar in Rosengen, the lower reaches of the Dalelv, the West Gothic and East Gothic ends of the Gota Canal as well as the majority of confluent lakes and streams. In this year deaths were also reported in the Tidern and the Osan and the Vikern and several other lakes (Alm 1929), as well as in the smaller water courses of the Kolbacksa, where populations had begun to thrive again after their demise between 1916 and 1918 (Vivier 1965 : Figure 8).

In the Baltic states (Figure 4) further outbreaks of the disease occured this year. In Lettgallen, lake Schausat, lake Rukopoler and the River Leitisch were attacked and between 1928 and 1929 the Istra, Audio, and Dsilias group of lakes also fell victim. Lakes Nezsa, Maschenowo, Spornoje, Maloje and Snidsa were also infected.

In Courland mortalities occured in the Bangau and the Ehnau (both affluents of the Windau or Venta) and in the Loride (Mannsfield 1942).

Between the years 1928 and 1929, the crayfish of the River Ock in England suffered a further rapid decline in numbers, having re-established themselves since 1884 (Duffield 1933 : Figure 5).

1929

In Sweden, the disease spread from the Gota Canal area to the Stang An and Lake Asund (Nyblin 1931). Crayfish deaths also occurred in Lake Nashulta in Sodermanland, the Addarn waterway system in eastern Uppland and lakes and tributaries in western Ostergotland (Alm 1929), and in the minor waterways of the Dalev.

In Livonia the spread of the disease appears to have slowed down in 1929. Mansfield (1942) cites only one river becoming infected, the Isleenne, a tributary of the Ewst.

However in Lettgallen, the disease was still in full swing and lake Dunakla, lake Olowez and the River Malte were infected in this year, whilst between 1929 and 1930, the River Itscha, a tributary of the Ewst, also fell victim

to the disease.

In Courland the epizootic appeared in the Melkenbach, the Neuhofscher Bach, the Masseres See and the Disheres See but after this date few incidences of the disease were recorded in this area (Vivier 1965).

In Finland it was detected in the Rannikkoalue system (Westman et al 1972 : Figure 7).

1930

In 1930 the disease front moved further up the Stangan (Nyblin 1931). In Lettgallen, it attacked the lakes Auleja, Zerpa, Porkal, Kurman and Bicha and the River Dubna (a tributary of the Duna). The infection was passed from the Duna basin to the Walikaja, and it was from a tributary of the Walikaja, the Utroja, that it was passed to the River Kuchva, probably by infected crayfishing equipment, in 1930 (Mannsfield 1942).

In Livonia the disease was reported in a side stream of the Duna, the Sussei, and in the Odse See, a lake on the River Perse, as well as in the Stroppen See near Dunaburg and the Swenten See (Mannsfield 1924).

In Finland the disease spread to the Hiitolanjoki and Ahvenanmaa water systems (Westman et al 1972).

1931

This year, the most productive lake remaining in Sweden, lake Erk in Upper province, was devastated by the crayfish plague (Nyblin 1931).

In Lettgallen the epizootic spread to lakes Pilda, Sajedneje, Sursa, Birsegola and Tschumanowo (Mannsfield 1942).

1932

The Crayfish Plague was detected in the Vehkajoki waterway in Finland this year (Westman et al 1972).

1933

In Livonia the Crayfish Plague was recorded in the Marienburger See (Mannsfield 1942), whilst the spread of the disease continued in Finland, reaching the Vantaanjoki system (Westman et al 1972).

1934

This year the disease was recorded in the Waidau, a tributary of the Aa in Livonia (Mannsfield 1942).

1935

In 1935 Crayfish Plague was reported for the first time in the Lapuanjoki river system in Finland (Westman et al 1972).

1936

This year both the Insersee and the Pullan-See in Livonia fell victim to the disease (Mannsfield 1942).

1937

Mortalities occurred this year in the Lehtisch, a left tributary of the Windau, in Courland (Mannsfield 1942).

1938

In 1938 the Wisle, a left tributary of the Aa, and the Schwarzbeckhofsche Seen in Livonia were attacked by crayfish plague, and in Courland, mortalities occured in the Rehsche, a right tributary of the Windau (Mannsfield 1942).

1939

In Finland, the first outbreak of the disease in the Halikonjoki, Lapinjoki and Sirppujoki river systems was noted (Westman et al 1972).

1940

Crayfish Plague was first reported in the Siuntionjoki and Kyronjoki waterways in Finland this year (Westman et al 1972).

1943

In this year the disease broke out once more in Latvia, in Lake Shventilis (Tsukerzis 1964).

1947

The appearence of the disease in the Isojoki and

Oulujoki water systems in Finland was noted this year (Westman et al 1972).

1951

In a further Latvian outbreak of the disease, the crayfish in Lakes Galstas and Zapsys were killed (Tsukerzis 1964).

In Finland, the crayfish of the Pyhajoki river system were affected by the disease for the first time this year (Westman et al 1972)

1952

This year saw the first identification of Crayfish Plague in the Valperinjoki river system in Finland (Westman et al 1972).

1954

In Finland the disease spread to the Virojoki water system (Westman et al 1972).

1955

The disease continued to spread in Finland this year, reaching the Ilolanjoki waterway amoung other rivers (Westman et al 1972).

1956

This year saw the first out break of mortalities in Spain (Figure 9), in the River Duero in the Valladolid

district (Cuellar and Coll 1983).

The disease was also detected in the Aurajoki, Siikajoki and Kalajoki water systems of Finland for the first time this year (Westman et al 1972).

1959

This year the disease spread to the Hounijoki, Vilajoki, Urpalanjoki, Letijoki and Tervajoki systems in Finland (Westman et al 1972).

1960

In 1960 the crayfish of lake Ungurinis in S.E. Latvia sucumbed to the disease (Tsukerzis 1964), and in France a spate of deaths in the tributaries of the Vienne was believed to be due to crayfish plague, but this was never varified (Vivier 1965).

This year also saw the first reported outbreak of Crayfish Plague in the Purmonjoki and Kiiminkijoki river systems in Finland (Westman et al 1972).

1961

This year the disease first affected crayfish in the Ahtavanjoki river system, Finland (Westman et al 1972).

1962

The spread of the disease in Latvia continued with the demise of the crayfish in lake Gavis (Tsukerzis 1964), whilst in Finland the disease spread to the Perhonjoki

river system (Westman et al 1972).

In June, the disease affected the crayfish of the River Czarna and its tributaries in the Kielecki voivodship, Poland (Kozlowski 1968).

1963

In 1963 lakes Shlavinas, Sutrinis and Shiekshtys in Eastern Latvia were affected by the disease (Tsukerzis 1964).

1964

This year the disease spread to the Halmaa, in the Vaalimaanjoki system in Finland and also to the Sipoonjoki system (Westman et al 1972).

1965

This year, a further large mortality of crayfish occurred in Spain (Figure 9), in the River Ucero, in the Soria district. This infection may have spread from Astacus leptodactylus introduced from Germany (which had already suffered badly from the disease), although Aphanomyces astaci was never positively identified (Cuellar and Coll 1983).

In Finland Crayfish Plague spread to the Iijoki river system (Westman et al 1972).

1966

The disease spread to Tohmajoki water system in

Finland this year (Westman et al).

1967

In 1967 the disease was diagnosed in lake Spindzius and the Trakai district of Lithuania (Mazylis and Sestukas 1968, Mazylis and Grigelis 1979).

1968

This year saw the first recorded outbreak of the disease amongst the crayfish of the Kuivajoki waterway in Finland (Westman et al 1972).

1978

This year saw the first positive identification of Aph. astaci in Spain, in animals from the Riaza river, the infection was believed to have originated from illegally imported Ast. leptodactylus. Deaths occuring in 1975 and 1976 in the River Iregua were attributed to stress caused by rainwater run-off or pollution, since the fungus could not be detected.

The disease spread to the Guadiana river in Cuidad Real region and later to other rivers in the area (Cuellar and Coll 1983 : Figure 9).

1979

In Spain, the disease spread further this year, reaching the Rivers Cadagua in Vizcaya province, Leizaran Y Araxes in Guipuzcoa province and Omecillo and Bairax Y

Ayuga in Alava province in June. In July and August it spread to the Rivers Ega, Cidacos, Araquil, Leizaran, Elorza, Salazar and Erro Y Larrain in the province of Navara, to the Rivers Guadalaviar and Jiloca Y Alfambra in Truel province and the River Pisuerga in Valladolid province. In August and September Aph. astaci was identified in the Ebro and its affluent waters in Zaragosa and in the Iregua river in Logrono (Cuellar and Coll 1983).

1980

In 1980 the disease was detected in many more Spanish rivers. In May it was observed in the Rivers Jucar (Albacete province), Guadiana (Cuidad Real province) and Guadarrama, atributary of the Tajo (Toledo) and later in the Duero (Zamora) the Carrion (Palencia), the Bernesga a tributary of the Esla (Leon), the Eresma and Esgueva (Valladolid), the Omecillo and Ayuga (Alava), the Ega, Cidacos and Erro (Navarra), the Iregua (Logrono) the Riaza and Duraton (Segovia),the Riaza and Esgueva (Burgos) the Jalon (Zaragosa) and the Ucero (Soria province).

1981

In 1981 the first suspected cases of Crayfish Plague were reported in the UK (Figure 5). The first major mortality occurred in the upper reaches of the Sherston branch of the Bristol Avon at Hyam, Whiltshire and in July a mortality was observed in the River Lea, at Ware in Hertfordshire (Lowery et al 1986.)

1982

In the UK, mortalities occurred in the River Whitewater in Hampshire but Aph. astaci was not confirmed. During the spring, mortalities spread from the River Lea to the Rib. A crayfish mortality was also observed in the Tetbury branch of the River Avon (Lowery et al 1986).

This year also saw the first reported outbreak of the disease in Greece (Figure 10), in the River Kalamas. The mortality occured shortly after a large scale mortality amongst juvenile Pacifastacus leniusculus introduced into the head waters of the river (Theocharis 1986).

1983

In the UK, crayfish in the northern branch of the River Wey succumbed to the disease over a 35 km stretch of the river around Bently and Aph. astaci was isdated from these animals. The southern branch of the river was also affected this year. The disease also spread to the Hampshire Avon, whilst in the Bristol Avon, animals continued to die. In Hertfordshire, the disease spread to the River Beane, a tributary of the Lea (Lowery et al 1986).

In four UK rivers, the Lodden, the Eamont, the Gade and the Kennet, crayfish populations disappeared around this time. The causes of the disappearances were not ascertained (Lowery et al 1986).

In Greece, the disease appeared in the River Louros,

in the Hipiros region, and in lake Ioannina nearby (Theocharis 1986).

1984

A further outbreak of the disease in England was confirmed this year in the Tees at Darlington (Alderman pers. com.), and in Summerhouse brook, a tributary of the Tees (Lowery et al 1986).

1985

This year saw the demise of crayfish in the River Chess in the UK (Alderman pers. com.)

1986

This year saw the disappearance of the last known population of crayfish in the River Rib in the UK. The cause was not ascertained. Crayfish plague outbreaks were also reported in unspecified rivers in northern Portugal, Greece and Asiatic Turkey this year (Alderman pers. com.).

1987

A crayfish mortality was reported in the River Misbourne in the UK this year. Aph. astaci was isolated from one of these animals.

CRAYFISH FLUCTUATIONS IN ENGLAND.

It is not clear when the first outbreak of crayfish plague occurred in England. A number of violent fluctuations in crayfish populations in certain English rivers have been reported since the 1880s, however, whether these fluctuations were the result of crayfish plague outbreaks is uncertain.

Duffield (1933) on the basis of interviews with local people, reported dramatic declines in the crayfish populations of the Rivers Ock, Thame, Windrush, Kennet and Colne between 1884 and 1889. The crayfish populations in these rivers often recovered only to crash again and mortalities were said to spread upstream, a phenomenon seen in crayfish plague outbreaks. The cause of these declines was never ascertained although factors such as pollution, disease or overfishing were suggested.

In the Thames a mass mortality of crayfish occurred in the last years of the 19th century (Cornish 1902). The mortality began around Staines and spread to the Windrush and Cherwell. Animals were said to turn red and emerge from their burrows before dying (Cornish 1902) The animals died in such numbers as to cause a red border to the river bank. These symptoms are similar to those described during a mass mortality of crayfish in Northern Italy in the 1860s (Cornalia 1860) and also to those described by Hofer (1900), who ascribed such deaths to crayfish plague.

However, such red colouration at death is not

typical of recent outbreaks of crayfish plague, nor is it a feature regularly described during earlier outbreaks (Schikora 1905, Unestam 1964a, Alderman and Polglase 1984, Alderman et al 1984). Futher, no animal dying after infection with Aph. astaci in experiments described later in this thesis showed any signs of red colouration. Even animals deliberately allowed to decay for a week only turned slightly orange. However, the degree of red colouration may be due to post mortem microbial activity: I have no information on this point. The cause of the mortalities described by Cornish, therefore remains uncertain.

By the time Duffield's work was published, Schikora (1903) had already identified the causative agent of crayfish plague as Aph. astaci, Duffield however, did not appear to be aware of the work, and discussed the possibility that the mortalities were due either to B. pestis astaci or to Thelohania. The only symptoms of the English mortalities that he described were those of crayfish dying in an artificial pond in Tubney near Oxford. These animals turned white before death and may well have died from Thelohania infection.

Both Duffield and Cornish, at different times, independantly described population crashes among the crayfish of the Windrush which occurred at around the same time, however, since the symptoms described by Cornish were unlike those usually associated with crayfish plague today, the cause of the population crash reported by Duffield must

remain in doubt.

Confusion over the cause of these mortalities is highlighted further by a report of Pixell Goodrich (1953). In an attempt to determine the cause of the earlier English mortalities, she examined crayfish from streams in the Wootton area near Oxford and concluded that a number of these animals were infected with microsporidians and a yeast, probably Cryptococcus gammari (a yeast causing mortalities in Gammarus). However she did not report any mass mortalities in this area at the time of her study and made no mention of Aph. astaci as a possible cause of mortality in crayfish, despite the fact that the organism had been recognised as such by a number of workers (Schikora 1903, Nyblin 1931, Rennerfelt 1936). It seems unlikely that these findings explain the violent fluctuations in crayfish numbers seen in the rivers around Oxford at the turn of the century. Crayfish mortalities around Oxford were also mentioned in an earlier report by Calman (1911) who associated these with the death of European crayfish. He too believed both the Oxford mortalities and those in Europe to be due to infection with microsporidians.

In 1981 mass mortalities of crayfish occurred in the upper reaches of the Bristol Avon near Malmsbury and in the River Lea, plague was suspected but no relevant pathogen was isolated. In 1982 mortalities were reported in the River Whitewater and the River Rib. In 1983 further mortalities occurred in the River Wey in Hampshire and Aph.

astaci was at last isolated from affected crayfish. This was the first time Aph. astaci had been isolated from crayfish dying in British waters, although it had been looked for since the first mortalities in 1981.

The route by which Aph. astaci reached Great Britain remains a contentious issue (Richards 1986, Lowery 1986, Marren 1986). It may have spread from the continent at the end of the last century, either via the introduction of infected crayfish or by other means, or it may have arrived more recently. No record of crayfish introductions at the turn of the century is available and it seems unlikely to have spread across the Channel on infected crayfishing equipment.

In recent times, Signal crayfish (Pacifastacus leniusculus), a species which can carry the disease (Unestam 1972 and 1974), has been introduced into English waters. The first Signal crayfish introductions were made in 1976. These animals were imported from Sweden where crayfish plague is certainly present (Alm 1929, Unestam 1964a), although the introduced animals were claimed to be free of the disease (Richards and Fuke 1977). The following year at least 25 further implantations were made (Richards and Fuke 1977). Within five years of the first introductions, large scale crayfish mortalities that were almost certainly due to crayfish plague, were occurring in the West Country and in the Thames valley. Both of these areas currently support large numbers of Signal crayfish farms (Holditch and Reeve pers. com. see also chapter 2).

Thus, although large scale crayfish mortalities and population crashes occurred towards the end of the last century in the Thames valley, workers searching for the cause were unaware of the existence of Aph. astaci. This, coupled with the atypical symptoms exhibited during some outbreaks means that these occurrences cannot be attributed to crayfish plague with any certainty. However the present outbreak of the disease in England seems likely to be connected with the implantation of Signal Crayfish carrying crayfish plague.

Figure 1. Northern Italy.

This figure shows a number of river systems in Northern Italy, whose crayfish populations suffered large scale mortalities between 1859 and 1862. A number of the rivers mentioned by Cornalia (1860) and Martinati (1862) were not traced. These may either have been small streams, and consequently not marked on maps, or their names may have changed.

SGL = Sangiovani Lupatoto

Bu = Buttapietra

Op = Oppeano

Z = Zevio

P = Palu

R = Raldon

V = Vallese

The rivers Olglio and Adda are not mentioned in either of the original texts, however, they were included since towns on their banks were said to have suffered crayfish mortalities.

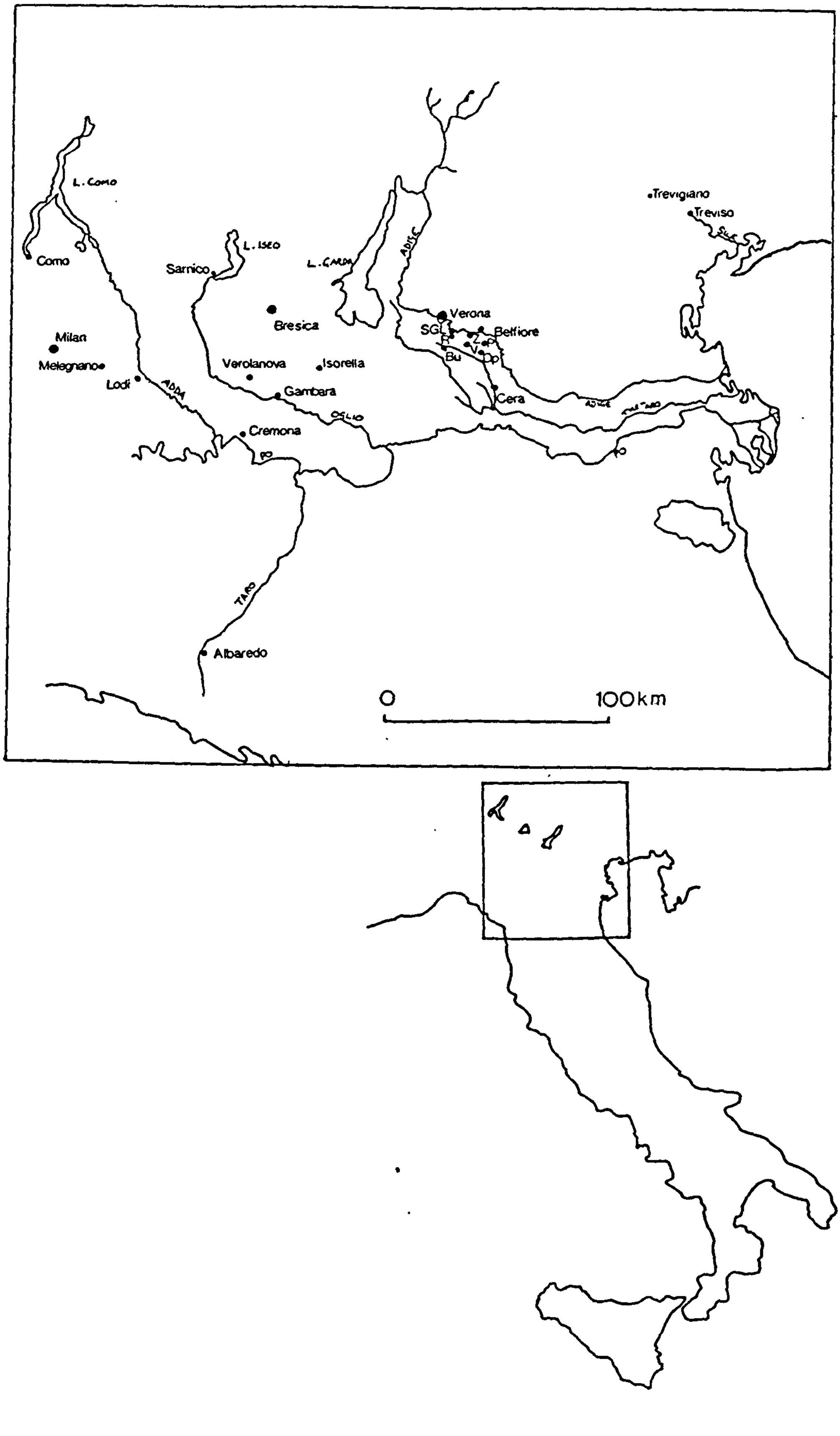

L. Como
Como
Sarnico
L. Iseo
L. Garda
Adige
Trevigiano
Treviso
Sile
Verona
Bresica
Milan
Melegnano
Lodi
Adda
Verolanova
Isorella
Gambara
Oglio
Cremona
Po
Cera
Adige
Tartaro
Albaredo
0
100km

Figure 2. France and Western Central Europe.

This figure shows rivers in France and Western Central Europe for which crayfish mortalities are included in this report. For the sake of space a number of towns have been omitted, however, in the case of France, the reader is refered to modern atlases of the area for these since place and river names have changed little in this area.

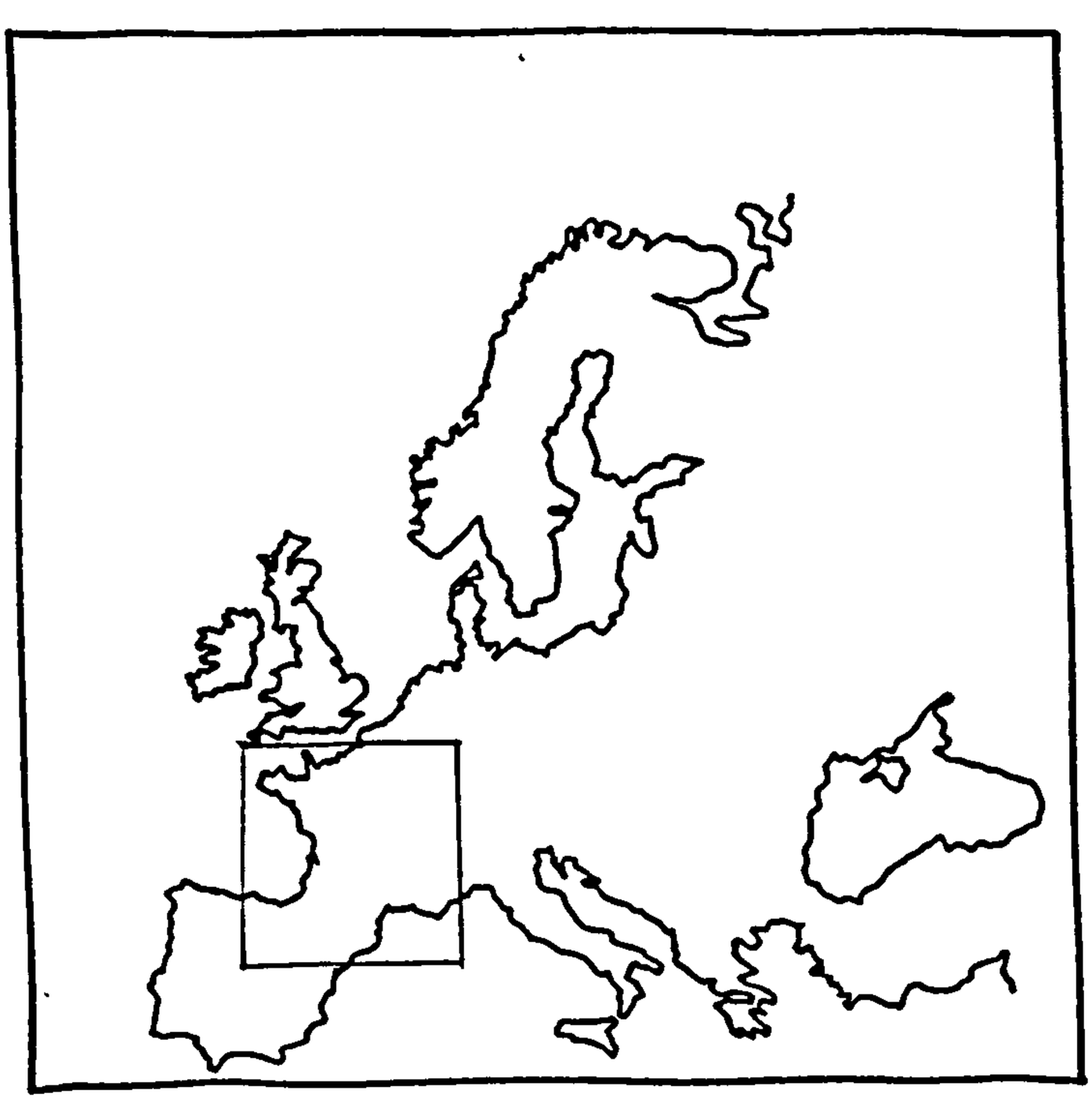
OR = R. Ornaine

GEM = R. Gemenelle

VING = R. Vingeane

B. = R. Blaiseron

FUS = R. Fusan



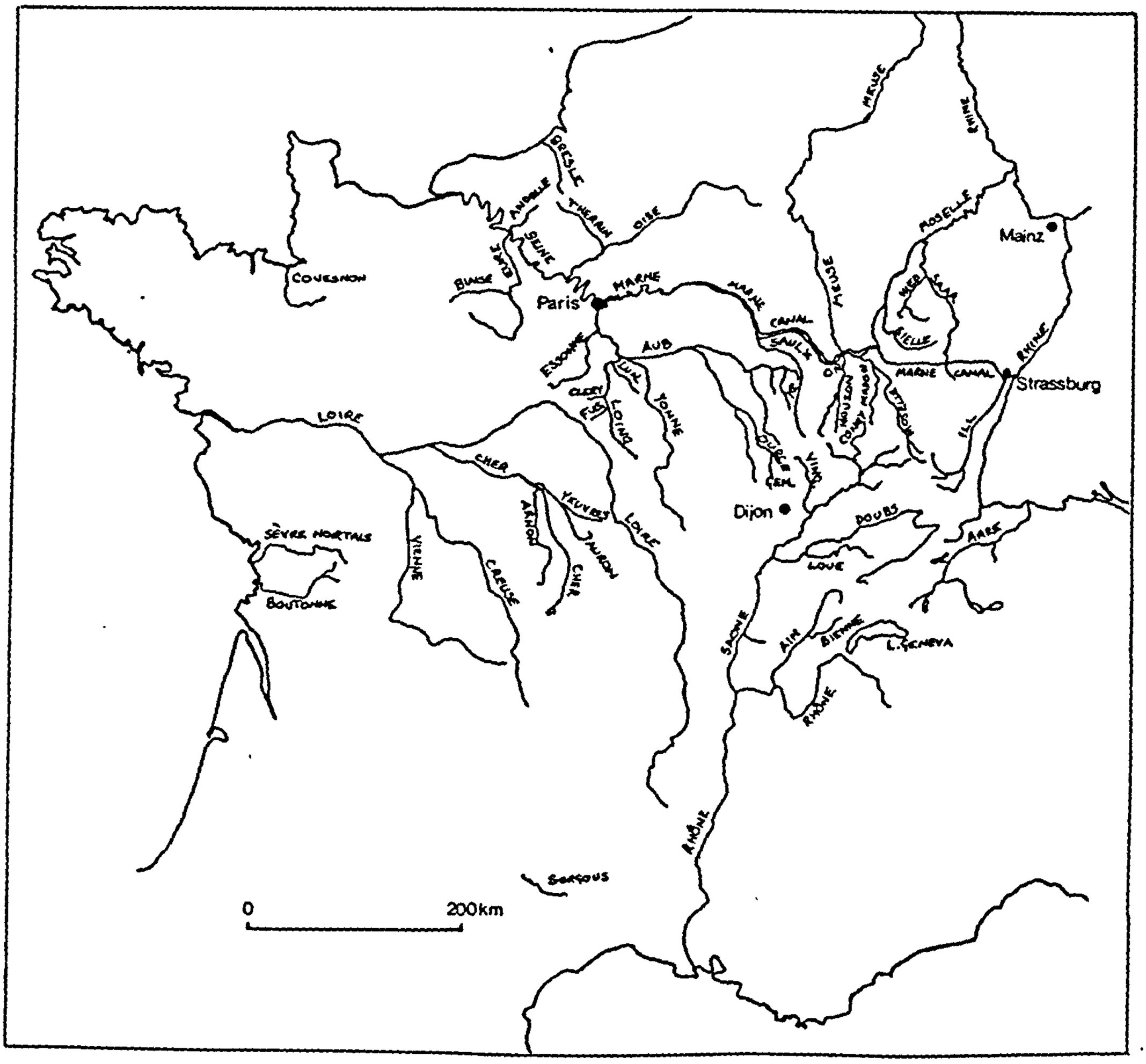

BRESLE
ANCRE
SOMME
THERAIN
OISE
COUESNON
EURE
BIEVRE
Paris
MARNE
MEUSE
MOSELLE
Mainz
NIED
SARRE
RHINE
CANAL
SAULX
AUB
ESSONNE
LOING
YONNE
LOIRE
CHER
YEVRES
ARNON
Strassburg
ILL
Dijon
DOUBS
AARE
LOUE
SAONE
AIN
BIENNE
L. GENEVA
RHONE
SEVRE NORTAIS
BOUTONNE
VIENNE
CREUSE
0
200km

Figure 3. Northern Central Europe.

This figure shows the rivers of Northern Central Europe for which crayfish mortalities are included in this report.

The acetate overlay shows the extent of certain areas whose inclusion on the main map would have been intrusive.

Ang = Angermunde

B = Beskow

L = Lubben

Gm = Gmunden

Dt Krone = Deutche Krone

Lb = Lubjana

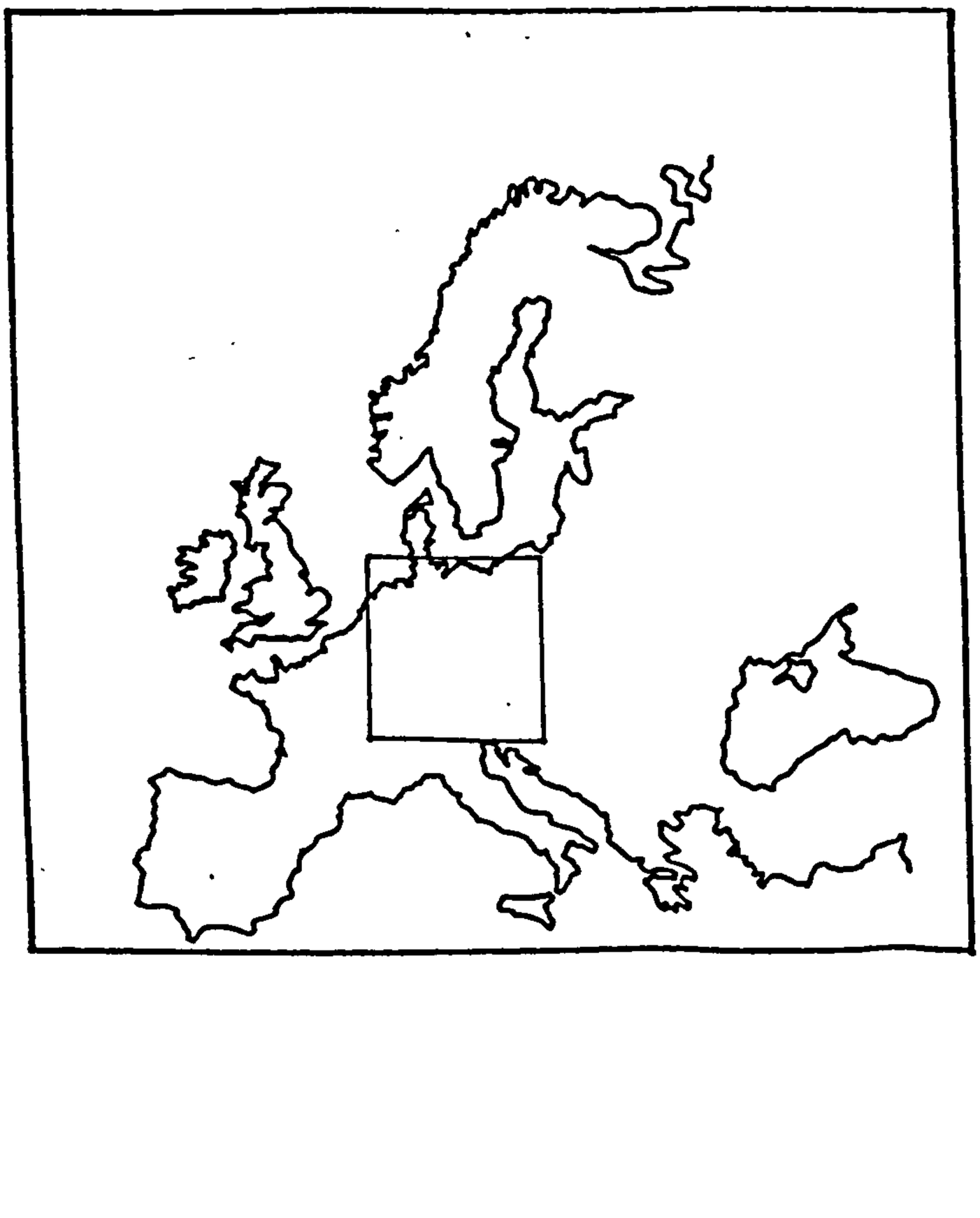
Em = Emskuchen on the R. Aurach

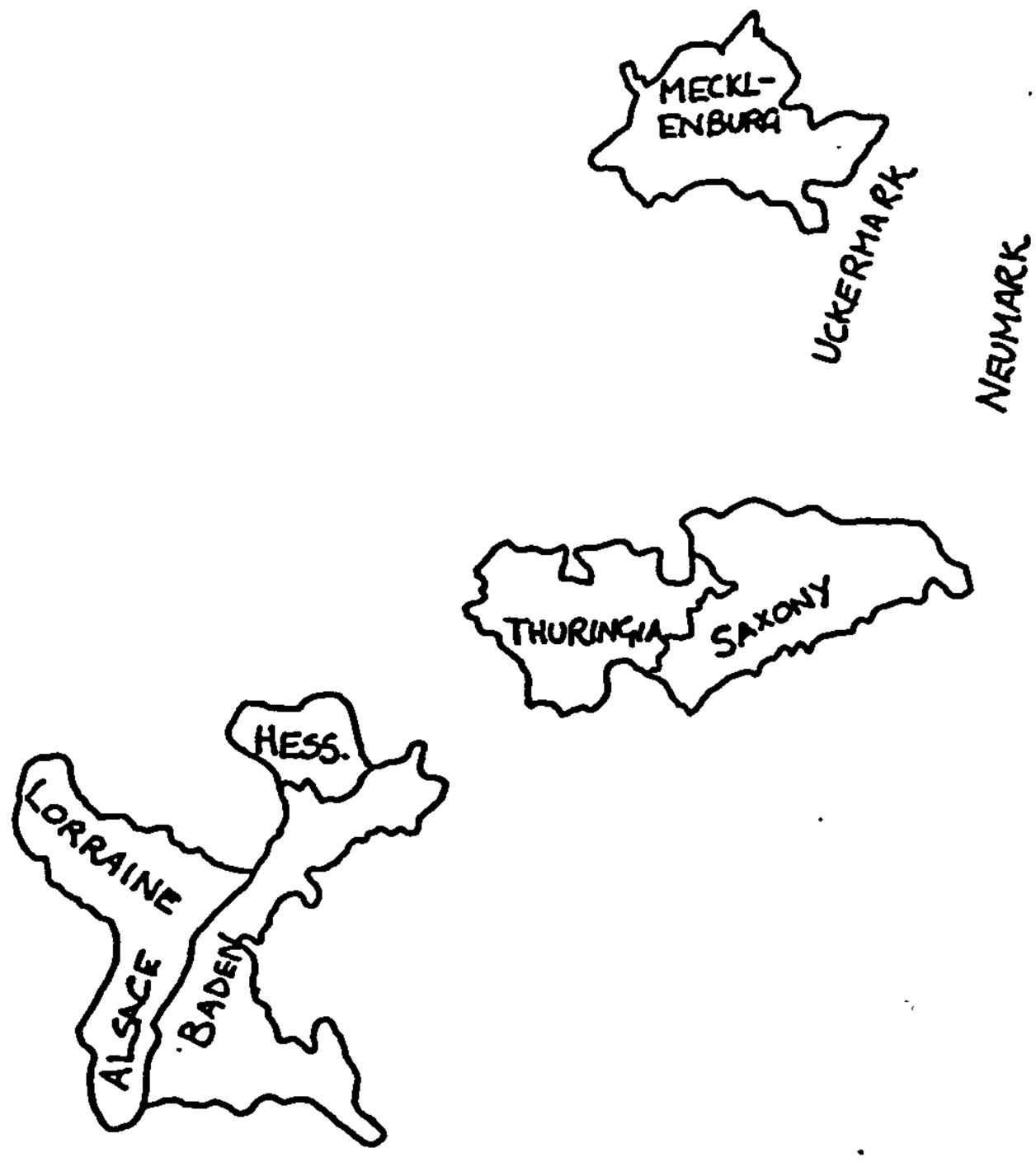
C = Cernembl

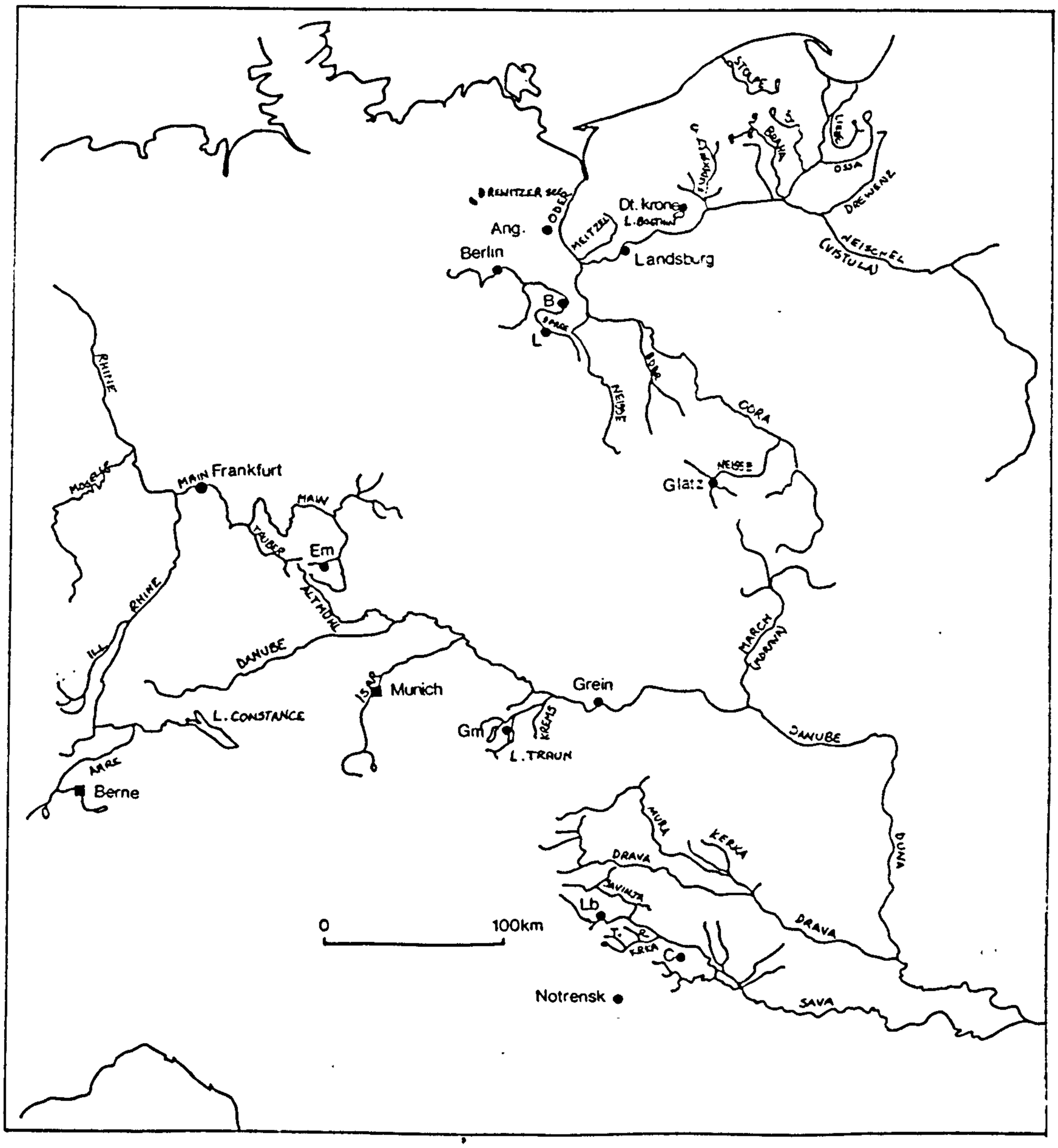
S. = R. Schwarzwasser

T = R. Temienka

R = R. Radulbach




MECKL-
ENBURG
UCKERMARK
NEUMARK
THURINGIA
SAXONY
HESS.
LORRAINE
ALSACE
BADEN


STOLPE
OSIA
DREWENZ
NEISCHEL
LUBSTULA
RENITZER SEE
ODER
Dt. krone
L. BOCHOW
Ang.
MEITZEL
Berlin
Landsburg
B
L
NEISSE
BOBR
QUORA
Glatz
MARCH (THAYA)
RHINE
MOSELLE
MAIN
Frankfurt
MAIN
TAUBER
Em
ALTMUHL
ILL
DANUBE
ISAR
Munich
L. CONSTANCE
Grein
Gm
KREMS
L. TRAUN
DANUBE
AARE
Berne
MURA
KERKA
DRAVA
SAVINJA
DRAVA
DUNA
Lb
SAVA
Notrensk
0
100km

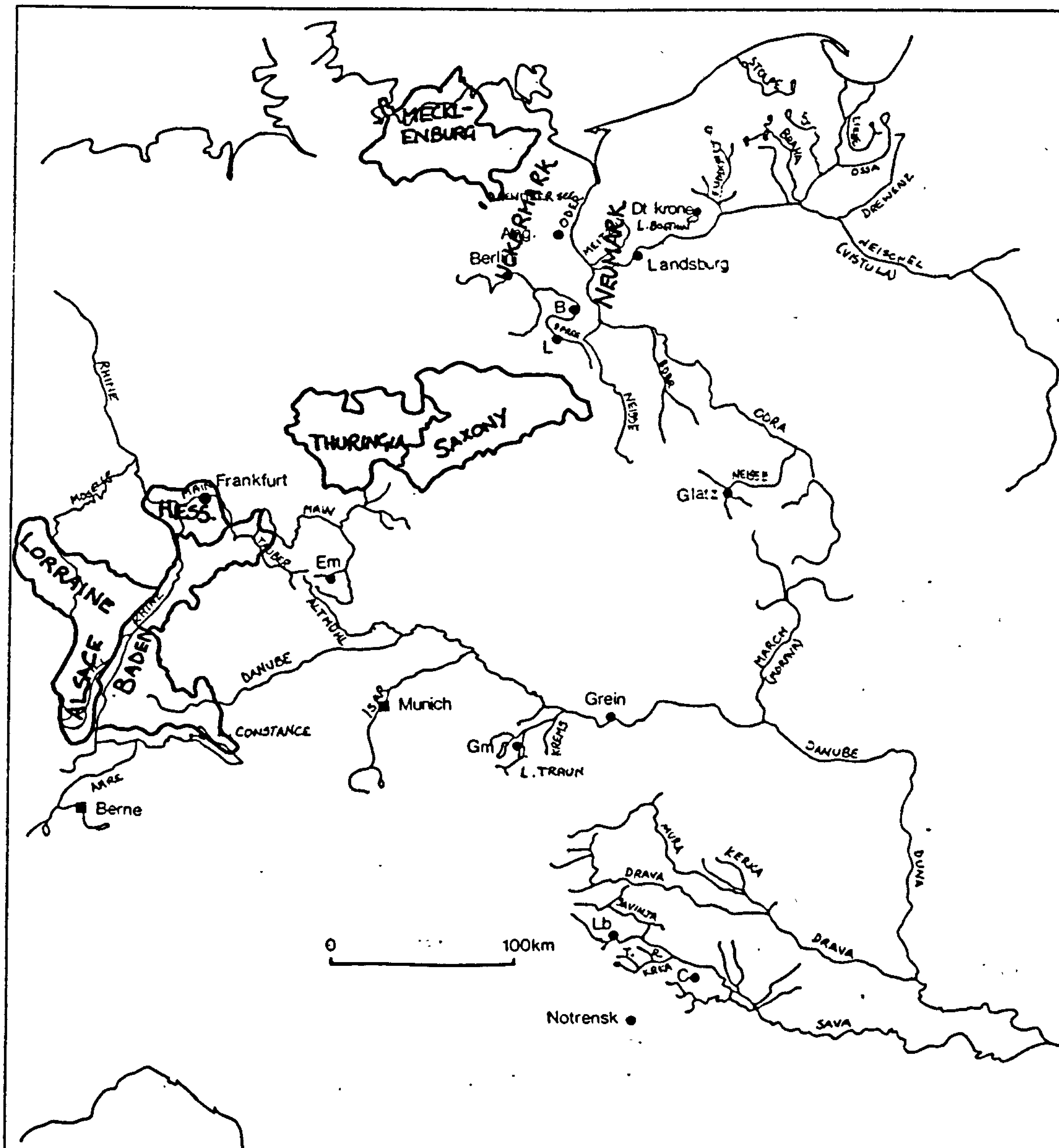
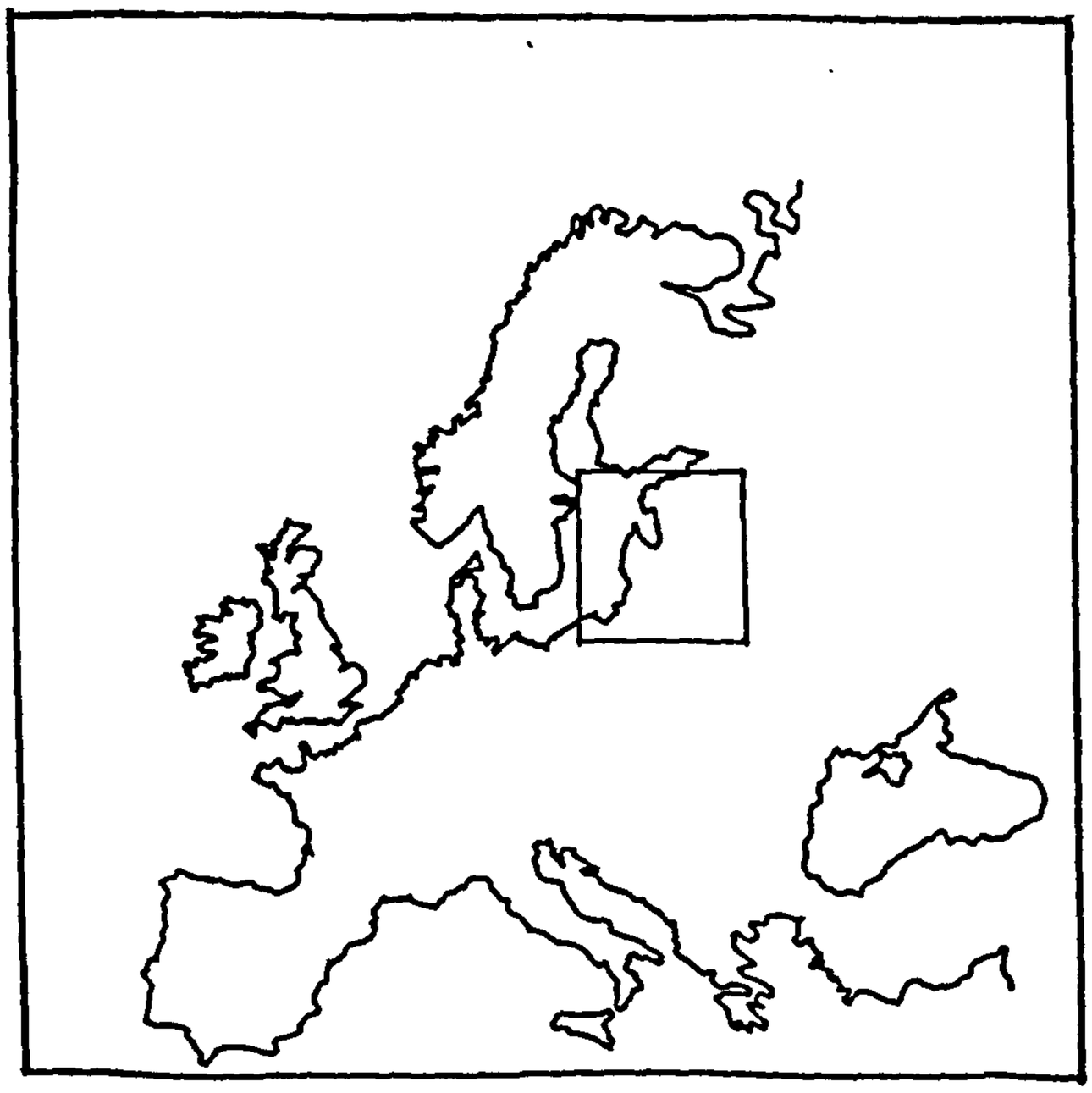

MECKL-
ENBURG
UCKERMARK
ODER
Ang.
Dt krone
Landsburg
NEUMARK
B
L
STOLPE
OSSA
DREWENZ
(VISTULA)
BOBR
NEISSE
Glatz
THURINGIA
SAXONY
Frankfurt
MAIN
HESS.
MOSELLE
RHINE
LORRAINE
ALSACE
BADEN
TAUBER
Em
ALTMUHL
DANUBE
CONSTANCE
AARE
Berne
ISAR
Munich
Gm
L. TRAUN
KREMS
Grein
MARCH
(MORAVA)
DANUBE
DUNA
MURA
KERKA
DRAVA
SAVINJA
Lb
KRKA
C
Notrensk
SAVA
0
100km

Figure 4. The Baltic States.

This figure shows the rivers and lakes of the Baltic States for which crayfish mortalities are included in this report.

1. Waddin See W. = R. Waidau
2. Wedder See
3. Sallain See
4. Inser See
5. Marienburger See

A number of the rivers and lakes mentioned in reports of crayfish mortalities from the Baltic States do not appear on this figure, since they have not been identified.



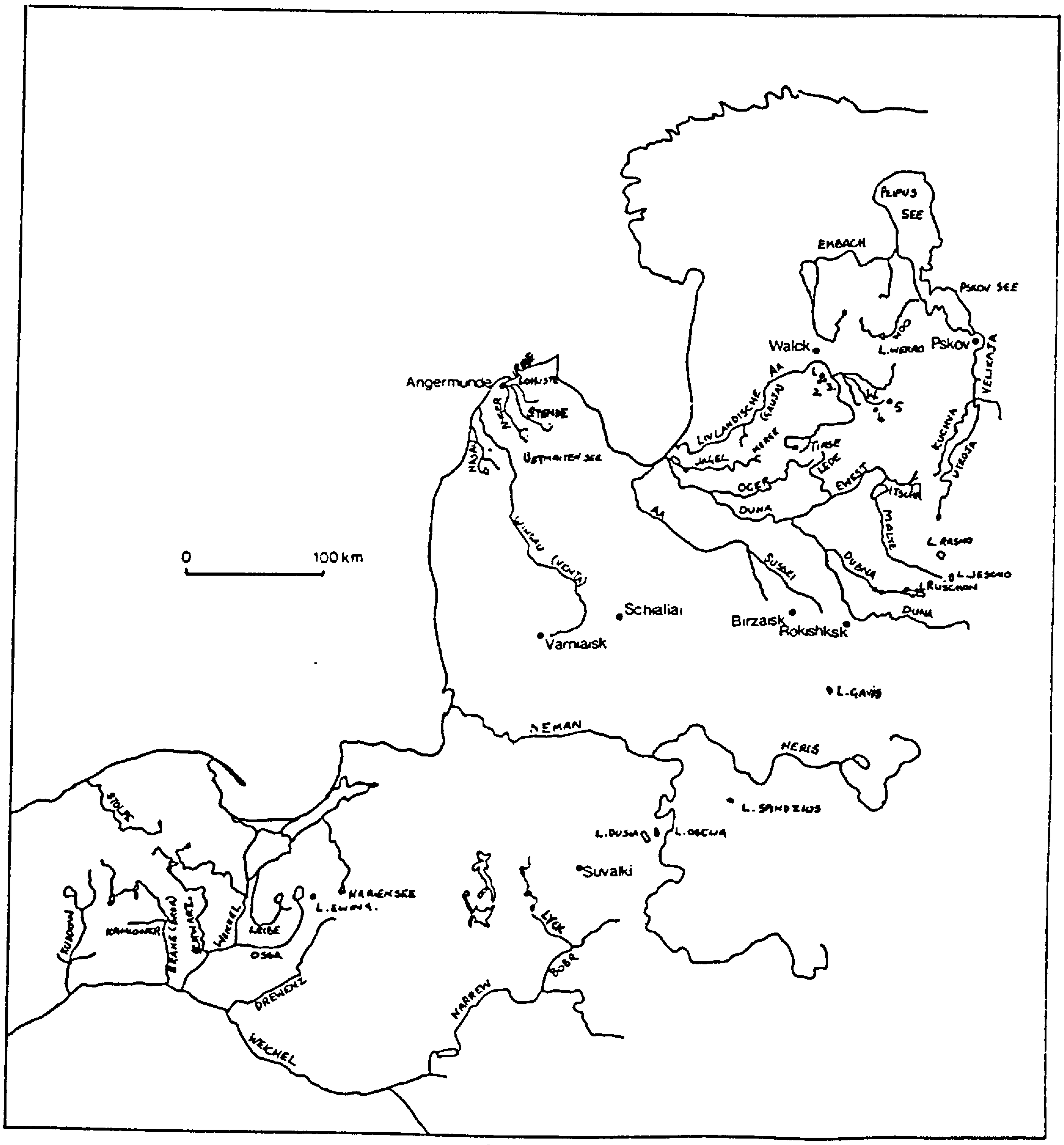
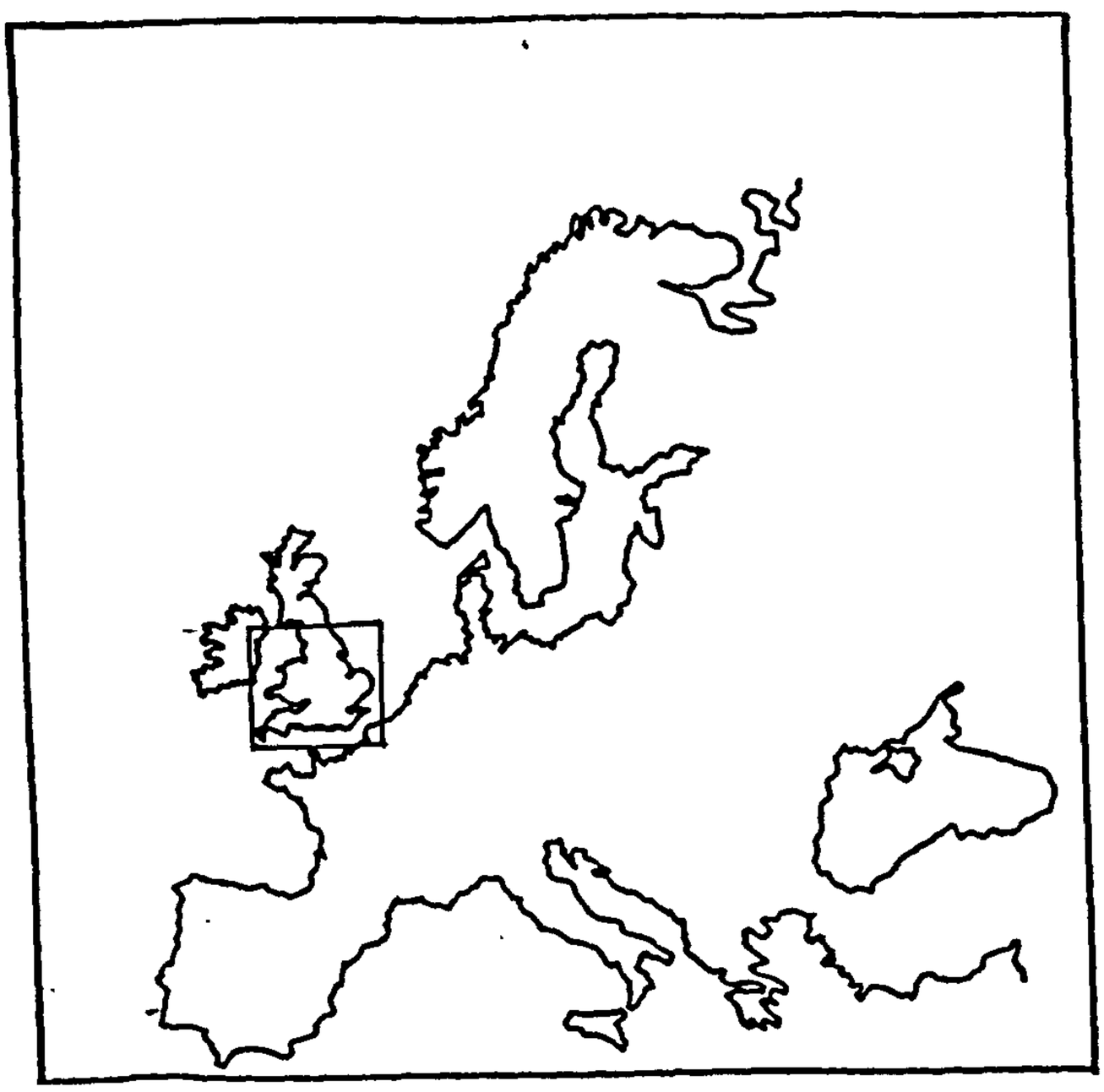

0
100 km
Angermunde
STENDE
WINDAU (VENTA)
Varniaisk
Schialiai
Birzaisk
Rokishksk
Walck
Pskov
PEIPUS SEE
EMBACH
PSKOV SEE
L.WERRO
VELIKAJA
LIVLANDISCHE
AA
(GAUJA)
TIRSE
LEDE
EWEST
OGER
DUNA
SUSSEI
DUBNA
MALTE
L.RASNO
L.JESCHO
L.GAVIS
NEMAN
NERIS
L.SANDZIUS
Suvalki
LYCK
BOBR
NARREW
WEICHEL
DREWENZ
OSSA
LEIBE
STOLPE

Figure 5. Great Britain.

This figure shows the river systems of Great Britain that have been affected by crayfish plague, as well as some rivers that have suffered crayfish mortalities possibly attributable to the disease. The occurrence of crayfish plague in the tributaries of the River Lea is discussed in chapter two.

B. Avon = Bristol Avon
T = Tetbury branch
S = Sherston branch
H. Avon = Hampshire Avon
E. = River Eamont
MIS. = River Misbourne
CH. = River Chess
Gd. = River Gade
SB = Summerhouse Brook
W = River Whitewater



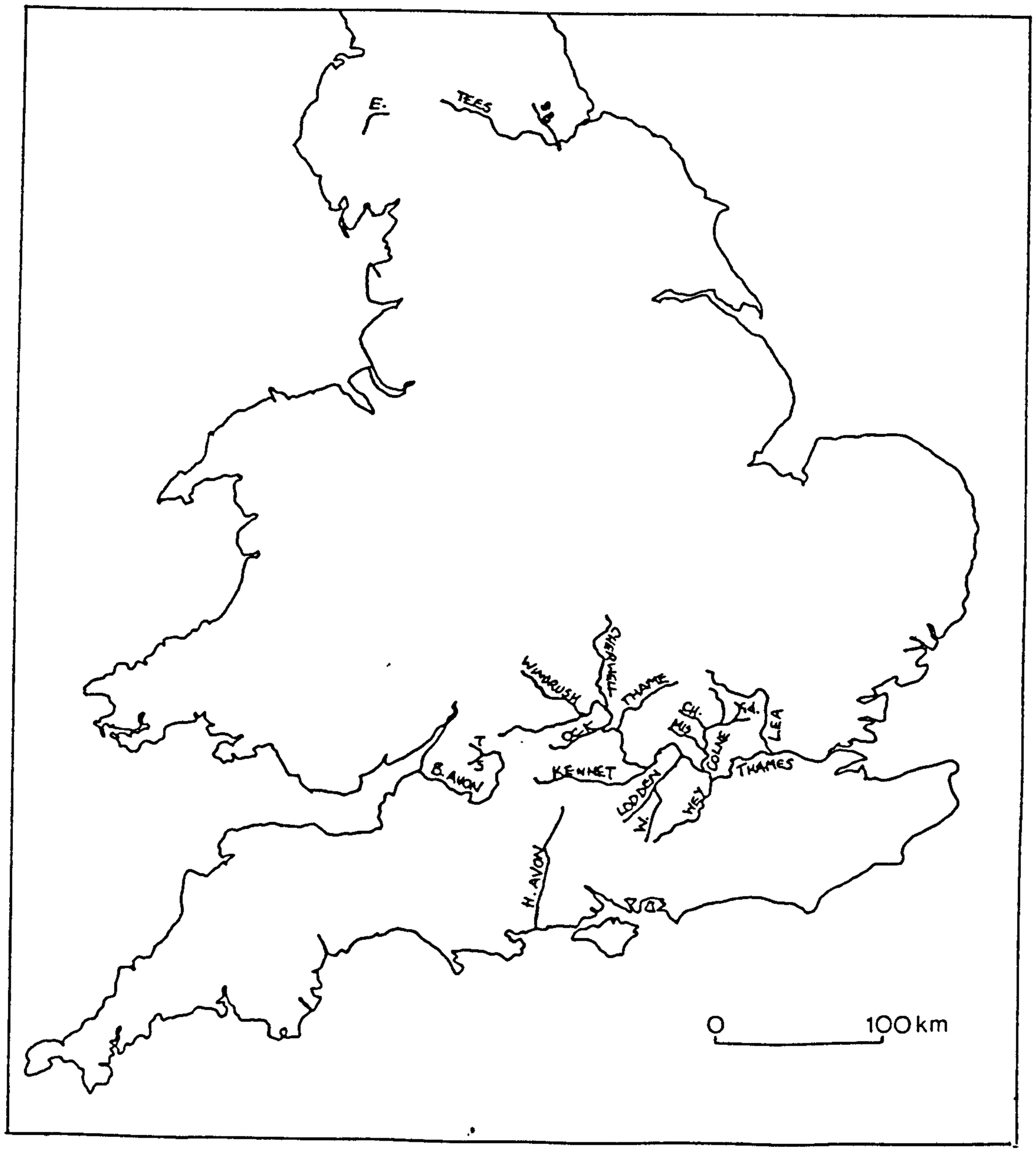
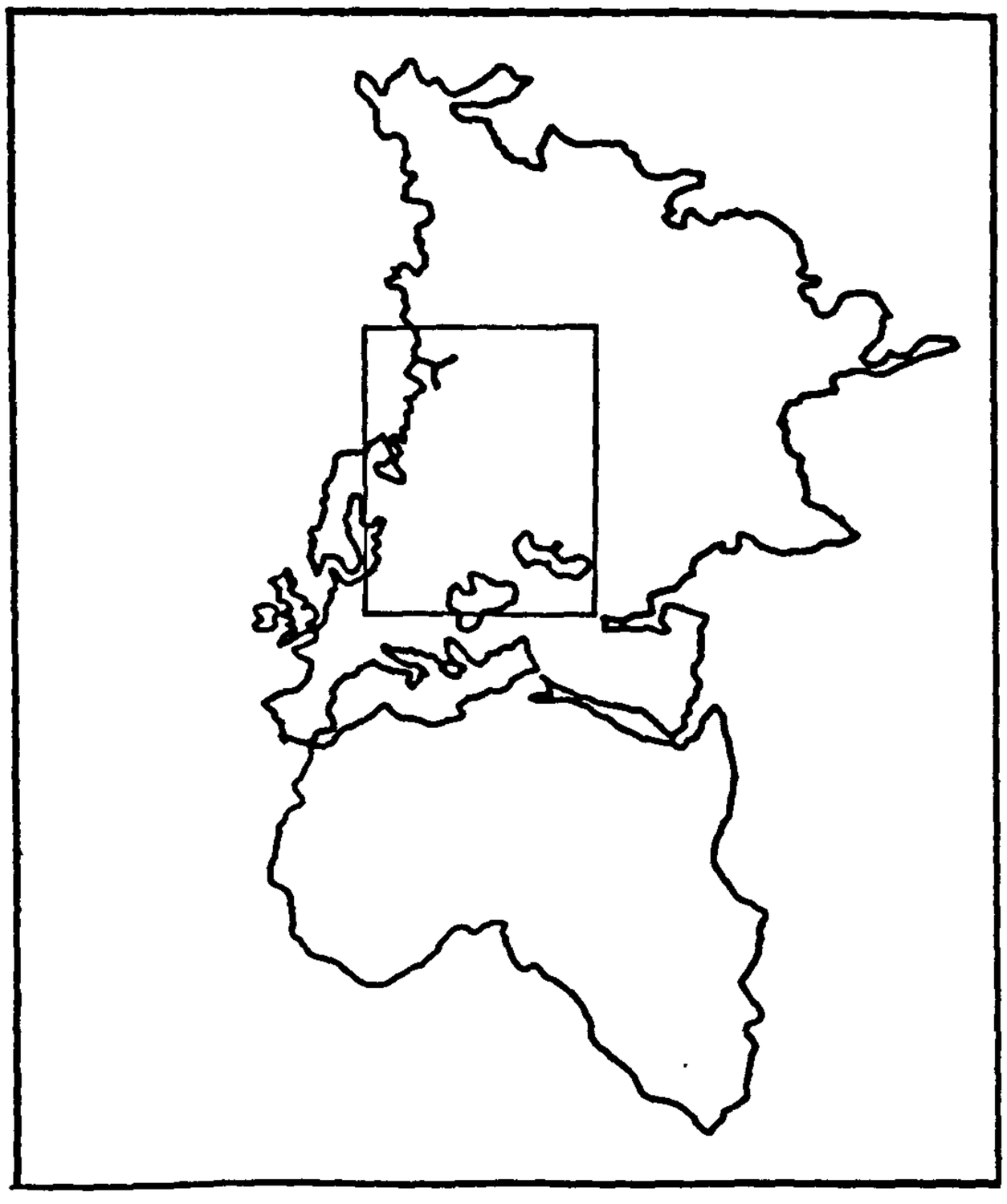

E.
TEES
CHERWELL
WINDRUSH
THAME
OCK
LEE CH.
LEA
COLNE
LEA
T.
S
B. AVON
KENNET
LODDON
W.
WEY
THAMES
H. AVON
STOUR
0
100km

Figure 6. Eastern Europe and the USSR.

This figure shows the rivers of Eastern Europe and the USSR for which crayfish mortalities are included in this report.

Bi = Birzaisk

Tr = Trakaisk



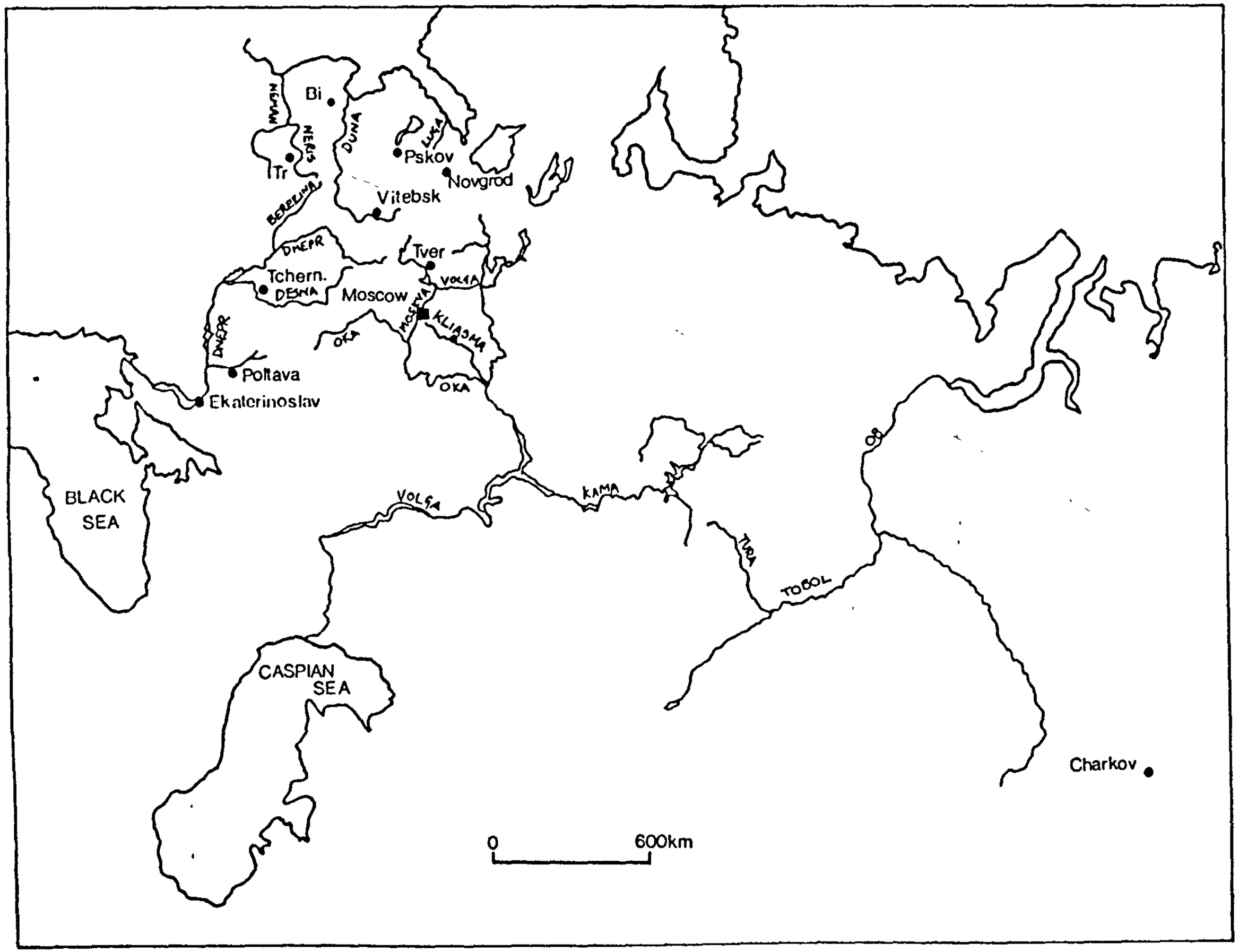
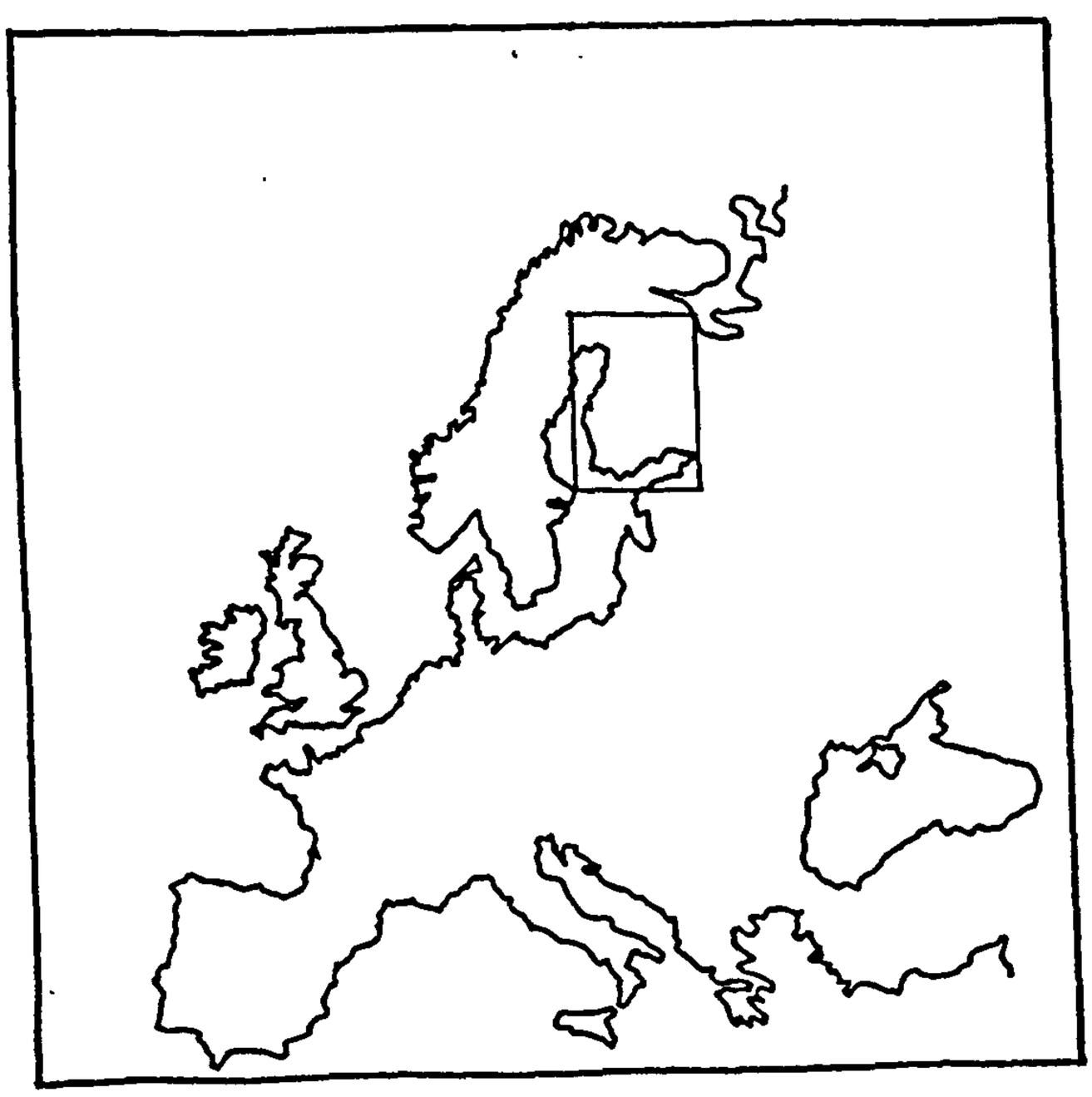

Bi
NEMAN
NEBIS
Tr
DUNA
LUKA
Pskov
Novgrod
Vitebsk
BEREZINA
DNEPR
Tver
Tchern.
DESNA
VOLGA
Moscow
MOSKVA
KLIAZMA
OKA
DNEPR
Poltava
Ekaterinoslav
OKA
BLACK
SEA
VOLGA
KAMA
TURA
TOBOL
OB
CASPIAN
SEA
Charkov
0
600km

Figure 7. Finland.

This figure shows the river systems and lakes of Finland for which crayfish mortalities are included in this report. For simplicity only the major water coures are shown.



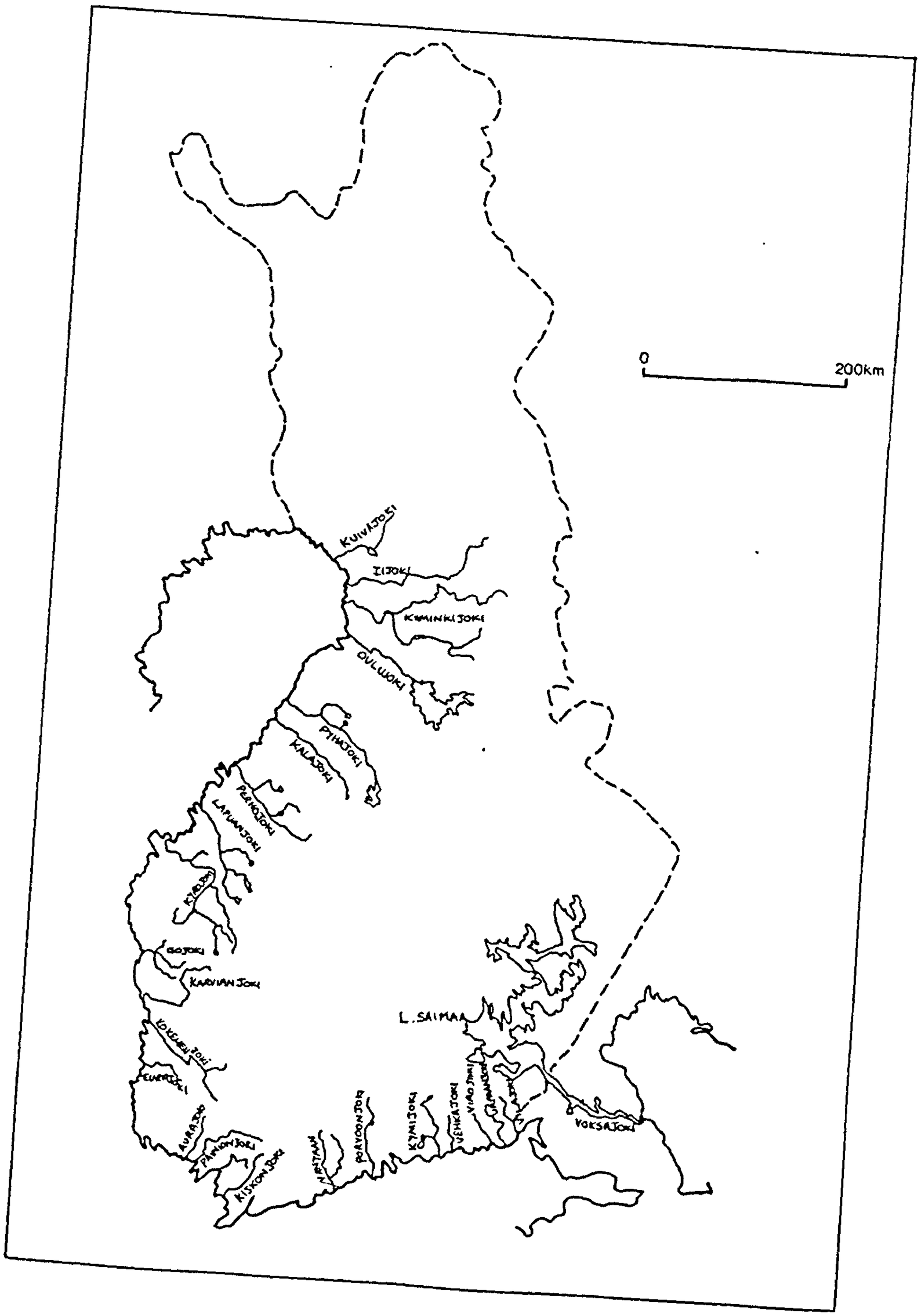
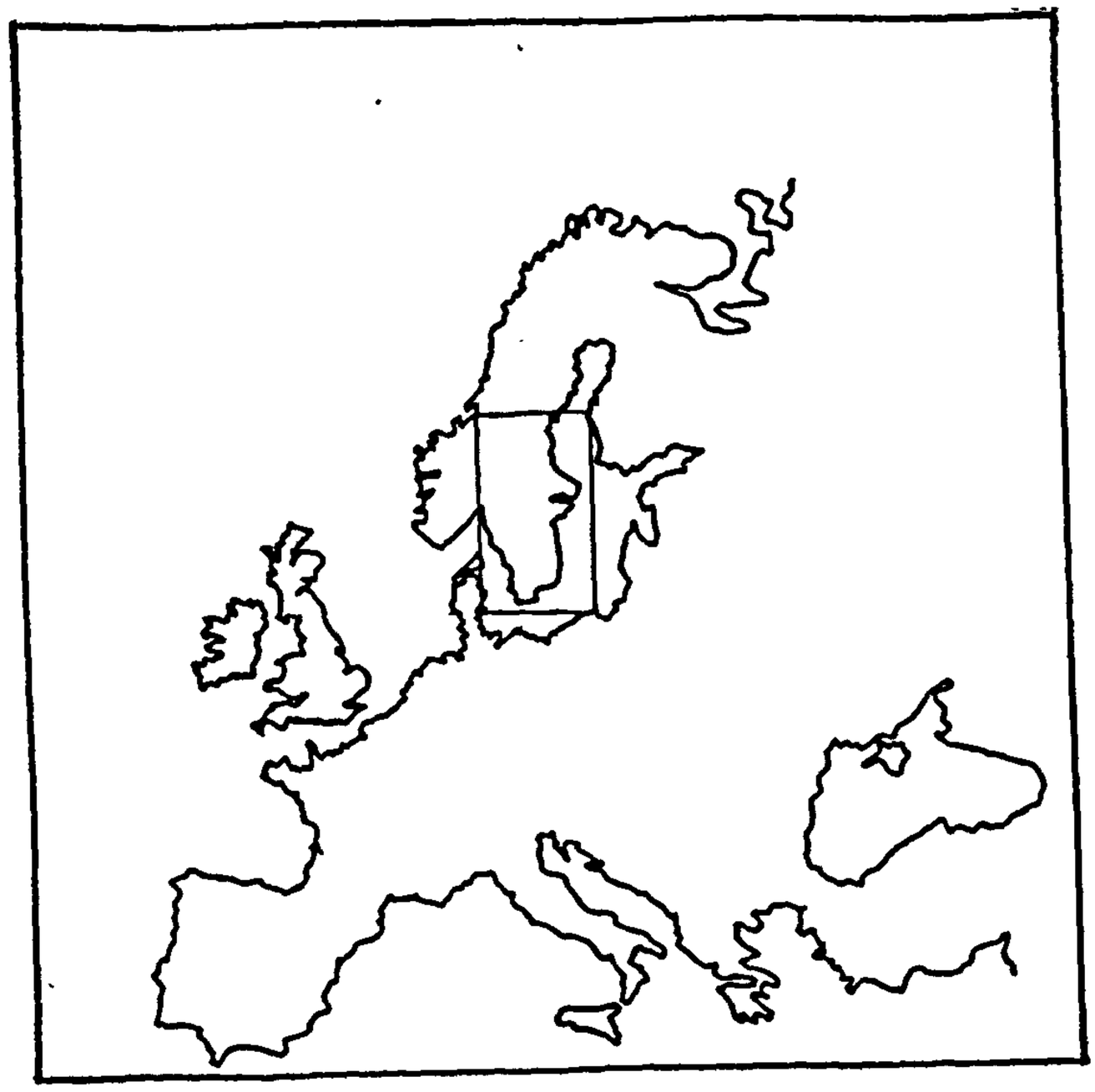

0
200km
KUIVAJOKI
IIJOKI
KUUSINKIJOKI
OULUJOKI
PYHÄJOKI
KALAJOKI
PERHOJOKI
LAPUANJOKI
KYRÖJOKI
OULUJOKI
KAROJANJOKI
L. SAIMAA
VOKSAJOKI
KYMIJOKI
VEHKAJOKI
PORVOONJOKI
KISKONJOKI
PÄRNÄJOKI
AURAJOKI

Figure 8. Southern Sweden.

This figure shows rivers and lakes of Southern Sweden for which crayfish mortalities are included in this report.



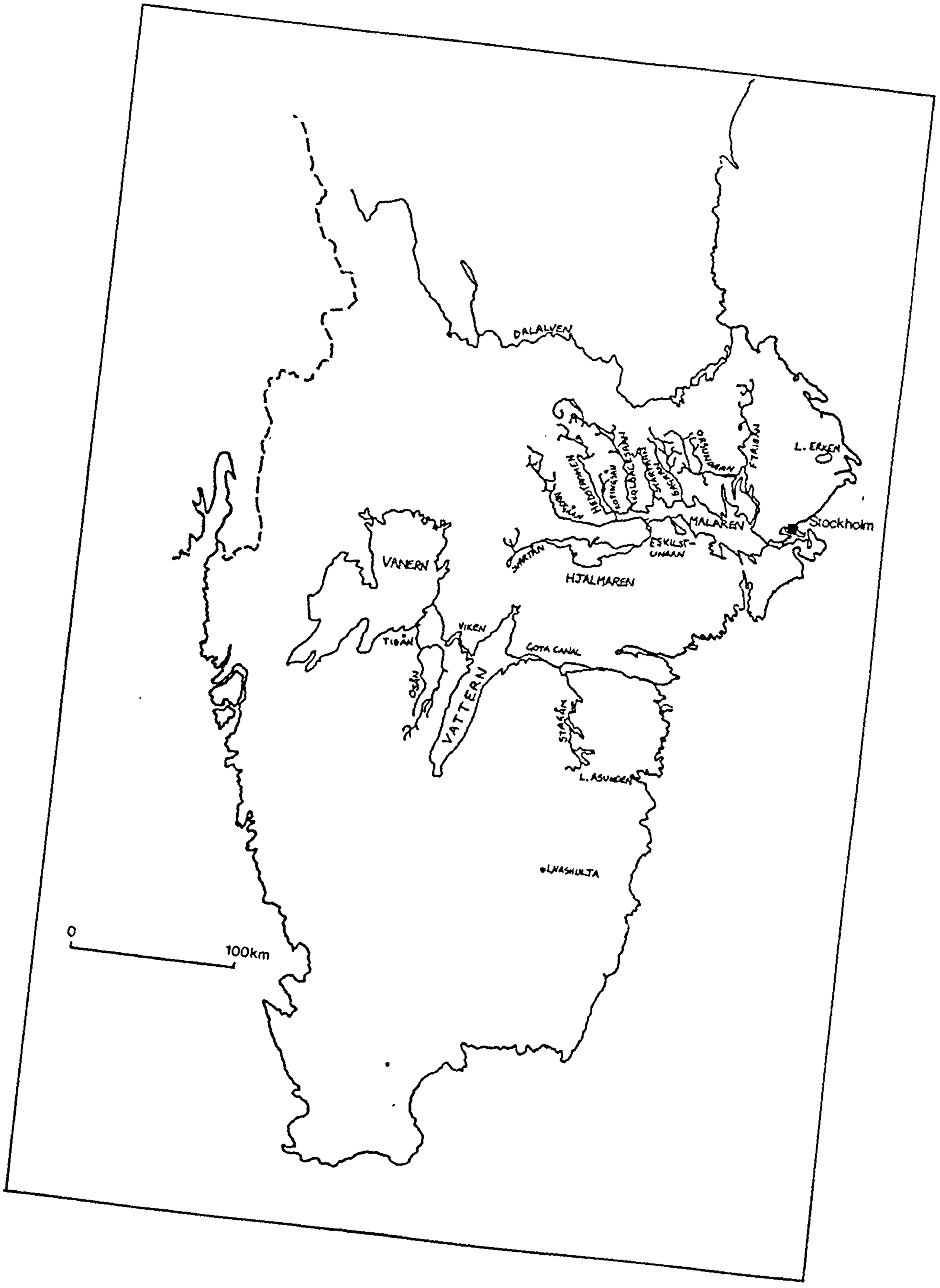
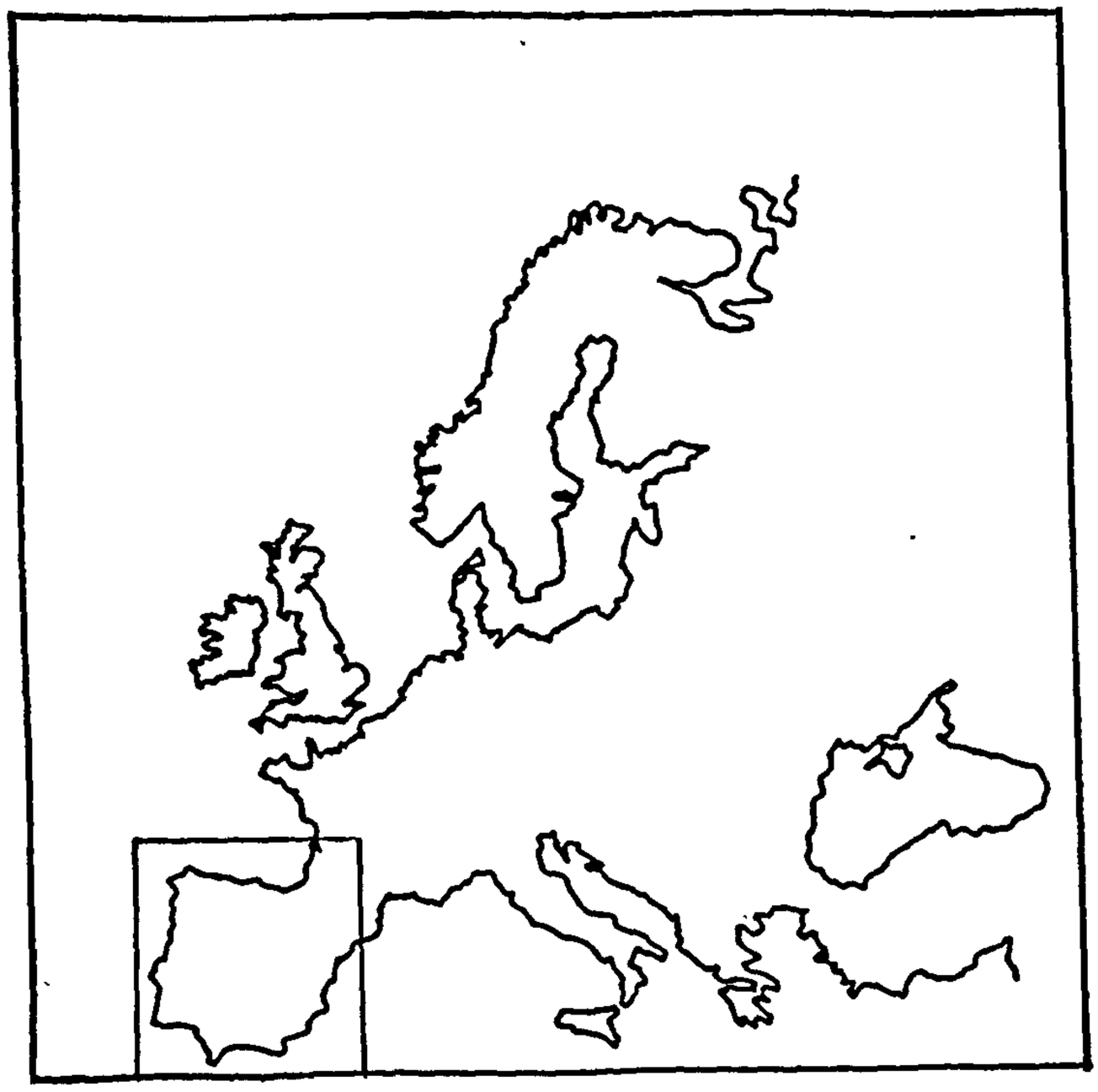

DALALVEN
L. ERKEN
ÖRSUNDAN
FYRISÅN
MÄLAREN
Stockholm
ESKILST-UNAAN
HJALMAREN
VÄNERN
TIDAN
VIKEN
GÖTA CANAL
ÖSAN
VÄTTERN
STRÖMMEN
L. ASUNDEN
LNASHULTA
0
100km

Figure 9. Spain.

This figure shows the major river systems of Spain for which crayfish mortalities are included in this report.



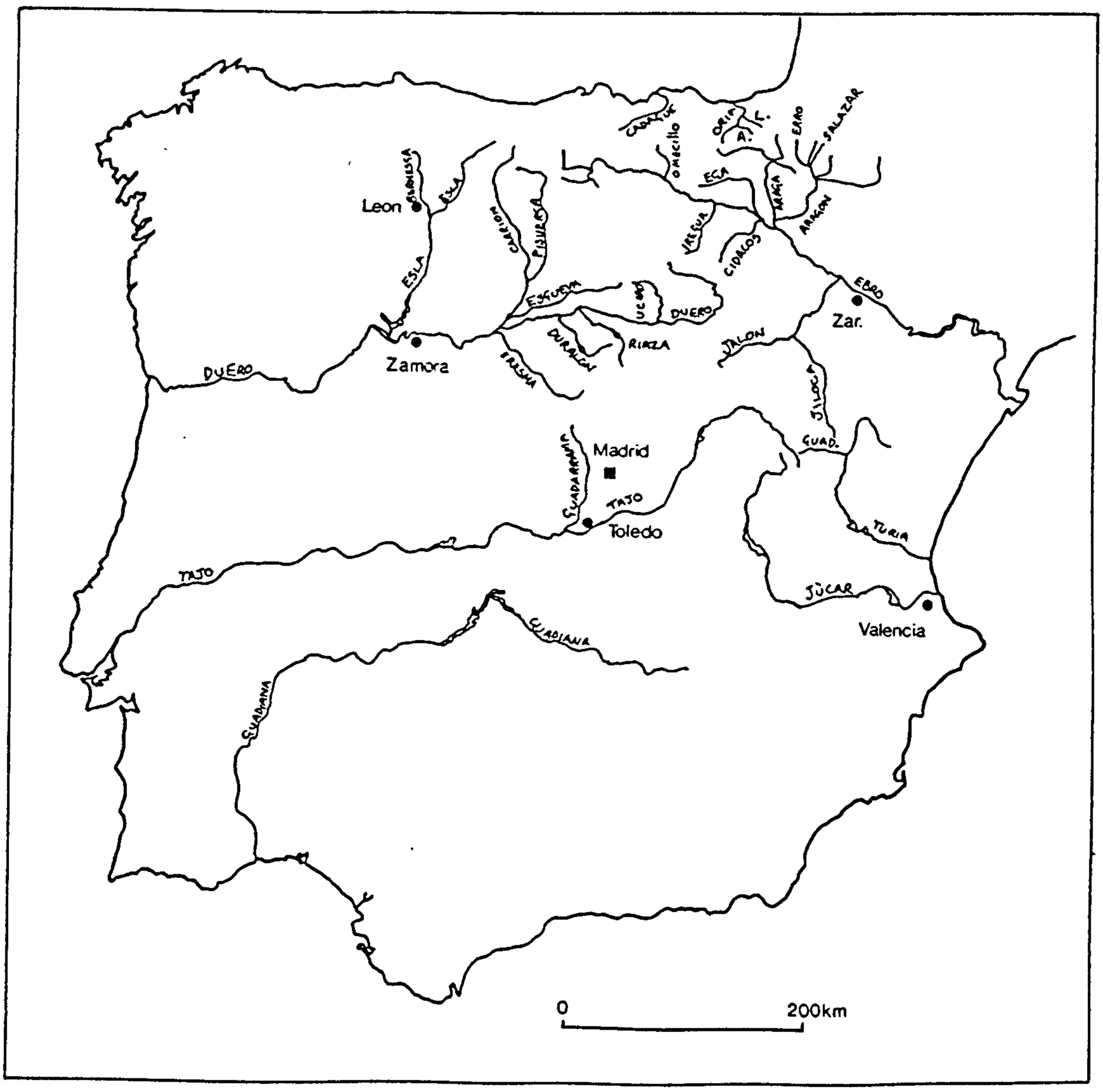
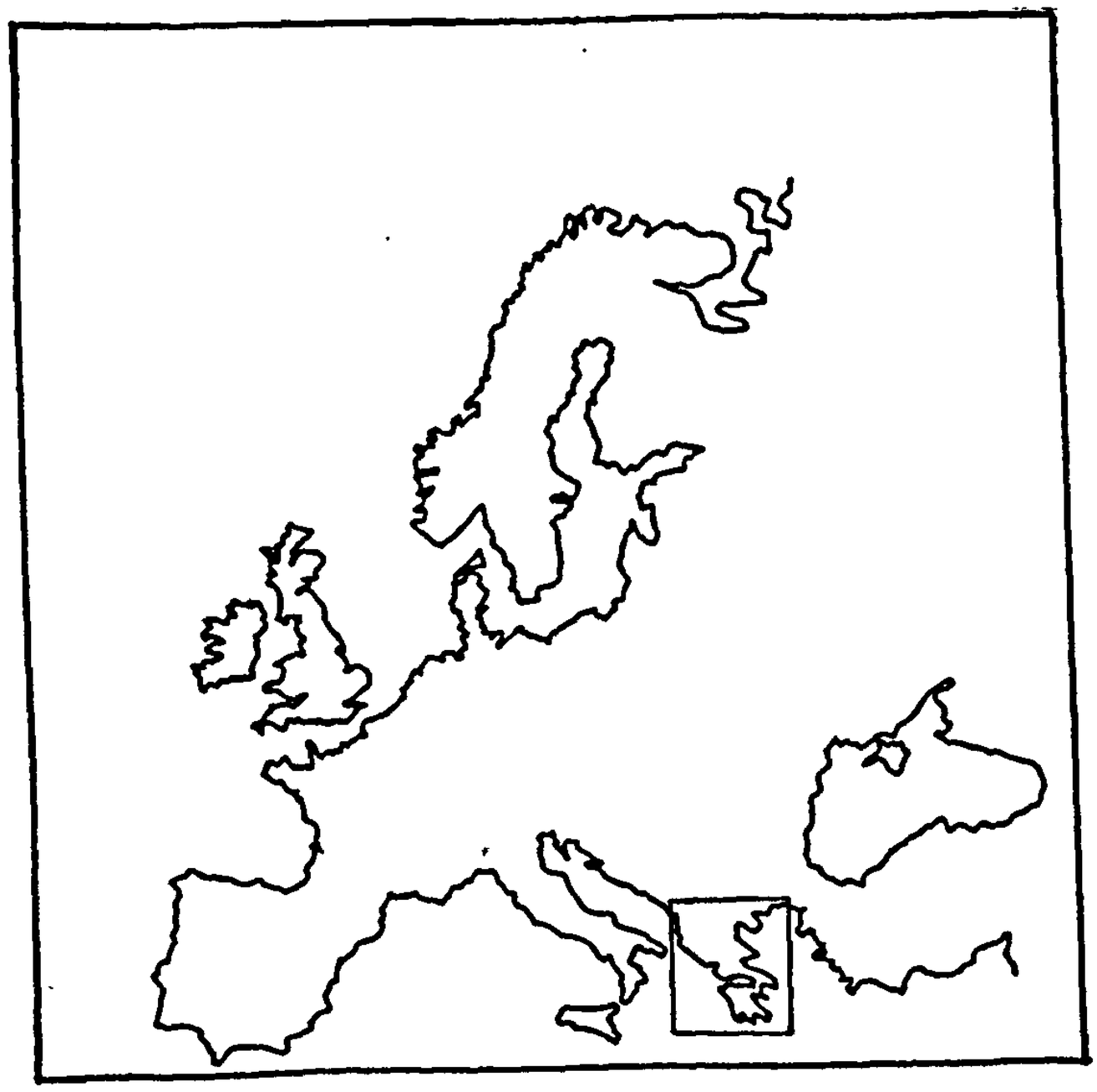
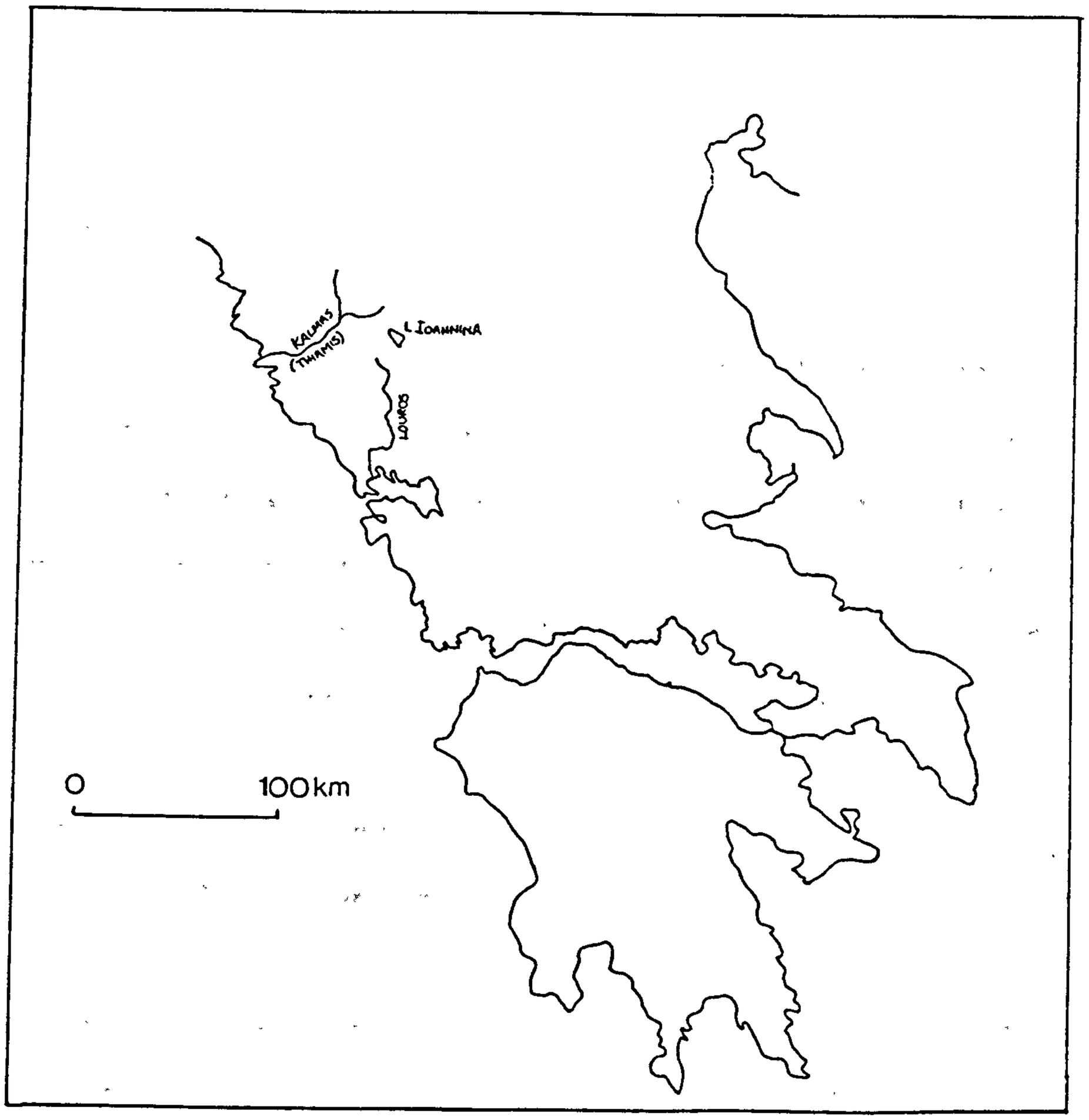

Leon
ESLA
ESLA
CARRION
PISUERGA
ESLA
ESGUEVA
UCERO
DUERO
DURATON
RIAZA
Zamora
DUERO
TERA
ORBIGO
ORNECILLO
ORIA
A.
EGA
ARAGA
EBRO
SALAZAR
ARAGON
VERGUA
CIDACOS
EBRO
Zar.
JALON
JILOCA
GUAD.
GUADARRAMA
Madrid
TAJO
Toledo
TAJO
GUADIANA
GUADIANA
TURIA
JÚCAR
Valencia
0
200km

Figure 10. Southern Greece.

This figure shows the two rivers and one lake in Greece that are known to have been infected with Aphanomyces astaci up to the present date.




KALAMAS
(THAMIS)
L IOANNINA
ARACHTHOS
0
100km

THE SPREAD OF CRAYFISH PLAGUE AND THE DISTRIBUTION OF RESIDUAL CRAYFISH STOCKS IN THE RIVER LEA AND ITS TRIBUTARIES.

INTRODUCTION.

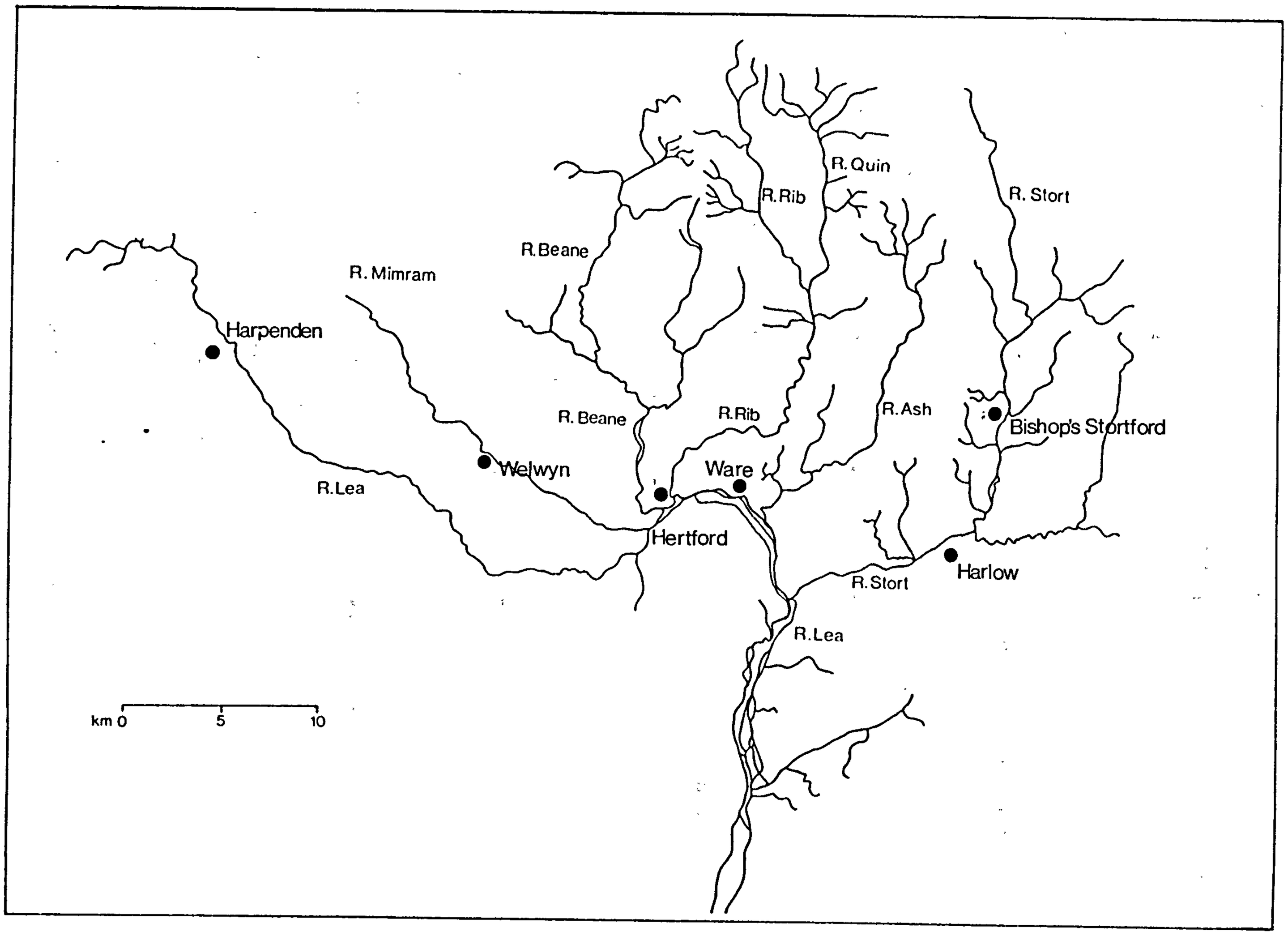
The River Lea catchment system consists of seven rivers, the Mimram, the Beane, the Rib, the Quinn, the Ash, the Stort and the River Lea itself, as well as a number of smaller tributaries (figure 1.). Until recently, the River Lea catchment has been a productive system as far as crayfish are concerned. Many riparian owners have reported large crayfish catchs in the past and have noticed the recent decline of populations.

The first mass mortality of crayfish was observed in July 1981 at Ware Lock on the River Lea itself, at this time the cause was undiagnosed. Since then the cause has been identified as infection with the oomycete fungus Aphanomyces astaci.

Since 1981 the populations of crayfish in this system have been monitored. This study is a continuation of that monitoring and aims to chart the spread of the disease through the river system and ultimately to identify and monitor any residual populations of Austropotamobius pallipes.

This report details the work carried out by previous workers (Deardsley pers. com., Lowery pers. com. and Southgate pers. com.) in addition to the results of

Figure 1. The River Lea and its tributaries.


R. Mimram
Harpenden
R. Beane
R. Rib
R. Quin
R. Stort
R. Ash
Bishop's Stortford
Welwyn
Ware
R. Lea
Hertford
Harlow
R. Stort
R. Lea
km 0
5
10

the present study, in order that a complete report of the effect of crayfish plague on the crayfish of the River Lea system could be presented.

METHODS.

The study was carried out in the spring, summer and autumn$_{\wedge}$ (1984–1987) using netting and trapping techniques in conjunction with caging experiments in order to test for the continuing presence of the disease.

1. Caging experiments.

Ten animals of mixed sex, were caged for a period of time in the river in question. Cages were made of quarter inch galvanized chicken wire and were 1m x 1m x 0.5m. The cages contained a number of bricks or flower pots as hides, and vegetation as food for the animals. The cage was secured in place by a rope tied to a tree or anchored to a heavy metal rod. The health of the animals was monitored and if any showed symptoms of Aph. astaci infection all animals were returned to the laboratory where$_{\wedge}$ they were observed. Any animals dying subsequently were subjected to post mortum examination and attempts were made to isolate the pathogen from them.

2. Post mortum and isolation techniques.

Dead animals were examined for external symptoms of Aph. astaci infection, such as brown or creamy discolourations of the soft cuticle, or whitening of the

eyes. Small pieces of arthrodial membrane, especially from the ventral abdomen, were excised and examined under the microscope for signs of fungal hyphae.

Pieces of soft cuticle approximately 1 mm square were removed aseptically, especially from discoloured areas, ventral abdomen and proximal leg articulations, and incubated on either Unestam's glucose peptone agar (GP agar, Appendix 3) or on river water agar (RW agar, Appendix 4). Both media incorporated 10 ppm. oxolinic acid to supress bacteria.

Isolation of the fungus was carried out according to Alderman and Polglase (1986). Small pieces of other tissues such as nerve cord, muscle and the eyes were also removed and incubated in a similar manner. After 12 to 48 hrs at 20°C it was possible to excise small pieces of agar from the growing edge of fungal colonies. These were then incubated over night in sterile distilled water and the sporulation pattern of the isolate was noted. Motile zoospores of isolates with Aphanomyces-like sporulation pattern were plated out on either GP or RW agar with 10 ppm oxolinic acid and single sporelings removed to separate agar plates.

After 4 - 5 days incubation at 20°C, a number of small pieces of agar from the colony edge were incubated overnight in 50 ml of sterile distilled water at 20°C. The resulting zoospore suspension was then added to an aquarium containing 4 - 6 Astacus leptodactylus in 15 litres of water. The animals were examined daily and dead

or moribund animals removed for post mortum.

If the test animals died showing signs of Aph. astaci infection and the fungus could then be reisolated from them, the original isolate was considered to have satisfied Koch's postulates of a pathogenic organism and was considered to be Aph. astaci.

RESULTS.

The River Lea.

1981

A mortality was first noticed in this system at Ware Lock (TL 353143) on the 19th of June 1981 (Lowery et al 1987, see Figure 2). Two days later animals were found dead downstream at Ware weir (TL 364142) and further downstream at Dobb's weir (TL 384083). Between these two sites, at Rye house (TL 385098), no animals were found. Twelve kilometers further downstream, dead animals were found in the Corn Mill Stream at Waltham Abbey (TL 375005) on the 27th of June. On the 20th of June animals were found dead upstream at Dicker Mill (TL 334134), Hertford (Deardsly pers. com.). Subsequently, many live animals were seen at Hertford Basin (TL 328130) and Hertford Castle (TL 326125). One dead animal was found 2 km up stream from here on the 14th of June.

Subsequent searches upstream from Hertford revealed no animals at Water Hall (TL 298097), Letty Green (TL 283100) and downstream at Pickets Lock (TQ 364940) near Enfield North London.

Figure 2. The River Lea.

This map shows the River Lea and its tributaries between Pickets and the source. The enlargement below shows the area around Ware and Hertford in more detail. S1 and S2 are Signal crayfish introductions near Stanstead Abbotts, exact locations unknown. S3 and S5 are further Signal crayfish introductions (see text).

WH = Water Hall

NS = Newgate Street

1. Hertford Basin

2. Site where a dead crayfish was found in 1981

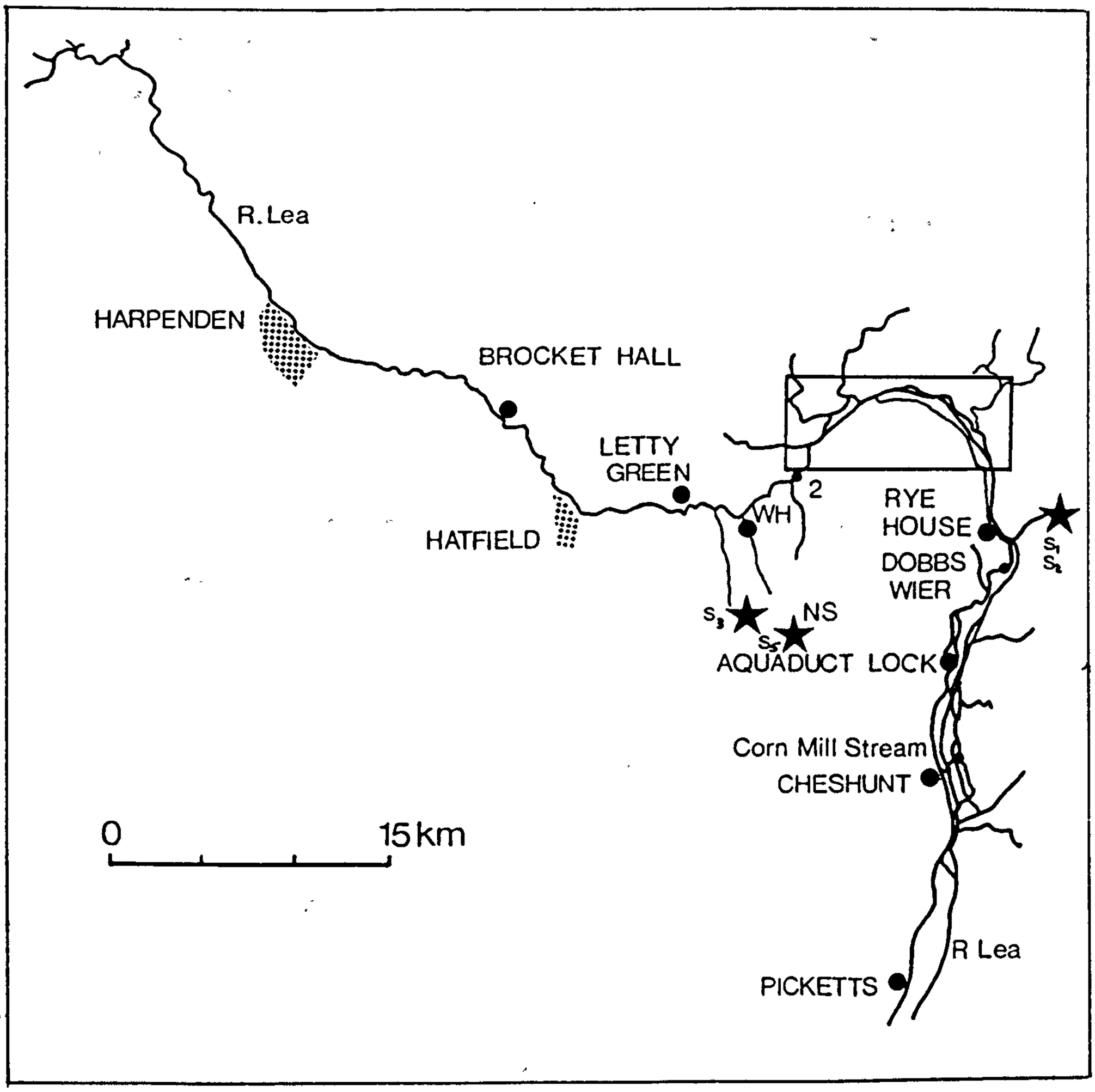
 = Unsubstantiated residual population

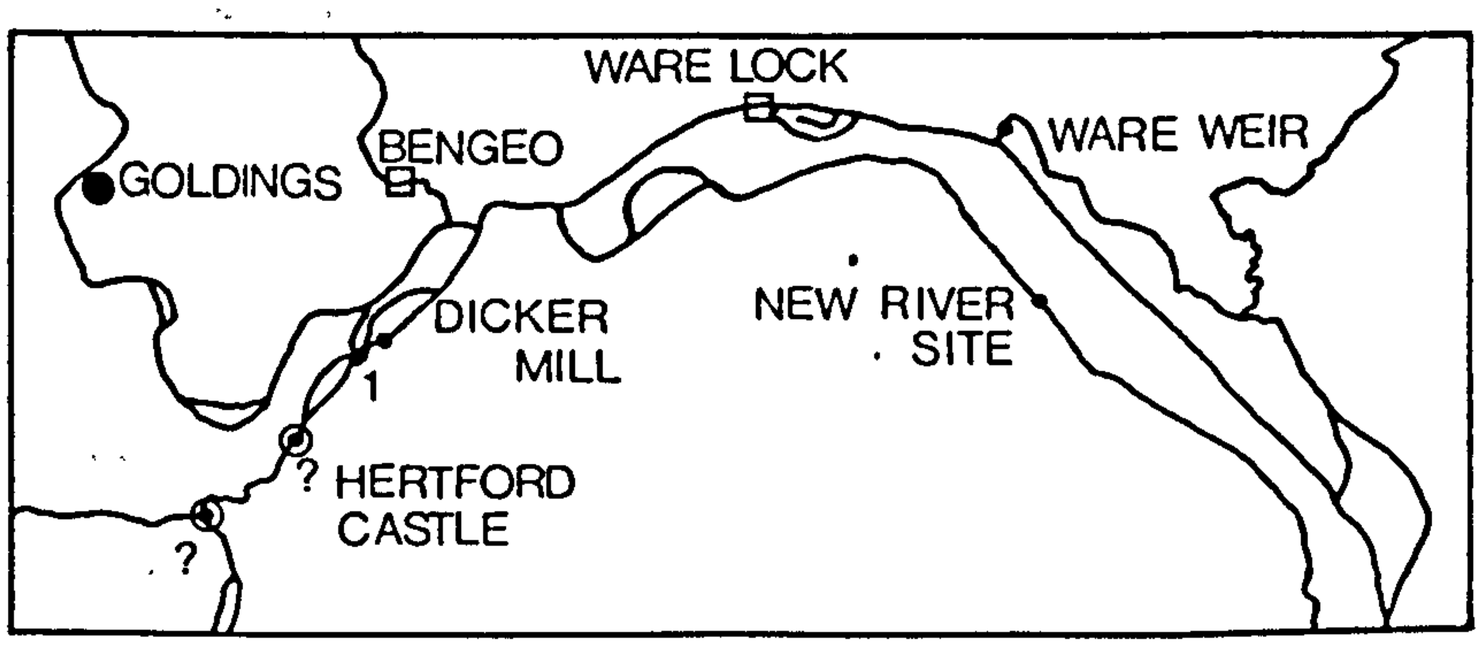
 = Sites where dead crayfish were found.

 = Sites where caging experiments were carried out.

 = Sites of Signal crayfish introductions.

● = Other sites.


R.Lea
HARPENDEN
BROCKET HALL
LETTY GREEN
HATFIELD
WH
2
RYE HOUSE
DOBBS WIER
NS
AQUADUCT LOCK
Corn Mill Stream
CHESHUNT
0
15 km
R Lea
PICKETTS


WARE LOCK
WARE WEIR
BENGEO
GOLDINGS
DICKER MILL
1
NEW RIVER SITE
? HERTFORD CASTLE
?

Healthy animals caged at Ware Lock on the 2nd of September all died within 8 weeks. In a repeat experiment on the 12th of October, at the same site, all the animals again perished. Aph. astaci was not identified in either case.

1982

A search of the River Lea at Hertford basin revealed no live animals, although many were found here the year before.

1983

Animls caged at Ware lock survived for 11 months before being returned to the laboratory. None subsequently died of crayfish plague.

1984

On the 19th of June a search of a gravel pit at Cheshunt (TL 369025) which lies less than 10 meters from the course of the Lea revealed no crayfish although fishermen reported that they had previously been abundant here.

Further searches were made at Harpenden (TL 149148), at Brocket Hall weir (TL 215125) several kilometers downstream and at Hatfield (TL 255098). No crayfish were seen at any of these sites.

1985

Crayfish were reported in the river, at the confluence of the Lea and the Mimram (TL 320122) on the 16th October but the reports were never substantiated.

1986

On the 6th October, members of the public reported catching about 30 crayfish in nets in the River Lea at Hertford (TL 325127). However, trapping on the 8th failed to locate any animals.

The River Mimram.

No crayfish mortalities have been observed in the River Mimram although its confluence with the Lea occurs close to sites where mortalities have been observed. Searches of the river as far upstream as Panshanger (TL 281133) have revealed no animals. In 1983 animals were found in the river near Tewin (TL 278134) and for a distance of 5km upstream. Farther upstream than this, no animals have been found (Figure 3.).

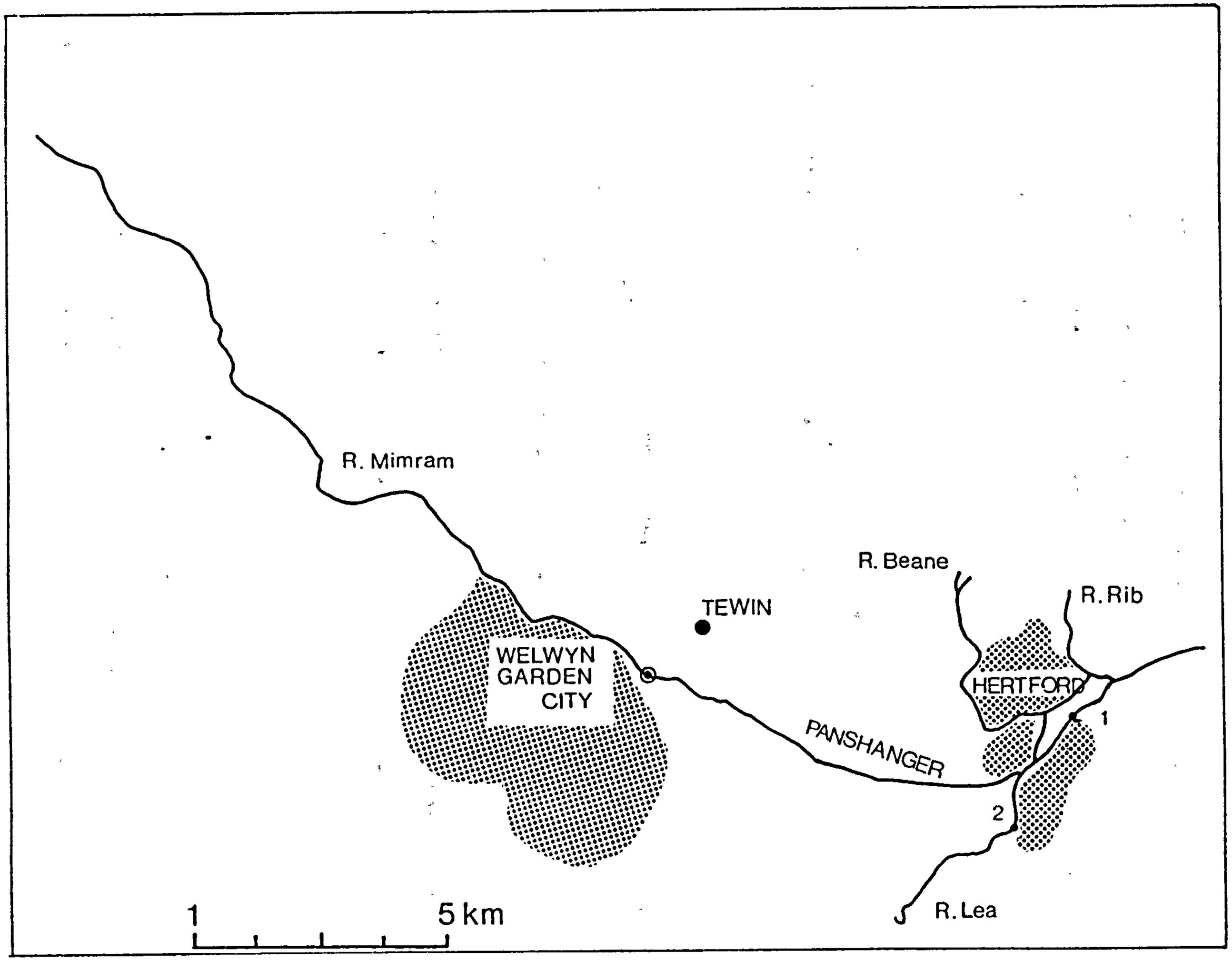
This population has been monitored up to and including 1986, at which time the animals were still thriving and a large number of one year old crayfish were caught by netting in amongst the roots of trees. This stretch of water is owned by a trout farm and is protected from human interference. Its isolation and protected status bodes well for the survival of this population.

Figure 3. The River Mimram

1. Dicker Mill where dead crayfish were found in 1981.

2. Site where a dead crayfish was found in 1981.

 = Residual population of native crayfish.


R. Mimram
TEWIN
WELWYN
GARDEN
CITY
R. Beane
R. Rib
HERTFORD
PANSHANGER
1
2
R. Lea
1
5 km

The River Beane.

1981

The first dead animal was found in the River Beane at Stapleford (TL 312179) in August 1981. although Aph. astaci was not confirmed. No further dead animals were seen this year. Live animals were found in September downstream from here at Goldings (TL 314141). and close by. at Horseshoe Weir (Deardsley pers. com., Figure 4.).

1982

A search of the Beane at Stapleford revealed no animals. where in 1981 one dead one was found.

1983

Healthy crayfish were caged in the river above the confluence with the Lea (TL 317135) on the 10th of September. After 59 days they were returned to the laboratory. All later died (Southgate pers. com.). Aph. astaci was subsequently isolated from a Turkish crayfish Astacus leptodactylus that was placed in contact with dying animals. (Isolate 13-184(1)).

1984

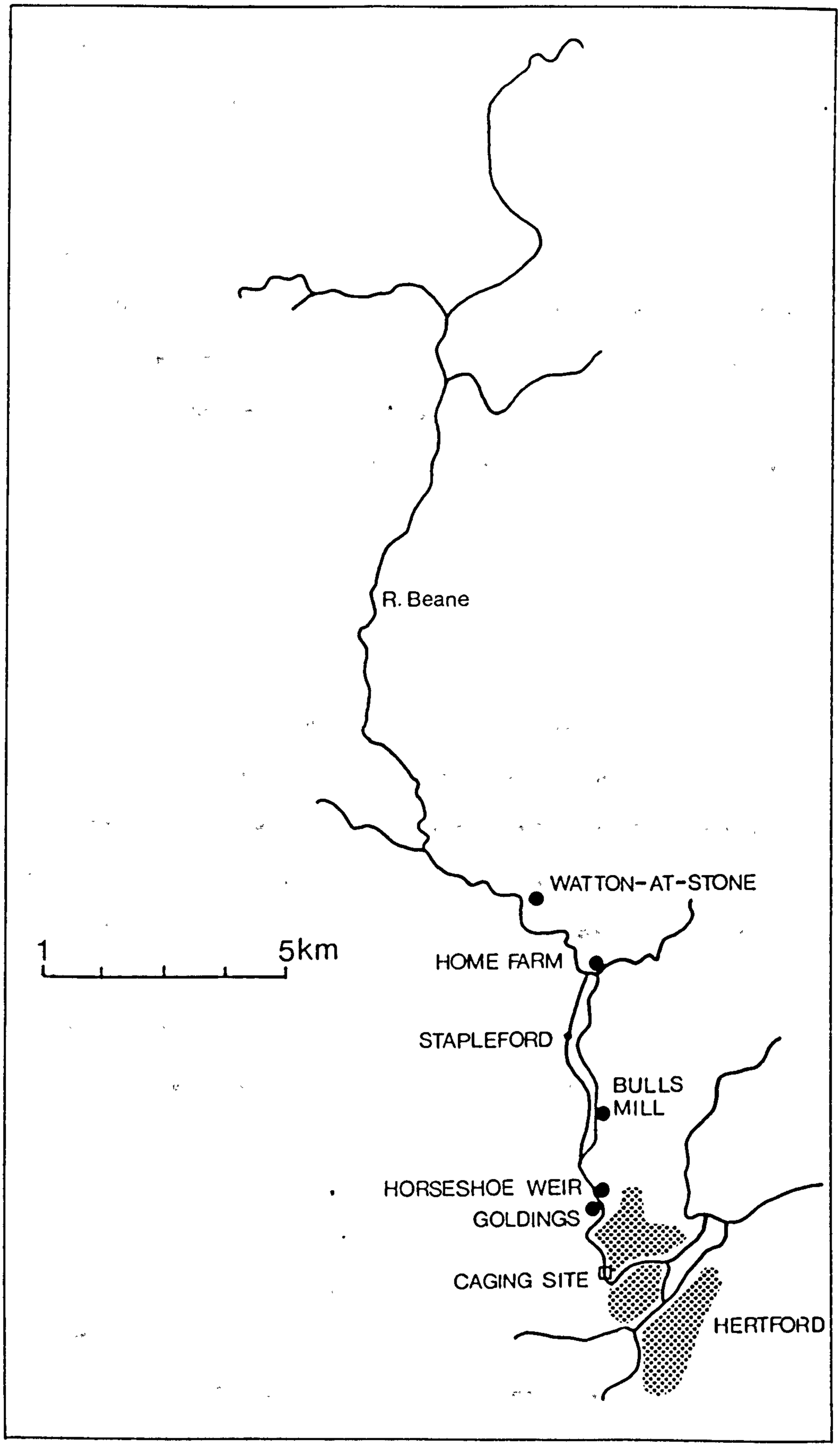
A search of the river at grid reference TL 317135. where caged crayfish had succumbed to crayfish plague the previous year. revealed no animals. Healthy animals were again caged here on the 1st June. The animals survived for 45 days after which time they showed no sign of disease.

Figure 4. The River Beane.

 = Sites where dead crayfish were found.

 = Sites where caging experiments were carried out.

● = Other sites.


R. Beane
WATTON-AT-STONE
HOME FARM
STAPLEFORD
BULLS MILL
HORSESHOE WEIR
GOLDINGS
CAGING SITE
HERTFORD
1
5km

Later all but one escaped. this animal was returned to the laboratory were it remained healthy.

1986

Searches of the river at Bull's Mill (TL 314157). and at Home Farm (TL 314182) by stone turning and netting revealed no animals, although the substrate conditions were favourable. Seven kilometers upstream at Watton-at-Stone (TL 307187), the situation was similar at several sites.

The River Rib.

1981

The first dead animals in the Rib were found in August 1981, shortly after the outbreak at Ware lock, at Bengeo (TL 333139), 1 km from the river's confluence with the Beane (Figure 5.). On the same day, animals were found dead at Payne's Hall (TL 337164) 4 km further upstream. However, a further 4 km upstream at Wade's Mill (TL 359175), live animals were found, whilst, 9 km furter upstream, a dead animal was found at Barwick Ford (TL 386189), however Aph. astaci was not diagnosed.

1982

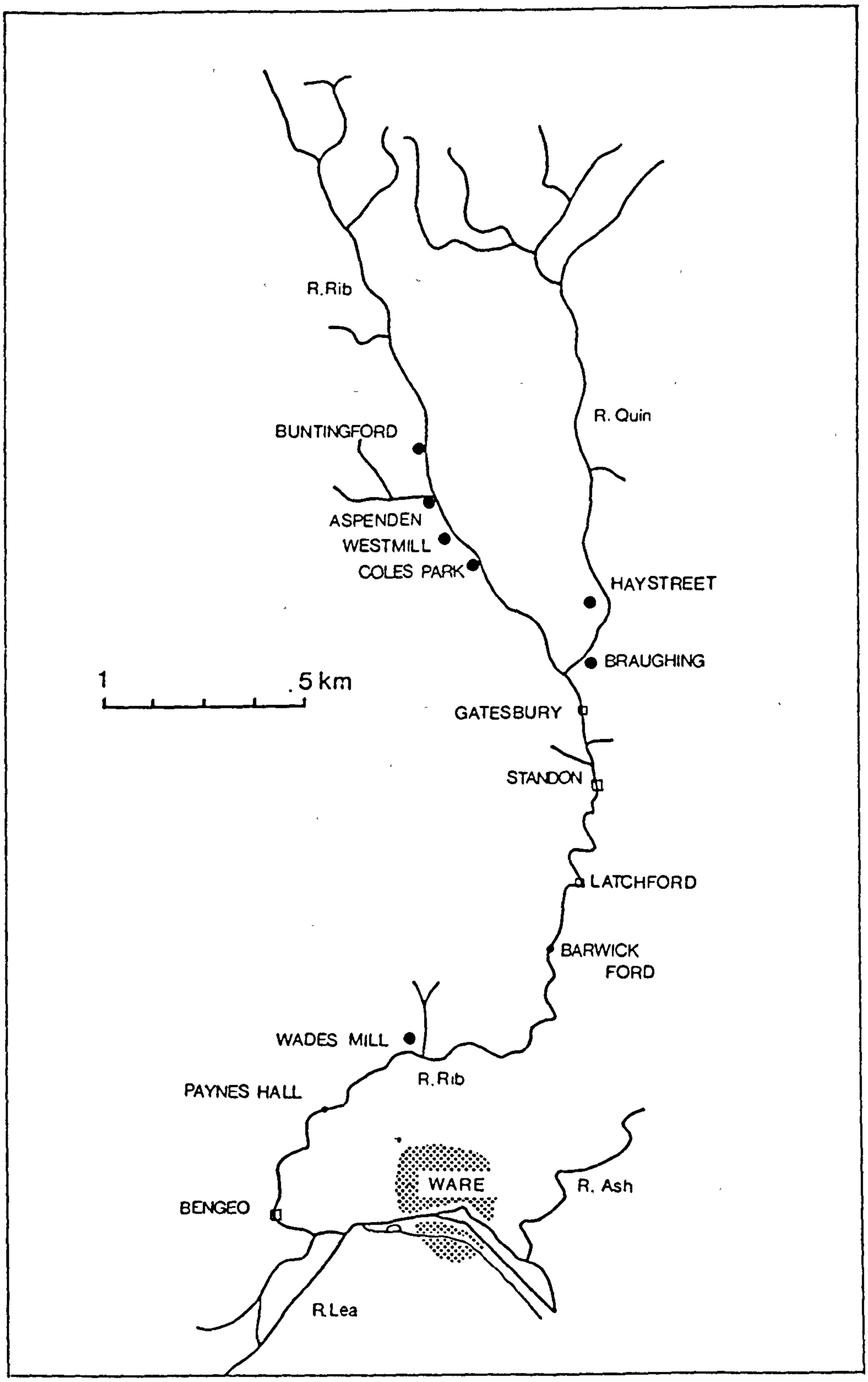
In 1982, dead crayfish were again observed at Barwick Ford, although 8 km further upstream at Standon (TL 394223), populations flourished. Later searches upstream from Barwick Ford for 1 km, failed to yield any

Figure 5. The Rivers Rib and Quin.

= Sites where dead crayfish were found.

= Sites where caging experiments were carried out.

● = Other sites.


R.Rib
R. Quin
BUNTINGFORD
ASPENDEN
WESTMILL
COLES PARK
HAYSTREET
BRAUGHING
1 .5 km
GATESBURY
STANDON
LATCHFORD
BARWICK
FORD
WADES MILL
R.Rib
PAYNES HALL
WARE
R. Ash
BENGEO
R.Lea

animals.

In November, a search of the river at Latchford (TL 394204) yielded a number of newly dead animals.

1983

Live animals were seen at Archer's Hall, Latchford (TL 392203), above Barwick Ford. Animals caged in the river at this point on the 8th September, later died although no Aph. astaci isolates were obtained. In a later experiment, healthy animals were again caged in the river at this point on the 12th of November (Southgate pers. com.). The animals again died. Two isolates were subsequently shown to be Aph. astaci (Isolates 1-1283(2) and 1-1283(3)).

Searches of the river at Standon, where the animals had been plentiful, suggested that there were none now present. Further searches of the river at Buntingford (TL 363293), several kilometers upstream, and at Westmill close by (TL 372272), revealed no animals. Beyond this point the river regularly dries in summer and is unlikely to support crayfish.

1984

A search of the river at Westmill revealed no animals and enquiries confirmed the continued absence of animals at Standon.

A cage containing 10 healthy Aus. pallipes was again placed in the river at Latchford on the 31st of May.

The animals were returned to the laboratory after 3 weeks and although a number of them subsequently died, they did not show signs of crayfish plague and no isolates of Aph. astaci were obtained upon post mortum.

Thirteen healthy animals were trapped above Gatesbury, at Coles Park (TL 380260) on the 6th of September.

1985

Ten Aus. pallipes were caged at Standon on the 29th July. After 22 days one was found dead and the rest were returned to the laboratory. Subsequently, animals died with symptoms of Aph. astaci infection and hyphae were seen in the ventral abdomen and the anal segments of several of these. Aph. astaci was isolated from one animal on 26th of August (Isolate 26-885(1)).

After a search using nets and traps revealed no animals, a cage containing 10 Aus. pallipes was placed in the river 2 km above Standon, at Gatesbury (TL 392238) on the 19th September. The first animal died with symptoms of Aph. astaci on the 7th October and the rest were subsequently returned to the laboratory. Aph. astaci was isolated from one of these animals.

Two kilometers upstream from Gatesbury, a search of the river at Coles Park, using traps, on the 8th August, yielded a single healthy male animal, suggesting the continued presence of a small population in this area despite the isolation of Aph. astaci from animals dying at

Gatesbury.

Further upstream from this site, at Aspenden (TL 362287) no animals were seen, suggesting that the population at Coles Park was isolated.

1986

In May, repeated trapping of the site at Coles Park failed to yield a single animal. It is therefore possible that the animals at this last site on the Rib have died out.

Animals were again caged at Gatesbury on the 16th May. All animals perished over the next 17 days. Aph. astaci was isolated from two of these animals (isolates 17-686 (1) 20-686(1)), indicating that the disease was still present in the river at this point, although the source of infection was not located.

The River Quinn.

1983

This year the River Quinn was surveyed a Braughing, 1 km from its confluence with the Rib (TL 392248), and at Hay street 1 km further upstream (TL 397261). No animals were seen (Figure 5.).

The River Ash.

1981

The confluence of the Rivers Ash and Lea occurs 2 km downstream from Ware weir, were animals were found dead

Figure 6. The River Ash.

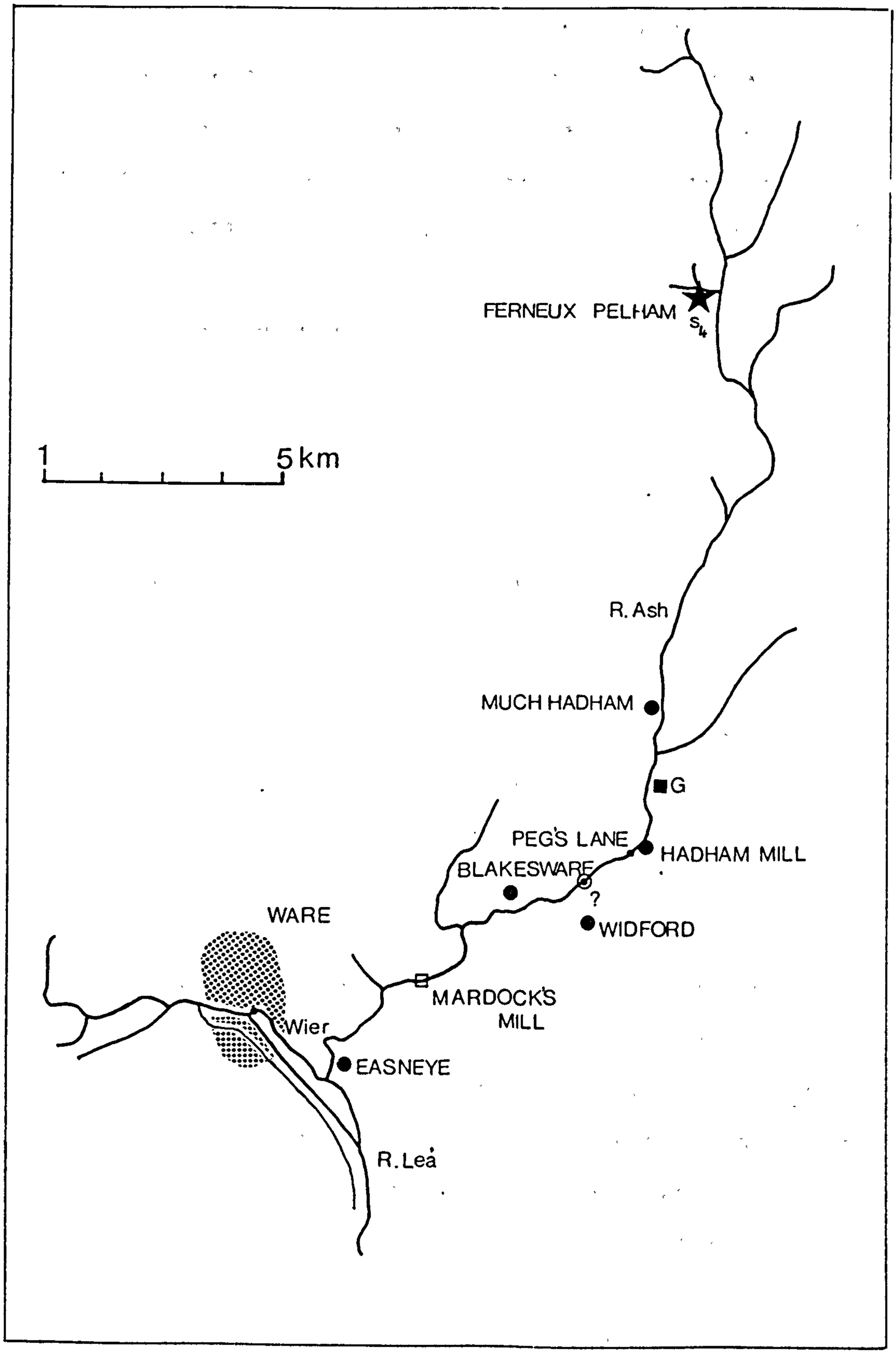
= Sites where caging experiments were carried out.

= Possible residual population of native crayfish.

★ = Site of Signal crayfish introduction (S4).

● = Other sites.

G = The Gingercress trout farm


FERNEUX PELHAM
S4
1
5km
R. Ash
MUCH HADHAM
G
PEG'S LANE
HADHAM MILL
BLAKESWARE
?
WIDFORD
WARE
MARDOCK'S
MILL
Wier
EASNEYE
R. Lea

in July 1981, and near the New River, where many animals were found dead on the 4th of August (see Figure 6 .). No animals were found in a search close to the confluence at Easneye (TL 377134) in August, but 3km upstream at Mardock's mill (TL 393148), two animals were found dead. Seven kilometers further upstream, at Peg's lane (TL 430185), another animal was found dead (Deardsly pers. com.).

1982

A search of the Mardock's mill site this year showed that the animals were still present.

1983

A search of the Ash at Much Hadham, revealed no crayfish, and Thames Water Authority Workers had no knowledge of residual populations.

1986

This year a survey of the river at Mardock's Mill did not reveal any crayfish, where previously they had been plentiful. A cage containing 10 Aus. pallipes was placed in the river at this point on 8th May. The animals remained in the river until the 20th of March 1987 when one animal was found dead. The remaining animals were returned to the laboratory but remained healthy.

Searches of the stretch of river 4 km upstream from here at Blakesware manor (TL 406159), did not locate any

animals where previously they were said by the gamekeeper to have been present. However, for 2 km upstream, the river bottom is bare, silty and anaerobic and would be inapropriate environment for crayfish.

One kilometer above this site, at Widford sewage works (TL 419166) one animal was found by Thames Water Authority workers. However, it is unclear whether this animal was Aus. pallipes or Astacus leptodactylus, since a fish farm close by. The Gingercress Trout Farm (TL 428175), recently aquired stocks of Astacus leptodactylus to re-sell.

Upstream, at Hadham Mill (TL 427170) and Much Hadham no animals were seen. Animals had apparently not been seen at Much Hadham for at least five years. Above this site the river dries from time to time and is unlikely to be suitable for crayfish.

Thus, the possibility exists that there is a residual population of Aus. pallipes in the Ash at Widford, although this awaits confirmation.

The River Stort

1981

At the time of the outbreak of mortalities in the River Lea, a survey was carried out in the Stort at Roydon 4 km from the confluence of the two rivers. One live animal was found. At the same time, several kilometers upstream at Bishop's Stortford causeway, live animals were also found, although 4 km further upstream a dead crayfish

Figure 7. The River Stort.

HMSt = Harlow Mill Station.

TF = Temple Fields.

S1 and S2 = Two Signal crayfish introductions near Stanstead Abbotts, exact location unknown.

1. = Bishops Stortford causeway.

2. = Bishops Stortford town centre, site where a single animal was caught in 1986.

3. = Site where a dead crayfish was found in 1981.

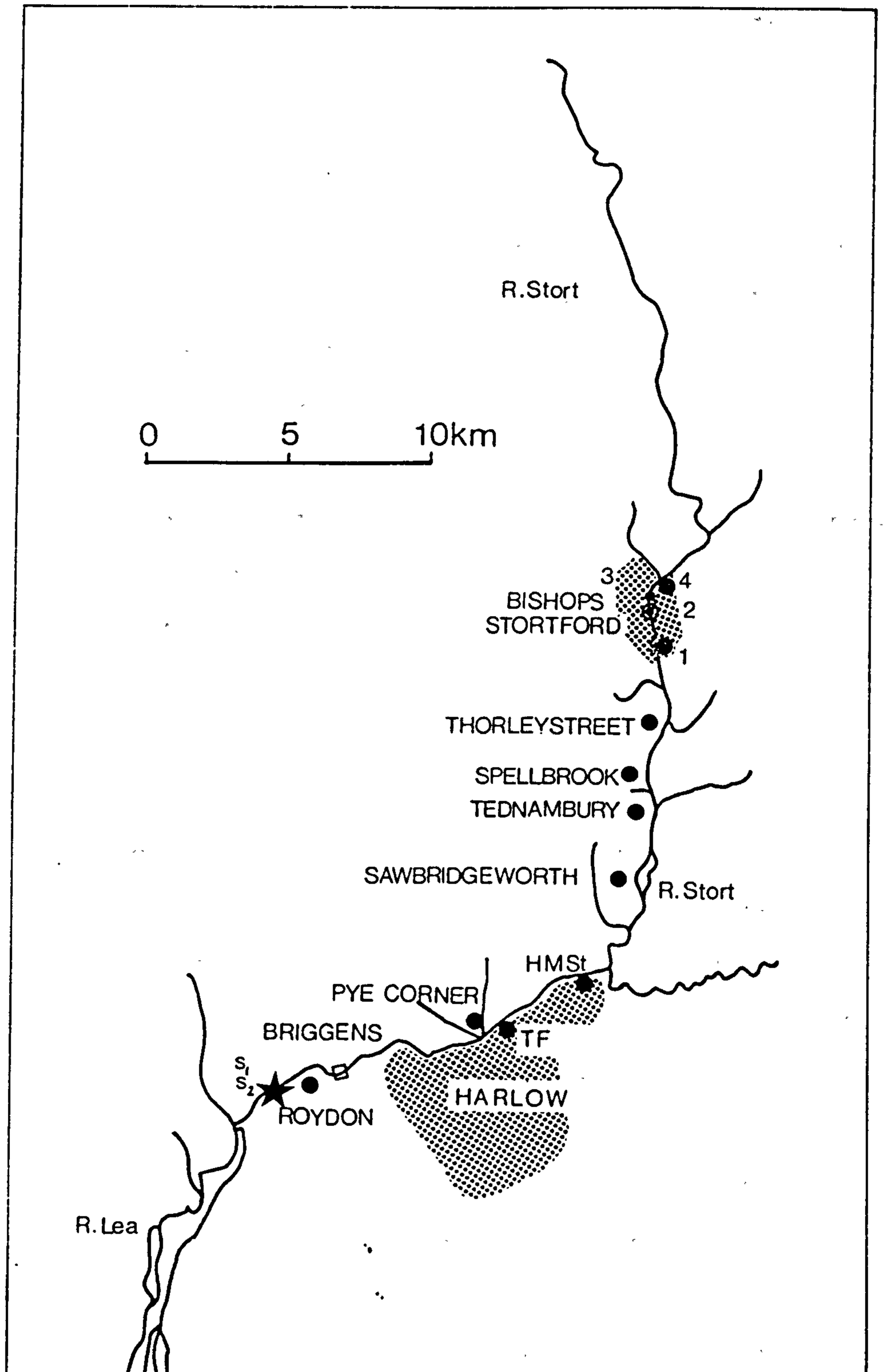
4. = Site in Northern Bishops Stortford where no animals were found in 1981.

= Sites where dead crayfish were found.

= Sites where caging experiments were carried out.

★ = Sites of Signal crayfish introductions.

● = Other sites.


R.Stort
0
5
10km
3
4
BISHOPS
STORTFORD
2
1
THORLEYSTREET
SPELLBROOK
TEDNAMBURY
SAWBRIDGEWORTH
R. Stort
HMSt
PYE CORNER
BRIGGENS
TF
S1
S2
HARLOW
ROYDON
R. Lea

was found. Aph. astaci was not confirmed (Deardsley pers. com. : Figure 7).

1982

In 1982, searches at Thorleystreet (TL 495198) and Spellbrook, (TL 490175) south of Bishop's Stortford, failed to locate any animals.

1983

This year, a search of the river at Roydon, where a live animal was found in 1981, failed to locate any animals, as did a search further upstream at Pye Corner, Harlow (TL454116).

1985

A search of the river 5 km upstream from the confluence, at Briggens (TL 421113), failed to locate any crayfish. Subsequently, a cage of 10 Aus. pallipes was placed in the river at this site on the 30th August. After nine weeks, three animals were found dead and the cage was removed to the laboratory. None of the remaining animals subsequently developed signs of Aph. astaci infection.

Further searches of the river around Harlow, at Pye Corner, Temple Fields (TL 463122) or at Harlow Mill Station (further upstream), revealed no animals, although members of the public claimed to have seen them in the area of the town centre.

At Tednambury (TL 496170), 7 km below Bishop's

Stortford causeway, where animals were found in 1981, no animals were found this year, nor were animals found in a search north of Bishop's Stortford (TL 494260).

1986

Two trapping sessions were carried out on the River Stort this year. One failed to locate crayfish where they had been reported the year before, near Harlow town centre, the other produced one animal in Bishop's Stortford town centre (TL 489223), indicating the possible presence of a small residual population of animals here.

Signal crayfish in the Lea tributaries.

Until the latter part of 1987, the origin of Aph. astaci infections in the Lea tributaries was unclear. Recently however, information regarding a number of Signal crayfish (Pacifasticus leniusculus) introductions in the area has come to light (Reeves and Holditch pers. com.).

Five introductions are known to have been made since 1978. The first involved 500 juveniles introduced into waters close to the course of the river Lea at Stanstead Abbotts in 1978, the precise location is not known. A further implantation of 500 juveniles was made in the vicinity of Stanstead Abbotts sometime after 1978.

Post 1978, 400 juveniles were introduced into the head waters of a small tributary of the Lea at grid reference TL 290063.

Pre 1983, an introduction of Signal crayfish was

made in the vicinity of Furneaux Pelham in Hertfordshire (TL 4227). This area lies close to the course of the River Ash. Finally, an implantation is believed to have occurred in the vicinity of Newgate Street in Hertfordshire (TL 302051) near a small tributary of the Lea, although no further information is available.

SUMMARY.

Crayfish mortalities were first noted in the Lea tributaries, in the vicinity of Ware and Hertford in June 1981, although not all populations of crayfish in this part of the river were affected at this time. The populations at Hertford basin and Hertford castle remained healthy.

In subsequent searches of the river upstream from Hertford no animals were found, although they were reported to be present at the confluence with the Mimram in 1985.

Searches downstream as far as Enfield yielded no crayfish even in a gravel pit close to the course of the river where they had previously been abundant.

Reports of crayfish being found again in the River Lea at its confluence with the Mimram in 1985 and at Hertford in 1986, suggest that the river is being recolonized. The source of the recolonizing animals is unknown but may be any one of the large number of backwaters around Ware and Hertford that may have remained unaffected by the disease.

Although one dead animal was found in the River Beane 4 miles from the confluence with the Lea in August 1981 Aph. astaci was not confirmed. Two miles down stream from here, a thriving population was still to be found, and it therefore seems likely that the disease had not spread far into the river at this time.

The disease was present in the Beane in 1983 as shown by caging experiments. Subsequently no live crayfish were found at any site investigated on this river. However, the disease was detected in the river by caging experiments in 1983.

By 1984, caging experiments suggested that the disease was no longer present in the river, certainly in the vicinity of its confluence with the Lea.

The first deaths in the River Rib occurred at around the time of the outbreak in Ware in August 1981. These first dead animals were found at Bengeo close to the confluence with the Lea and the mortality appeared to spread for two miles upstream. Populations above this wee healthy.

The disease then appeared to spread upstream from this site reaching as far as Latchford in 1982. After this the spread of the disease became less rapid, reaching Standon in 1983. By 1984 the plague front was located somewhere between Standon and Coles Park some three miles upstream.

Between September and October 1985, crayfish plague was still present in the river at Gatesbury, although the

source of the disease was never located. Live animals were still present upstream from here at Coles Park.

By May 1986 no animals could be found at Coles Park. The disease was again detected in the river at Gatesbury in May, suggesting that an undetected source of infection existed in the river, possibly residual, infected animals at Coles Park.

Sites above Coles Park had consistantly failed to yield crayfish, suggesting that the disease had now infected all known populations of crayfish in the river Rib.

No outbreaks of crayfish plague have been detected in the River Mimram and a thriving population of animals exists at Tewin. This population has probably remained unaffected because the lack of crayfish populations downstream from here has prevented the retrograde spread of the disease from the Lea where populations close to the confluence with the Mimram have been affected by the disease.

Although Aph. astaci has never been isolated from the River Ash, a number of crayfish populations have declined since 1981. Most notable of these was the population at Mardocks Mill which died out between 1982 and 1986, although crayfish plague could not be detected in the river in 1986.

A single population may still exist in the Ash around Widford although it remains to be confirmed.

There have been no substantiated cases of crayfish

plague in the River Stort, however, a number of populations have declined since 1981 such as as that around Bishop's Stortford causeway, which disappeared between 1981 and 1985. A single animal was found in Bishop's Stortford town centre in 1986, suggesting that a small population may exist here.

Thus crayfish plague has been confirmed in the Rib, and the Beane and was suspected in the Lea. No confirmation of the disease exists in the Stort, the Quinn or the Ash other than the decline of well known populations of crayfish.

Residual populations of crayfish remain in the Mimram at Tewin and in the Stort at Bishop's Stortford. Unconfirmed reports suggest that populations may also exist in the Ash at Widford and the Lea at two sites in Hertford.

Until recently the source of crayfish plague infection was unknown, however recent reports of Signal crayfish introductions (animals known to be carriers of the disease (Unestam 1972 and 1974)) in the Lea trubutaries may well explain the native crayfish mortalities (Reeves and Holditch pers. com.). The earliest of these introductions, in 1978 predates the first recorded mass mortalities of crayfish in the Lea by 3 years, although further, unrecorded mortalities may have occurred before then.

POSSIBILITIES FOR THE EXISTENCE OF A NATURAL RESERVOIR OF INFECTION.

INTRODUCTION.

When stocks of crayfish are destroyed by crayfish plague, attempts are often made to reintroduce the animals (Roch 1881. Kozlowski 1968, Herfort-Michiell 1973. Soderhall *et al* 1977). Such attempts are not always sucessful since reintroduced animals may fall victim to crayfish plague themselves (Franke 1894 Herfort-Micheill 1973).

The infection of reintroduced animals may arise in a number of ways. First, a residual stock of infected crayfish may remain in the water body (Franke 1894). This possibility may be avoided in still, slow running or enclosed water bodies by liming. This technique kills the remaining live crayfish, thus removing reservoirs of infection in infected populations. However this is often ineffective (Svensson *et al* 1976) and a combination of electrical barriers to prevent the migration of animals, and the elimination of remaining crayfish by judicious use of a large inoculum of *Aph. astaci* spores is considered to be more effective (Soderhall *et al* 1977).

Secondly the disease can be transported from place to place by the action of man (Alm 1927, Hastein and Gladhaug 1973). Thirdly, the disease may be introduced into a re-established population from a reservoir in the environment, such as an alternative or transport host, a

saprophytic stage or from resting stages.

Schikora (1906) suggested that the sexual stages of the fungus might exist in other arthropods such as Gammarus which therefore act as the natural reservoirs of infection. He was able to demonstrate that the fungus was capable of growing on live flies, killing them rapidly, without producing spores (Schikora 1903 and 1906).

Sexual or resting structures have not been described with certainty, however, putative sexual structures were decribed by Schaperclaus (1927) on crayfish and by Renerfelt (1936) in pure culture. Later Unestam (1969b) described certain swollen hyphal structures and swollen zoospores both with thickened walls, as well as structures similar to those described by Renerfelt (1936). Alderman and Polglase (1986) described coiled hyphae with highly refractile cytoplasm that may have been resting structures. Thus, although putative resting stages appear to be rare, the possibility of the existance of a true resting stage is tantalizing.

Benisch (1940) showed that the Chinese Mitten Crab (Eriocheir sinensis) could be infected with Aph. astaci, however attempts to infect other aquatic arthropods, including Mysis relicta, (Peracarida) have failed (Unestam 1969c and 1972).

Hall and Unestam (1980) demonstrated that Aph. astaci would grow on excised scales of Salmo salar and that the fungus produced spores under these conditions, which raises the possibility that fish may act as

transport hosts for the disease.

Early workers found it difficult to culture Aph. astaci in vitro, and used such media as crayfish blood and horse serum (Rennerfelt 1936). It was later demonstrated that the fungus could be grown on relatively simple media (Unestam 1965) indicating that it did not have the specific nutrient requirements often found in obligate parasites. Indeed the fungus can be cultured on agar containing only river water, 0.6% glucose and 0.1% yeast extract (Alderman and Polglase 1986). However the ability of a fungus to grow on simple media does not necessarily imply that the organism can exist as a saprophyte (Unestam 1969b).

Further, it has been pointed out (Unestam 1969b) that the ability of Aph. astaci to produce chitinase (Unestam 1966a) coupled with the chemotactic attraction of its zoospores to crayfish (Unestam 1969b) and the limited range of its utilizable carbon sources (Unestam 1965) suggests that it is a parasite, physiologically adapted to aquatic arthropods.

Thus, although no natural alternative hosts have been demonstrated in the past, the fact that fish scales, a fresh water crab (E. sinensis) and flies have been infected under laboratory conditions, suggests that, under certain conditions the host range may include organisms besides crayfish.

In these experiments a number of aquatic arthropods were tested for susceptibility to Aph. astaci infection. A

variety of leaves were also tested for their suitability as a natural substrate for the growth of the fungus and attempts were made to transmit the disease using two species of fish as vectors.

METHODS.

1. Possible animal reservoirs of infection.

A number of fresh water invertebrates were tested for susceptibility to infection by Aph. astaci.

1.1. Gammarus pulex.

Specimens of G. pulex were collected from the River Mimram at Tewin (a site known to be free of Aph. astaci) and kept at 8°C for short periods before use.

Zoospores of Aph. astaci (Isolate 28-1083(4)) were produced by incubating small pieces of an agar culture in sterile distilled water overnight at 16°C.

For test purposes, 15 animals were kept in 400 mls. of tap water in 500 ml. glass beaker and challenged with 11,000 swimming Aph. astaci zoospores. Gentle aeration was begun 1 day after challenge and animals were kept at room temperature (18 - 20°C) for the duration of the experiment. Control animals were kept under similar conditions but were not challenged with Aph. astaci zoospores. Infection experiments were repeated three times.

Dead animals were removed and examined under a binocular microscope for gross signs of infection. Squash

mounts were then made, stained with methylene blue and these too were examined under a microscope. A number of dead animals were placed in sterile distilled water with 10 ppm oxolinic acid added to prevent bacterial growth, and examined over a period of 1 week for signs of Aph. astaci hyphae or zoosporangia.

Twelve dead test animals were fixed in Davidsons fixative (Appendix 5, Shaw and Battle 1957) for 24 hours, then sectioned and histologically examined using Grocott's modification of Gomori's silver methylamine stain for fungi (Appendix 6).

Finally a number of G. pulex were challenged with Aph. astaci as above. Two weeks later they were divided into two batches of 10 animals each and placed in small gauze bags. These bags were introduced into 30 litre polythene troughs containing 4 Ast. leptodactylus each. Control crayfish were kept in the same manner but no gammarids were added.

1.2. Other invertebrates.

Specimens of Asellus sp., the water boatman Coryxa punctata and the water beetle Deronectes sp. were collected from the River Mimram at Tewin. Chaoberus crystalinus were acquired from commercial sources.

Several specimens of each species were kept in 400 ml. of tap water in separate 500 ml glass beakers and challenged with Aph. astaci zoospores at a dose of 10 000 swimming zoospores per beaker (Isolate as above). Table 1

details the number of animals of each species used and the duration of each experiment. Each experiment was carried out in duplicate.

Dead animals were examined under a microscope for signs of infection. They were then placed into a quantity of sterile distilled water containing 10 ppm oxoloinic acid and observed over a period of several days for signs of Aph. astaci hyphae or zoosporangia.

1.3. Fish as possible vectors of crayfish plague.

Bullheads (Cottus gobio) were collected from the River Beane and 10 - 12 cm long rainbow trout (Salmo gairdneri) were acquired commercially. Two 40 l glass tanks were set up containing four trout each, two 30 litre polythene troughs containing 4 bullheads each and two 30 litre troughs containing only tap water. After a weeks acclimatization, aeration was turned off and 300 000 swimming Aph. astaci (Isolate 28-1083(4)) zoospores were introduced into each tank. After 24 hours aeration was continued. Five days after introduction of the zoospores, four Ast. leptodactylus were introduced into each tank. The Bullheads were transfered to a small perforated plastic trough inside the polythene trough to protect them from the crayfish. The water in each tank was carefully changed every two to three days. The crayfish were examined regularly for signs of Aph. astaci infection.

2. Reservoirs of infection in plant material.

In these experiments fungal growth on cracked hempseeds and the leaves of four tree species was assesed.

Twenty sterile, cracked hemp seeds were incubated in either 25 mls of sterile distilled water, sterile river water or untreated river water, in a Petri dish. To each Petri dish 10 000 swimming Aph. astaci zoospores (Isolate 28-1083(4)) were added. Incubation continued for 1 week at 20°C, after which the seeds were examined for signs of colonization by Aph. astaci.

The experiment was repeated with dried Oak (Quercus robur), Ash (Fraxinus excelsior), Willow (Salix fragilis), and Alder (Alnus glutinosa) leaves, sterilized by exposure to gamma radiation using a Cobalt 60 source.

RESULTS.

1. Possible animal reservoirs of infection.

1.1. G. pulex.

The results of a representitive G. pulex infection experiment are detailed in table 1. It is clear that there is little difference in the death rates between control and challenged animals, even though the challenge dose was large.

There were no gross signs of Aph. astaci infection in dead animals nor were any hyphae visible in squash mounts, histological examination of fixed animals revealed no fungal hyphae. Similarly no Aph. astaci zoosporangia were ever seen on animals incubated in sterile distilled

water containing oxolinic acid, although a number of other fungi were seen to grow on the cadavers.

Table 1. Alternative hosts.

	Number	Duration	Spore conc.	Deaths control	Deaths test
Gammarus pulex	15	8 days	11 000/400ml	4	5
Coryxa punctata	10	14 days	10 000/400ml	1	1
Chaoberus crystalinus	10	14 days	10 000/400ml	1	1
Deronectes sp.	5	21 days	10 000/400ml	0	0
Asellus sp.	6	14 days	10 000/400ml	0	0

Finally when *G. pulex* challenged with *Aph. astaci* were placed in gauze bags, in tanks containing crayfish, the crayfish remained healthy for 6 months, although all the gamarids were dead after 8 days. After 6 months the crayfish were examined. No signs of *Aph. astaci* infection were seen and the experiment was terminated.

1.2. Other invertebrates.

The results of exposing the other tested invertebrates to suspensions of *Aph. astaci* zoospores are given in table 1.

Of the ten *C. punctata* challenged with *Aph. astaci* one died, athough no *Aph. astaci* hyphae were seen to grow from it during incubation in sterile distilled water.

The single Chaoberus crystalinus larva that died during these experiments showed no sign of fungal infection when examined under the microscope, nor did any Aph. astaci zoosporangia become apparent after incubation in sterile distilled water containing oxolinic acid.

Neither the Deronectes sp. nor the Asellus sp. challenged showed any sign of being infected by Aph. astaci.

1.3. Fish as possible vectors of crayfish plague.

There were no crayfish deaths in tanks containing trout previously exposed to Aph. astaci zoospores or in tanks containing similarly treated bullheads (Table 2.).

Table 2. Fish as vectors of crayfish plague.

	Duration	Deaths
Trout	4 Months	None
Bullheads	4 Months	None
Control (Spores only)	4 Months	One death

The single control animal that died was examined for signs of Aph. astaci infection and none were found. A post mortem examination involving plating out small pieces of arthrodial membranes from the legs, anal region, dorsal and ventral abdomen and the eyes onto river water agar (Appendix 4) yielded no Aph. astaci isolates.

The experiment was terminated after four months, after which time all animals were examined for signs of

Aph. astaci infection. None were found.

2. Possible reservoirs of infection in plant material.

Table 3 details the results of experiments to determine the suitability of natural plant substrates for the growth of Aph. astaci.

Table 3. The growth of Aphanomyces astaci on plant material.

	Sterile DW	Sterile RW	Non sterile RW
Hemp seeds	+	+	-
Leaves:-			
Oak	-	-	-
Ash	-	-	-
Willow	+	+	-
Alder	-	-	-

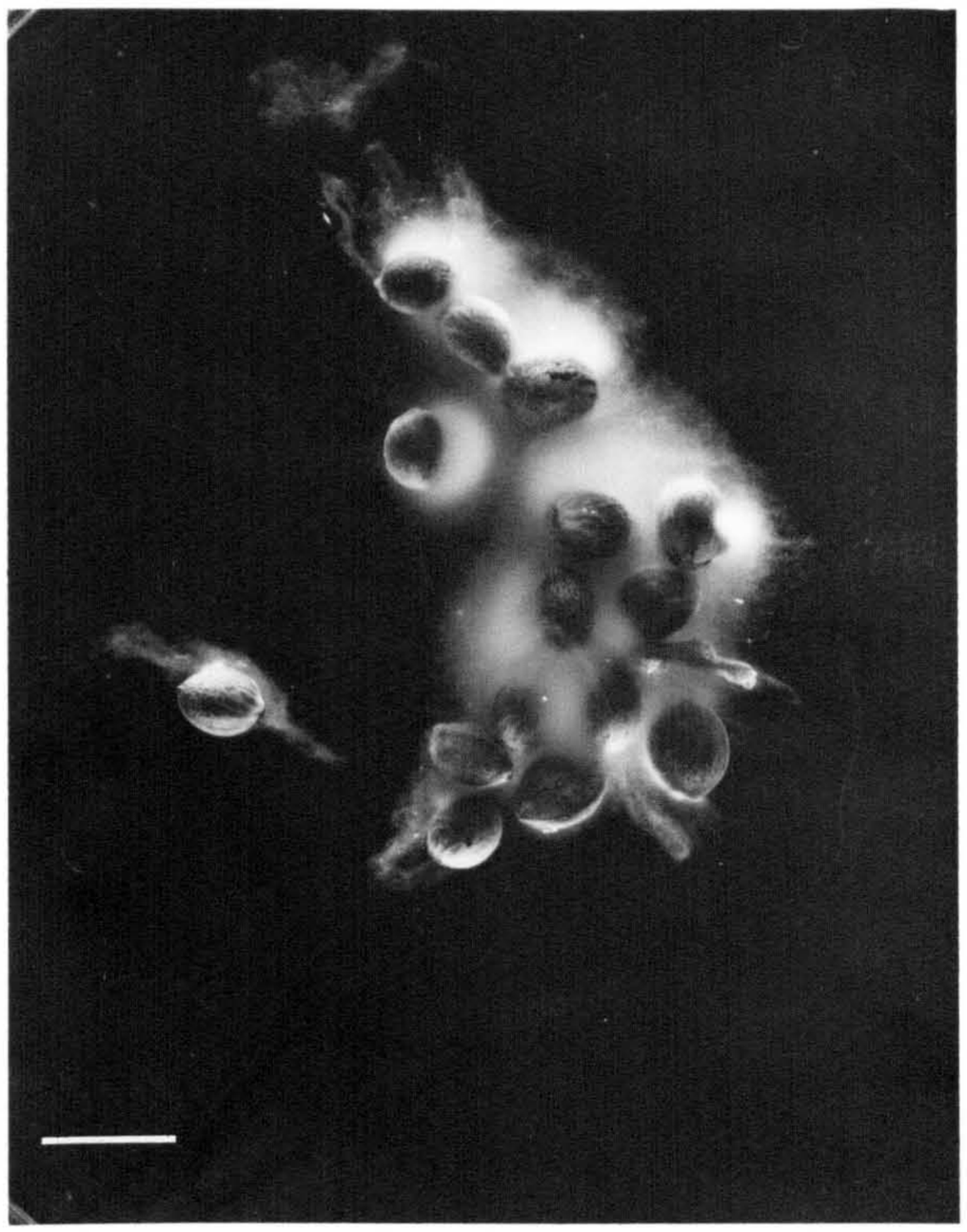
+ = Growth: - = No growth.

In both sterile river water and sterile distilled water Hemp seed cultures, tufts of fungal hyphae could be seen, both attached to the hemp seeds and in the surrounding water, after 24 hrs incubation (Figure 1). By 6 days the tufts were larger and microscopic examination revealed the presence of primary zoospore clumps typical of Aph. astaci, in both sterile distilled water and sterile river water. Disection of the seeds revealed that the fungal hyphae ramified over the inner face of the testa. When the experiment was repeated using seeds divided up into testa embryo and endosperm, the fungus was

Figure 1. The growth of Aphanomyces astaci on hemp seeds.

This photograph shows Aph. astaci growing on sterile cracked hemp seeds in sterile distilled water. The photograph was taken after 10 days incubation at 20°C

Scale bar = 5 mm



found to grow predominantly on the embryo.

No Aph. astaci zoosporangia were seen under non sterile conditions, although other species of fungi were present, predominantly Saprolegnia species.

Microscopic examination of one distilled water culture revealed a single structure as shown in figure 2. This structure was approximatly 50 um. in diameter with smooth, thickened walls. The cytoplasm was granular, more so towards one side, and a single large structure resembling a vacuole was present on the more granular side. The structure resembled previous descriptions of the oospore of Aph. astaci (Rennerfelt 1936), and of swollen hyphal structures illustrated by Unestam (1969b) which he was unable to demonstrate conclusively to be sexual stages. The structure was however not similar to descriptions of the sexual stages given by Schapperclaus (1927 and quoted in Rennerfelt (1936)) who suggested that oogonia were 16-20 um in diameter and bore spines.

Similar structures were seen in one laboratory infection of Ast. leptodactylus and are shown in figure 3. These structures were seen on the surface of infected perianal soft cuticle. Since such infections are unlikely to be pure cultures, it is not possible to attribute these structures to Aph. astaci with any degree of certainty.

After 7 days, examination of Willow leaf fragments exposed to Aph. astaci zoospores revealed the presence of tufts of fungal hyphae around the broken end of leaf veins, but only in sterile distilled water and sterile

Figure 2. Oogonium-like structure from Aphanomyces astaci grown on hemp seed.

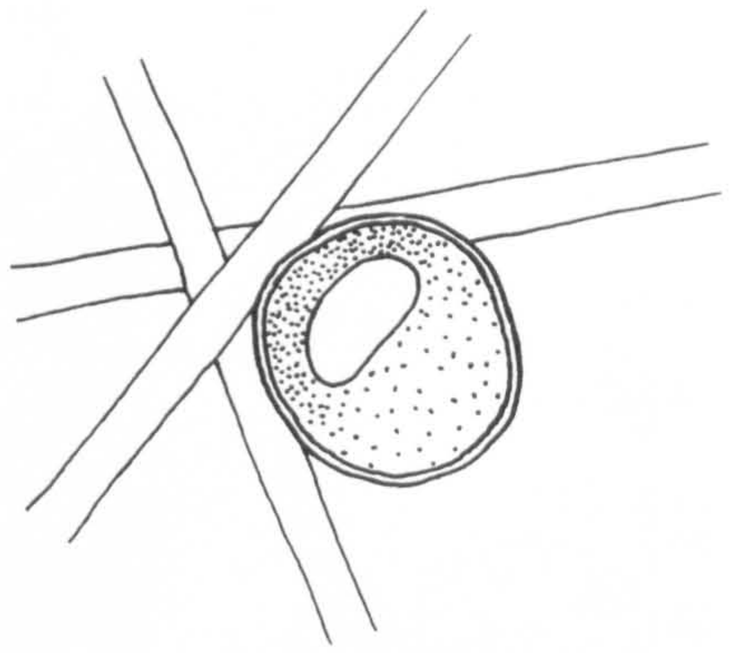
This oogonium-like structure was seen after six days culture of Aph. astaci isolate 28-1083(4) on sterile cracked hemp seeds at 20°C. The structure has a smooth wall.

Scale bar is approximately 50 um.

Figure 3. Structures resembling fungal resting stages on Astacus leptodactylus.

This photomicrograph shows structures resembling oomycete resting stages on the surface of infected cuticle from the perianal area of Ast. leptodactylus. These structures resemble that shown in figure 2., from a pure culture of Aph. astaci growing on hemp seeds.

Scale bar = 50 um.



50µm

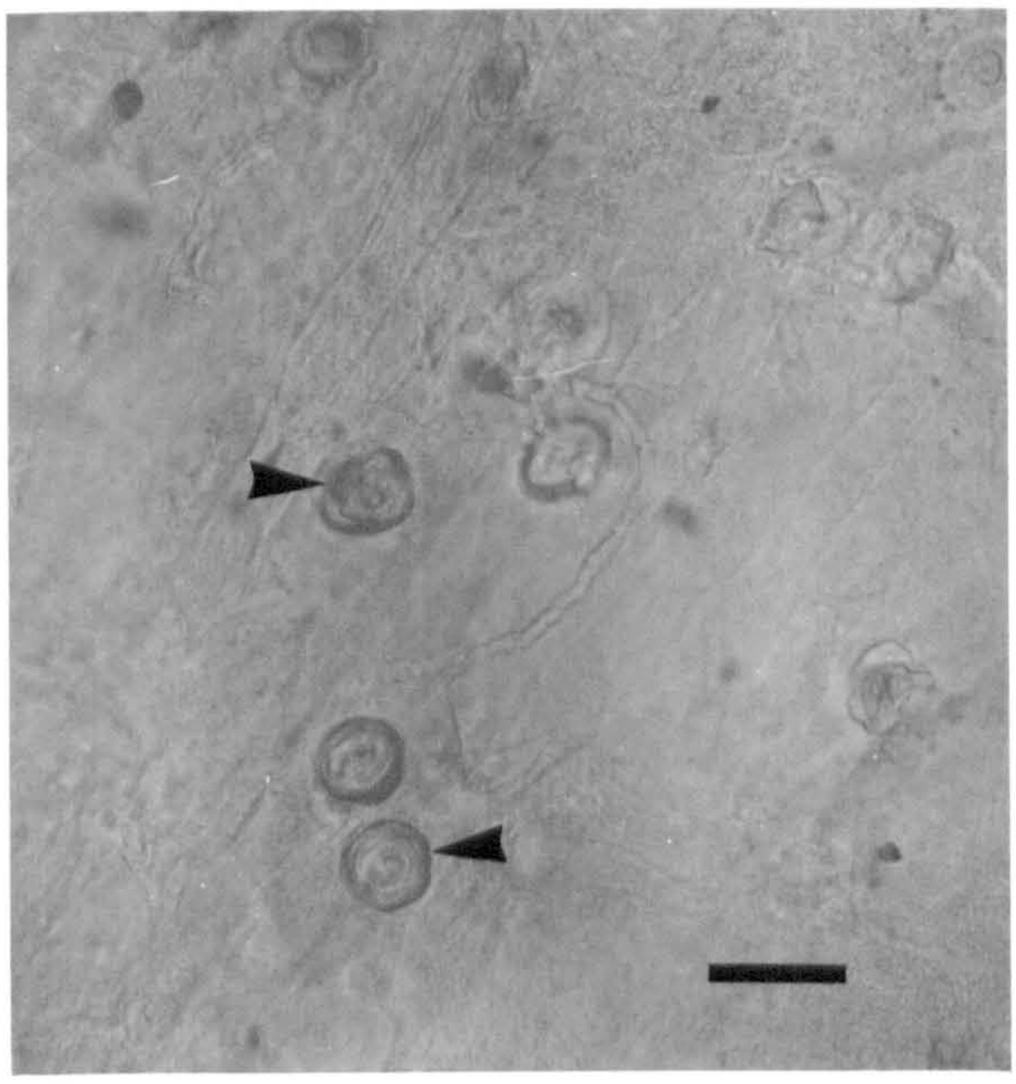


Figure 4a. Willow leaf fragment with Aphanomyces astaci growth.

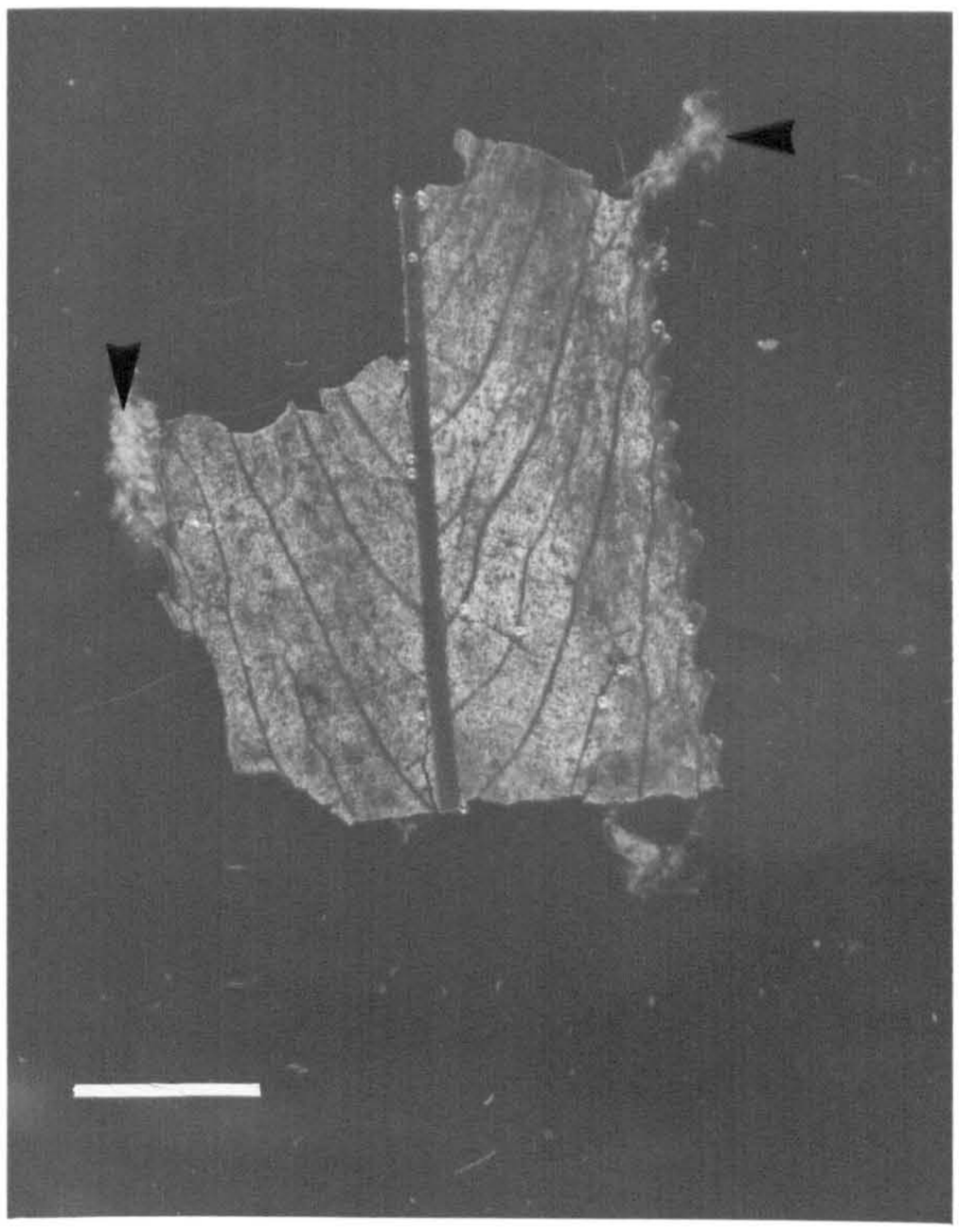
This photograph, taken after 17 days incubation at 20°C, shows a fragment of willow leaf on which can be seen tufts of Aph. astaci hyphae. The tufts of hyphae tend to eminate from the broken ends of the leaf veins.

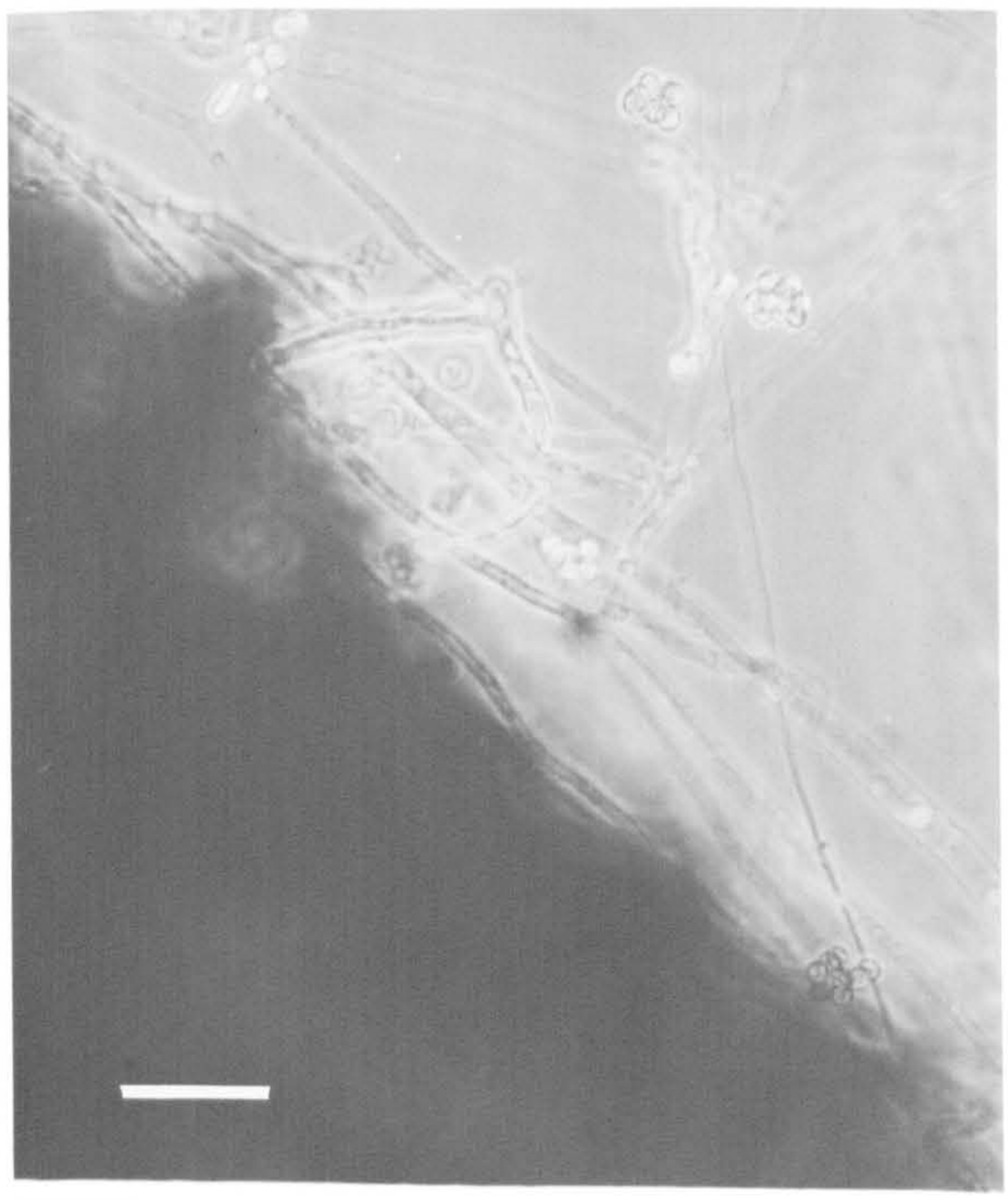
Scale bar = 10 mm

Figure 4b. Aphanomyces astaci growing on a willow leaf.

This photomicrograph of the material illustrated in figure 4a. was taken at a magnification of x 100 and shows Aph. astaci hyphae and primary zoospore clusters.

Scale bar = 50 um.





river water incubations (Figure 4a). Microscopic examination showed that primary zoospore clusters were present although they contained fewer primary cysts than sporulated agar cultures (Figure 4b). There was no evidence of growth on the leaves of other tree species. No Aph. astaci zoosporangia could be detected under non sterile conditions.

DISCUSSION.

The inability to demonstrate appreciably higher mortality rates in G. pulex challenged with Aph. astaci, compared to unchallenged controls; coupled with the failure to demonstrate fungal infection of dead test animals in both gross and histological examination, strongly suggests that the animals are not susceptible to the crayfish plague fungus. Further, the fact that Aph. astaci could not be demonstrably transmitted to crayfish from G. pulex previously exposed to Aph. astaci, suggests that this organism is unlikely to be a natural reservoir of infection.

Similarly, the amphipod Asellus did not appear to be susceptible to infection with Aph. astaci. Thus it is evident that resistance within the super order peracarida (of the class Malacostraca) is high, since neither the isopod G. pulex, the amphipod Asellus nor the mysid M. relicta (Unestam 1972) have been shown to be susceptible to the disease. Other tested invertebrates also proved to be resisitant to Aph. astaci infection.

Despite the fact that Aph. astaci has been shown to grow and sporulate on fish scales in vitro (Hall and Unestam 1980), it was not possible to demonstrate transmission of the fungus using either trout or bullheads as a vector. Alderman et al (1987) have however, demonstrated that fish (rainbow trout) exposed to zoospore suspensions for 24 hrs, could transmit Aph. astaci infection to healthy Ast. leptodactylus, even though they had been drained of water. Results presented in chapter 4 suggest that, in pond water, this is enough time for zoospores to become non motile at room temperature, however, it is unclear whether transmission in this case was due to the transfer of motile infective zoospores along with the fish or whether the fish acted as true carriers. The inability to demonstrate transmission of Aph. astaci infection via trout or bullheads in these experiments, after allowing the zoospore inoculum to become non motile suggests that under these conditions neither trout nor bullheads act as vectors of the disease.

Aph. astaci was shown to be capable of growing on sterile cracked hemp seeds, although, from the studies carried out it was not possible to determine whether the fungus penetrated the plant material, or whether it utilized nutrients leached from the seed. The same isolate of Aph. astaci was capable of colonizing sterile willow leaves, but not sterile oak ash or alder leaves. Zoosporangia of the fungus were not visible on hemp seeds or leaves under non sterile conditions.

To saprophytes growing on plant material the posession of cellulase and pectinase enzymes would be an advantage.

Previous studies have failed to demonstrate cellulase activity in Aph. astaci although a low pectinase activity was found (Unestam 1966a). Cellulase activity is found in other species of Aphanomyces such as Aph. eutriches as well as in other members of the Saprolegniaceae (Unestam 1966a, Thompstone and Dix 1985). The saprophytic species Aph. laevis however, is not thought to produce cellulase (Unestam 1966). Earlier studies (Unestam 1965) showed that, out of 25 compounds, Aph. astaci grew best on media containing glucose. The utilization of other carbon sources, such as amino acids, cellobiose and sucrose, was low and dependant upon the nitrogen source used.

Thus, whilst the cellulose and pectin degrading properties of Aph. astaci are similar to a closely related saprophyte, its poor utilization of carbon compounds other than glucose suggest that it would, at best, be a slow growing and possibly poor competitor for nutrients in anything other than a glucose rich environment. This is consistant with the results of growth studies on hemp seeds and tree leaves, in which the presence of the fungus was only demonstrated in sterile conditions.

The inability to demonstrate the presence of Aph. astaci zoosporangia in non sterile conditions does not preclude presence of the fungus under these circumstances,

since competition from other organisms may slow growth or prevent or delay sporulation.

The occurrence of a structure with thickened walls in one hemp seed culture suggests that the organism may be capable of producing some form of resting stage, albeit under sterile conditions.

Thus these experiments have confirmed earlier reports that susceptibility to Aph. astaci infection is low in invertebrate groups outside the decapod crustaceans and suggest that the likelihood of fish acting as vectors of the disease is small.

The ability of the fungus to grow on both hemp seeds and leaf material under sterile conditions suggests that it may possess the ability to exist outside the host as a saprophyte. The occurence of a structure reminiscent of a resting stage during growth on plant material hints at the possibility of dormant stages existing in the wild. Similar structures were seen in a single case of a laboratory infection but could not be confirmed as resting stages of Aph. astaci. The fact that Aph. astaci could not be demonstrated to be present on plant material under non sterile conditions suggests that it is at best a weak competitor on plant substrates.

Since the fungus is a chitinase producer (Unestam 1966a), and appears to be adapted to a chitin rich environment (Unestam 1969b) it may be pertinent for future studies to concentrate on the possibility that this organism may exist outside the host on chitinous detritus

such as exuviae.

OBSERVATIONS ON POLYPLANETISM IN APHANOMYCES ASTACI

INTRODUCTION

In previous studies involving the infection of crayfish with Aph. astaci, experiments were carried out in glass double distilled water (Unestam 1969, 1972) or pond water (Unestam and Weiss 1970). Before infection experiments with British isolates of Aph. astaci were carried out, the effectiveness of tap water as a medium for infection was studied. Zoospore suspensions in sterile tap water were examined to determine how long the spores remained motile. These studies revealed evidence of repeated encystment of the zoospores.

The results presented in this section are single observations of the phenomenon of repeated encystment (polyplanetism) of Aph. astaci zoospores under a variety of conditions and were initially intended to form the basis of a more detailed study which was not continued.

Methods.

Suspensions of Aph. astaci zoospores (isolate 6-1183(2) or 28-1083(4)) were prepared by incubating small pieces of agar (Appendix 4) from a vigorously growing culture, in sterile distilled water for between 15 and 19 hrs at 20°C. Under these conditions zoosporulation began after approximately 14 hrs and a large number of swimming

zoospores (typically 20,000 per/ml) were present after 15 to 16 hrs.

Five millilitres of zoospore suspension was then added to 20 mls. of either sterile distilled water, sterile tap water or sterile pond water, in a Petri dish. A wide bored, sterile plastic pipette was used to add the zoospore suspension in order to minimize encystment caused by mechanical disturbance. The zoospores were then observed hourly by dark field illumination and the number of encysted spores on the bottom of the dish was calculated from the mean of 10 fields at a magnification of 100 times.

RESULTS.

Figure 1. shows the number of encysted spores in distilled water and in tap water over a 12 hr. period. After one hour, 12 spores per field in the tap water culture and 11 spores per field in the distilled water culture were already encysted. The number of encysted spores in distilled water rose to a maximum of 24 per field after five hours and then declined again to 10.5 spores per field. The number of cysts then began to rise once more.

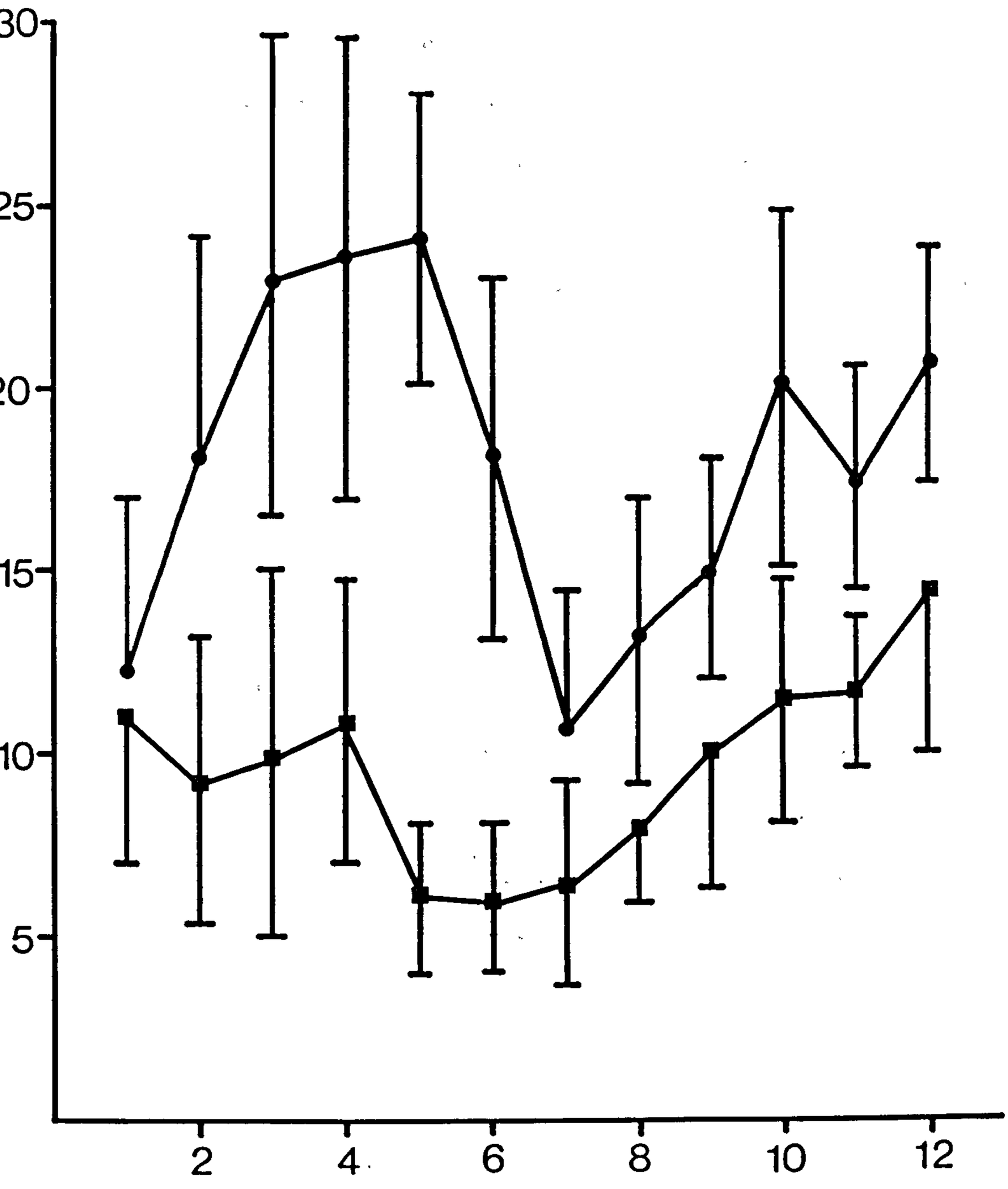
In tap water, an initial decline in the number of encysted spores was followed by a rise to 11 spores per field after four hours. A second, more pronounced decline in the number of encysted spores was then followed by a

Figure 1. The number of encysted zoospores in tap water and distilled water culture over a 12 hour period.

Figure 1. shows the number of encysted zoospores per field at x100 in both tap water and distilled water cultures. Each point is the mean of 10 fields and the bars represent standard deviations.

■—■ Tap water

●—● Distilled water


30
25
20
15
10
5
Number per field
2
4
6
8
10
12
Time (hrs)

further rise.

When the distilled water cultures were examined after 50 hrs. a number of empty cysts were observed (table 1.). Empty cysts were also present in tap water cultures but debris in the water made them difficult to count.

Table 1. Number of encysted zoospores and empty cysts per field, in distilled water culture after 50 hrs.

Number of encysted zoospores	Number of empty cysts	Implied number of encystment cycles
20.3 ± 7.4	84.5 ± 8.1	4.2

Figure 2. shows the results from distilled water and pond water (taken from a small garden pond) cultures followed for 24 hrs and 29 hrs respectively.

In the distilled water culture, a single peak of encystment occurred six hours after the start of the experiment, after which the number of encysted spores continued to rise until 22 hrs.

Empty cysts were visible two hours after the begining of the experiment, four hours before the fall in the number of encysted spores. The number of encysted spores continued to rise throughout the experiment.

The number of encysted spores and empty cysts was counted after 52 hrs. (table 2).

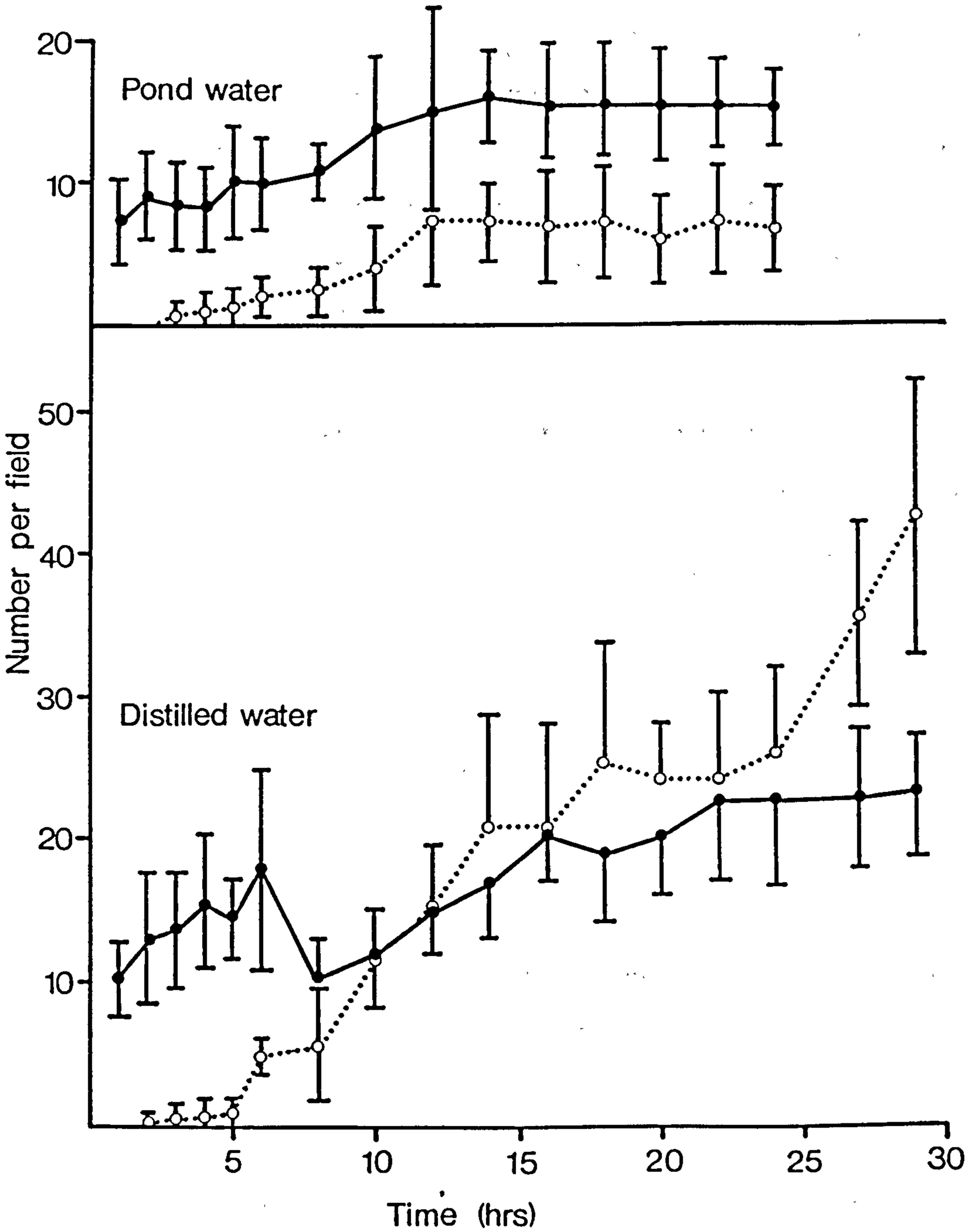
In the pond water culture, a small peak of encystment occurred 2 hrs after the begining of the observation period followed by a smaller increase after 5

Figure 2. The time course of encystment and appearence of empty cysts in distilled water and pond water cultures.

Figure 2. shows the number of encysted zoospores and empty cysts per field at x100 in both pond water and distilled water cultures. Each point is the mean of 10 fields and the bars represent standard deviations.

●—● Encysted spores

○··○ Empty cysts


Pond water
Distilled water
Number per field
Time (hrs)
20
10
50
40
30
20
10
5
10
15
20
25
30

hrs. After this the number of encysted spores continued to rise. Encystment appeared to be complete after 14 to 16 hours. After 16 hrs 13% of the zoospores had germinated.

Empty cysts began to appear after two hours, around the time of the first peak of encystment, and the numbers continued to rise up until 12 hours into the culture.

The number of encysted spores and empty cysts after 23 hours is detailed in table 2.

Table 2. The mean number of encysted spores and empty cysts per field in distilled water and pond water culture.

	Encysted zoospores	Empty cysts	Implied number of encystment cycles
Distilled water	28.1 ± 6.2	66.4 ± 9.8	2.4
Pond water	15.7 ± 2.8	7.3 ±3.9	0.46

Counts were made after 23 hours in pond water and after 52 hours in distilled water.

Figure 3. shows the results of replicate distilled water cultures followed for 22hrs. In this experiment the number of empty cysts were counted each hour as well as the number of encysted zoospores.

Both replicate cultures show two distinct peaks of encystment followed by a decrease in the number of encysted spores. The first peak occurred after three hours in one culture and after four hours in the other. The second peak occurred after 16 hrs in both cultures.

Shortly after the first peak of encystment, empty

Figure 3. The number of encysted zoospores and empty cysts in replicate distilled water cultures over a 23 hour period.

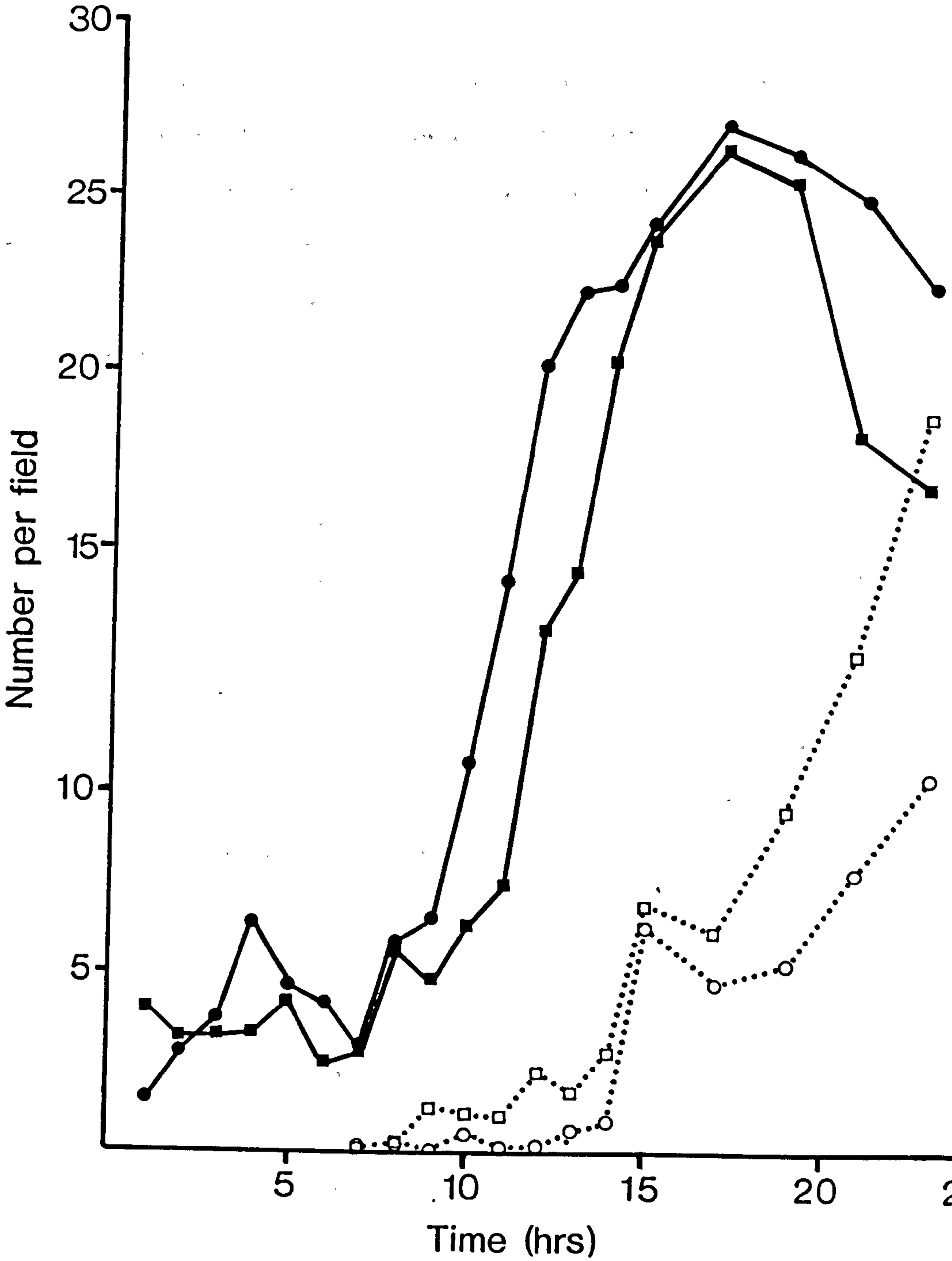
Figure 3. shows the number of encysted zoospores and empty cysts per field in replicate distilled water cultures at x100. Each point is the mean of 10 fields. Standard deviations are not given for the sake of clarity.

Encysted spores — Replicate 1. ■—■

Replicate 2. ●—●

Empty cysts — Replicate 1. □···□

Replicate 2. ○···○


30
25
20
15
10
5
Number per field
5
10
15
20
25
Time (hrs)

cysts began to be visible on the bottom of the petri dish. The numbers of empty cysts rose throughout the experiment and showed no sign of leveling out after 22 hrs.

In the above experiments, the temperature was not controlled and fluctuated between 18 and 21°C. Figures 4a and 4b. show the results of two experiments in which the number of encysted spores and empty cysts were followed in cultures maintained at 15°C and 20°C respectively, using a thermostatically controlled plate. The results of replicate cultures are presented in each case.

In cultures maintained at 15°C (Figure 4a), the first peak of zoospore encystment occurred after five hours in one culture and after six hours in the replicate. After this, further peaks of encystment, each one higher than the last, occurred after 11 hrs., 20 hrs., 29 hrs., 40 hrs., and 55 hrs. The peaks were somewhat more distinct in one culture than in its replicate. The final peak, after 55 hrs was the least distinct.

Two final counts of encysted spores and empty cysts were made after 72 hrs and 82 hrs. The two counts were compared statistically to determine whether any increase in either encysted spores or empty cysts had occurred over this period.

There was no significant difference ($P<0.05$) between counts of empty cysts or of encysted spores in either replicate beteen 72 and 82 hours. The numbers of empty cysts and encysted spores after 72 hrs. (± standard deviations) are given in table 3.

Figure 4a. The number of encysted zoospores and empty cysts in distilled water culture at 15°C over a 72 hour period.

Figure 4a. shows the number of encysted zoospores and empty cysts per field in distilled water culture at 15°C (Magnification x100). Each point is the mean of 10 fields. Standard deviations are not given for the sake of clarity.

Encysted spores Replicate 1. ■—■

Replicate 2. ●—●

Empty cysts Replicate 1. □···□

Replicate 2. ○···○

Figure 4b. The number of encysted zoospores and empty cysts in distilled water culture at 20°C over a 32 hour period.

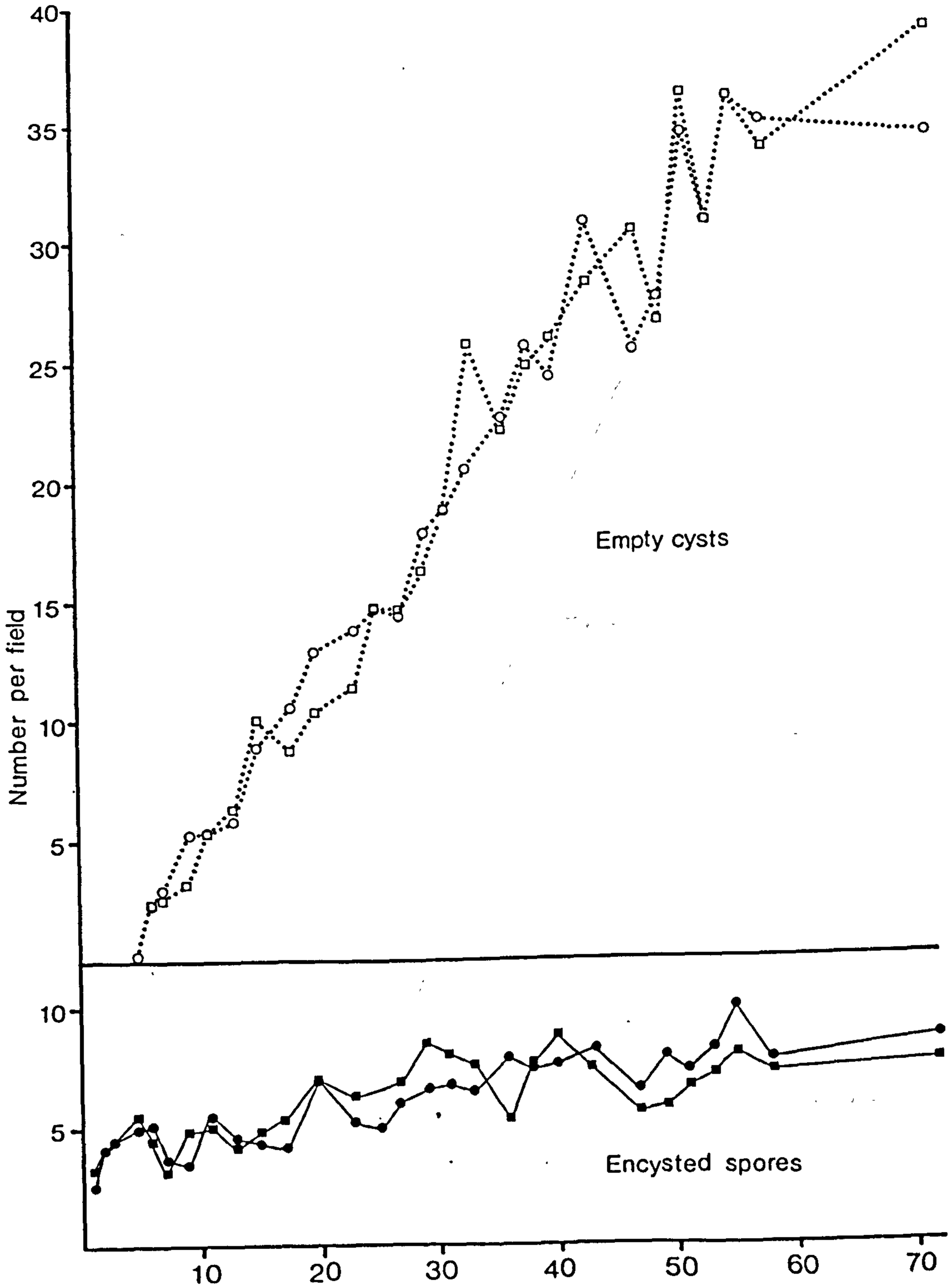
Figure 4b. shows the number of encysted zoospores and empty cysts per field in distilled water culture at 20°C (Magnification x100). Each point is the mean of 10 fields. Standard deviations are not given for the sake of clarity.

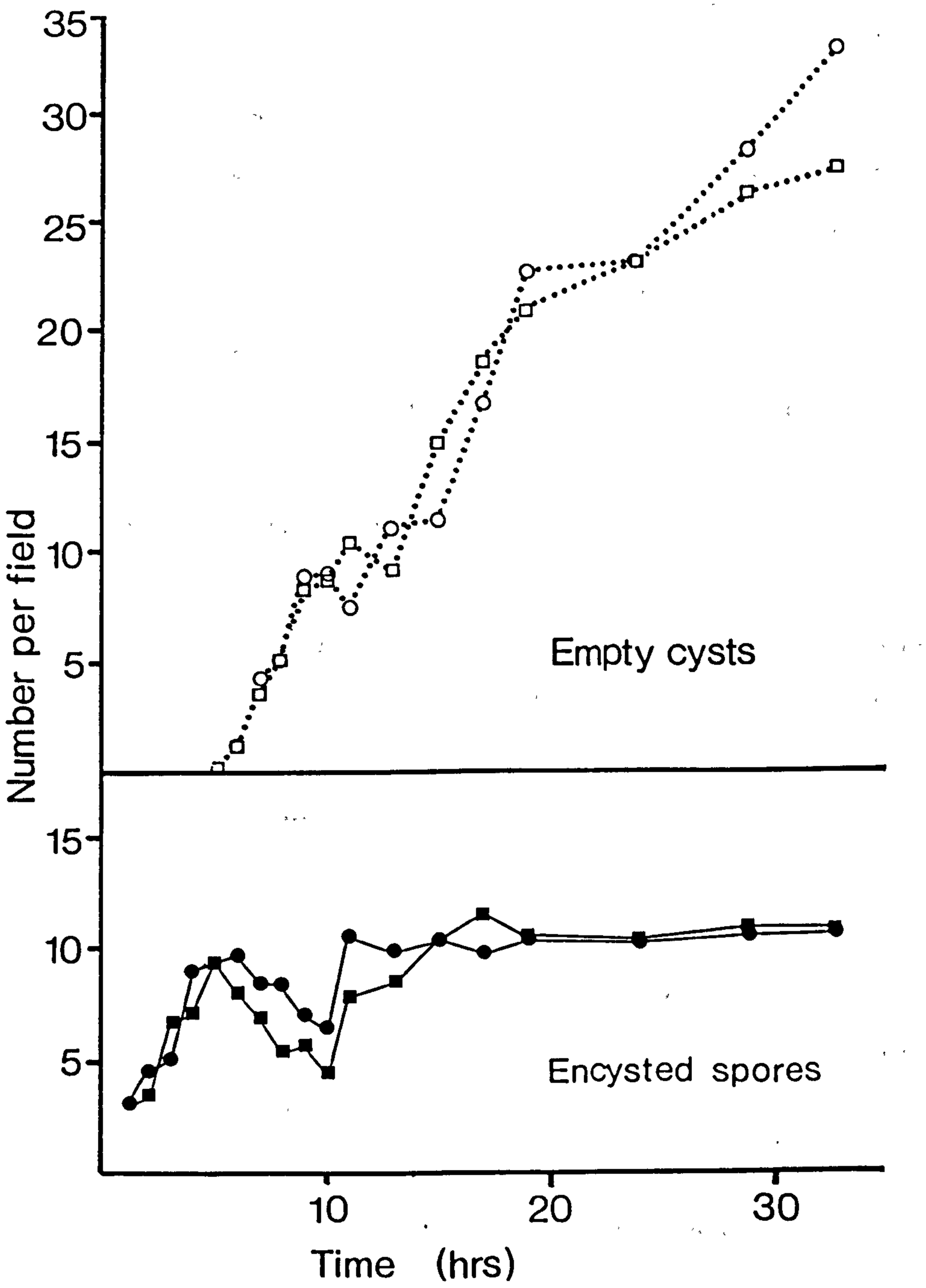
Encysted spores Replicate 1. ■—■

Replicate 2. ●—●

Empty cysts Replicate 1. ○···○

Replicate 2. □···□


40
35
30
25
20
15
10
5
Number per field
Empty cysts
10
5
Encysted spores
10
20
30
40
50
60
70
Time (hrs)


35
30
25
20
15
10
5
Number per field
Empty cysts
15
10
5
Encysted spores
10
20
30
Time (hrs)

At 20°C (Figure 4b), one distinct peak of encystment occurred after five hours in one culture and six hours in the other. There after the number of encysted spores increased, levelling off after 19 hrs.

The first appearence of empty cysts, after six hours, coincided with a fall in the number of encysted spores. The number of empty cysts then rose. However, a temporary slowing in the appearence of empty cysts occurred in both replicate cultures between 11 and 13 hours, this coincided with the presence of low number of encysted spores in the cultures. After this plateau, the number of empty cysts continued to rise, even after the number of encysted spores had reached a maximum, until 34 hrs. The number of encysted spores and empty cysts in each replicate were compared statistically after 33, 48 and 55 hrs to determine whether an increase in encysted spores or empty cysts had occurred.

No significant increase in the number of empty cysts or encysted spores could be demonstrated after 33 hrs, in either replicate ($P<0.05$). The number of empty cysts and encysted spores (± standard deviation) in each replicate after 33 hrs. is shown in table 3.

One drop of formalin was added to each culture to kill all zoospores and a further count was made to ensure that encystment was indeed complete.

In one experiment two zoospore cultures were set up as above, to one culture one drop of formalin was added to kill the zoospores. Both cultures were incubated for 24

hrs at 18°C and the numbers of encysted zoospores and

Table 3. The final number of encysted spores and empty cysts per field in replicate zoospore cultures maintained at 15 and 20°C.

	Encysted zoospores	Empty cysts	Implied number of encystment cycles
20°C rep. 1	10.8 ± 3.8	32.9 ± 8.9	3.04
rep. 2	11.0 ± 3.8	27.2 ± 9.1	2.47
15°C rep. 1	7.7 ± 2.7	41.3 ± 9.2	5.34
rep. 2	8.5 ± 4.2	34.4 ± 8.1	4.05

Counts were made after 33 hrs at 20 C and 72 hrs at 15 C.

empty cysts counted. The results are shown in table 4.

Table 4. The number of encysted spores and empty cysts in formalized and unformalized cultures after 24 hrs incubation.

	Encysted zoospores	Empty cysts
Formalized culture	14.1 ± 5.7	0
unformalized culture	6.4 ± 2.1	5.1 ± 3.6

Means of ten fields at x100 are given + standard deviation.

DISCUSSION.

An initial increase in the number of encysted spores was followed by a decrease which in most cases coincided with the appearance of empty cysts. This implied that a period of zoospore encystment was followed by a period of re-emergence. The phenomenon was apparent in sterile tap water, pond water and distilled water cultures. It was least obvious in pond water, the initial peak of encystment being small, however, the appearence of empty cysts again coincided with a fall in the number of encysted zoospores.

The fact that empty cysts were not seen in formalized fresh cultures indicated that they were not empty primary cysts transfered with the initial inoculum of swimming spores, which had subsequently settled out of solution.

An estimate of the number of encystment cycles that occurred was made by dividing the number of empty cysts by the final number of encysted zoospores. The figure for distilled water varied between 2.4 and 5.34 and appeared to be dependant upon temperature since the number of cycles at 15°C was greater than that at 20°C. The number of encystment cycles in pond water was less, 0.46 compared with 2.4 in the distilled water control.

Generally only one encystment/re-emergence cycle was apparent if the number of encysted spores was followed with time. This was probably due to a progressive

asynchrony of the zoospore cultures. However, in sterile distilled water, at 15°C, 5 cycles of encystment and emergence are clearly shown in replicate 1. (Figure 4a.). Calculations (table 3) imply 5.34 cycles.

Polyplanetism, the multiple re-emergence of zoospores, has been demonstrated in several aquatic fungi. Hohnk (1934) demonstrated polyplanetism in a Saprolegnia and an Achlya species and five successive cycles of encystment and emergence in an unidentified asexual Achlya species at 15°C (Salvin 1940). Recently multiple encystment of Aph. astaci zoospores has also been reported (Cerenius and Soderhall 1984a and 1985). In these studies encystment was induced either by vortex mixing or by addition of growth medium. Induction of zoospores emergence was achieved by replacing encystment media with fresh 1 mM calcium chloride. In this manner three successive generations of zoospores could be produced at 13°C (Cerenius and Soderhall 1984a).

In the present experiments, no artificial stimulus was given to encourage emergence of the zoospores, and, although the initial encystment may have been the result of handling, no further stimulus was applied to induce encystment.

Saprophytic members of the genus Aphanomyces cannot be induced to exhibit polyplanetism under the same conditions as Aph. astaci, and it has been suggested that polyplanetism in Aph. astaci is an adaptation to a parasitic way of life (Cerenius and Soderhall 1985).

Certainly periods of encystment may allow for dispersion between patchy nutrient sources but such behavior raises several important questions. Firstly, are endogenous substrates used to repeatedly fabricate new cyst walls? It has been demonstrated that the soluble glucan fraction of Phytophthora palmivora zoospores decreased upon encystment whilst insoluble carbohydrate increased correspondingly, suggesting that endogenous glucans were utilized during cyst wall synthsis (Tokunaga and Bartnicki-Garcia 1971). Earlier it was noted that successive generations of Achlya sp. zoospores contained less globular material (Salvin 1940). Further, encystment of Aph. astaci zoospores has been shown to involve the disappearence of all the peripheral vesicles with granular contents from the zoospore, and the fusion of several types of vesicle with the plasmalemma. (Cerenius 1985). It therefore appears likely that endogenous reserves are indeed utilized during encystment of zoospores and it may therefore be these reserves that ultimately limit the number of encystment cycles possible.

Secondly, what is the advantage to the organism of multiple encystment cycles? The distribution of host organisms is patchy and it may be the case that short periods of encystment, in this case of between three and five hours, enable the zoospore to be transported by water currents with some degree of protection, to a new area.

Thirdly, are zoospores of Aph. astaci likely to exhibit multiple encystment cycles under natural

conditions? From the limited observations made, it appears that both the total period of activity and the number of encystment cycles are reduced in pond water compared to distilled water, suggesting that under natural conditions the number of encystment cycles may well be less than that observed in distilled water. Further, 13% of the zoospores were found to have germinated after 16 hrs.

A number of ions are capable of inducing encystment of Aph. astaci zoospores at low concentrations (Unestam 1966, Svensson and Unestam 1975) including sodium at 3 mM and calcium at 5mM. Thus ions in the medium may contribute to the reduced period of activity and also to the lower number of encystment cycles in pond water.

In previous studies on repeated zoospore emergence in Aph. astaci, zoospores have been produced by incubation of washed mycelia in 1 mM calcium chloride (Cerenius and Soderhall 1984a) or in lake water (Cerenius and Soderhall 1985). Zoospores produced in this way could be induced to encyst by additions of peptone or tryptone solutions (Cerenius and Soderhall 1985). In the present studies, zoospores were produced by incubating small pieces of agar from the edge of fungal colonies, in sterile distilled water. Thus, peptone from the agar blocks leaching into the spore suspension may have been involved in the encystment of the spores, although to what extent it contributed to the phenomenon of polyplanetism is unclear.

VIRULENCE AND HISTOPATHOLOGICAL EXAMINATION OF APHANOMYCES ASTACI INFECTION IN ASTACUS LEPTODACTYLUS.

INTRODUCTION.

The earliest accounts of crayfish plague (Cornalia 1860, Martinati 1865, Anon 1879), report that the disease often spreads rapidly through crayfish populations, killing infected animals in a matter of days. In a study of crayfish plague in the River Werro in Livonia, Hofer (1900) demonstrated that healthy crayfish caged in waters infected with the disease died within eight days, whilst in laboratory infections, Schikora (1903) observed symptoms of the disease between 8 and 14 days after infection. Unestam (1969c) showed that in laboratory infection experiments Astacus astacus exposed to Aph. astaci zoospore suspensions, died between five and seven days after infection, although only three out of five Ast. leptodactylus were killed. The virulence of Aph. astaci may, however, become attenuated in laboratory culture (Unestam and Svensson 1971).

As early as 1883 fungi of the genus Aphanomyces were described in the tissues of crayfish suffering from crayfish plague (Van dem Bourne 1883) The fungus was susequently mentioned in studies by Hilgendorf (1884), Leuckart (1884) and by Rauber and Magnus (in Schaperclaus 1922). However it was not until Schikora (1904) identified Aph. astaci as the causative agent of crayfish plague that reports of the types of tissue involved were made.

Major sites of infection were found in the arthrodial membranes (Schaperclaus 1934, 1935 and 1954a & b, Nyblin 1934, Unestam 1970 & 1973), especially those of the ventral abdomen and the walking legs. The hyphae ramify both within and between the layers of chitin fibril deposition (Nyhlen and Unestam 1975) and grow out through cuticle at the time of death to sporulate (Schaperclaus 1954b). Infections spread to the connective tissue and occasionally to muscles (Schaperclaus 1927, 1934 & 1954b) and gills (Schikora 1906). The only other tissue that is reported to be infected is nerve (Schaperclaus 1927, 1954a & b, Nyblin 1934 Rennerfelt 1935, Unestam and Wiess 1970). Schaperclaus (1954a) suggested that the organism reached the nerve cord having infected nearby arthrodial membranes and spread along it, eventually reaching the brain and causing the death of the animal. These observations were supported by Nyblin (1931) who demonstrated fungal infections of the brain in all but two animals studied, whilst other organs were spared. The total number of animals studied was not mentioned.

Eye infections are also reported (Schaperclaus 1934 & 1954, Unestam 1970). Hyphae are thought to enter the eye via the optic nerve from the brain and, on death, erupt through the cuticle, often completely covering the eye (Schaperclaus 1954a).

The animals are capable of mounting a cell mediated defence reaction against the invading fungus. Both cytotoxic (Soderhall et al 1985) and humeral defence

mechanisms exist. In response to soluble factors (β 1-3 glucans) released from the fungal cell wall (Unestam and Ajaxon 1976, Unestam and Beskow 1976, Unestam and Soderhall 1977, Soderhall 1978), the semi-granular haemocytes degranulate, releasing prophenoloxidase (PPO). This sticky enzyme binds to the fungal cell wall (Soderhall et al 1979) where it becomes converted to phenol oxidase (PO). Phenol oxidase catalyses the conversion of phenolic compounds - specifically O-diphenols (Unestam and Nyhlen 1974) - to melanin, an orange pigmented compound that encapsulates the fungus preventing it from growing. Intermediates in the pathway of PO activation and melanin synthesis have been demonstated to have fungistatic (Soderhall and Ajaxon 1982), lytic, cell degranulating (Smith and Soderhall 1983) and opsonic (Soderhall et al 1986) properties.

In vitro, haemocytes have been shown to adhere to hyphae in a stream of crayfish hemolymph, encapsulating and eventually melanizing them (Unestam and Nylund 1972), whilst in vivo heavy infiltration of infected regions by haemocytes can readily be demonstrated. It is believed that a combination of encapsulation and melanization of hyphae by haemocytes plays a major role in the animal's defence against attack by Aph. astaci, indeed the haemolymph of resistant species, such as Pascifastacus leniusculus, is capable of stronger melanization of fungal hyphae in vitro than that of susceptible species and this may partly explain their resistance to the disease

(Unestam and Weiss 1970, Unestam and Nylen 1972, Unestam 1975).

Studies such as those of Schaperclaus (1954a), which demonstrate the presence of hyphae within the brain suggest that invasion of nervous tissue by the fungus plays a major role in the death of the animal. However, invasion of the brain is not the only postulated cause of death from aphanomycosis. Nyblin (1931) suggested that secondary invasion of the infected tissues by opportunistic bacteria, which subsequently invade other parts of the body, may ultimately cause the death of animals already weakened by fungal invasion. He also pointed out that crayfish are susceptible to the effects of inoculation with a wide range of bacteria. Moreover, Schaperclaus (1927) revealed the presence of numbers of bacteria in the hepatopancreas of animals infected with Aph. astaci.

Although the disease has been widely studied in the past, no study of the spread of the fungus within the body of whole crayfish has been reported. Nor has data on the size and location of individual foci of infection been published, although Unestam and Weiss (1970) pointed out that after infection with small numbers of zoospores, fungal involvement was often limited. No concise explaination has been made of the cause of death in animals with such apparently limited infections. Information on the progress of fungal invasion at the time of death may be useful in determining the primary cause of

death in animals infected with Aph. astaci.

This study aimed to determine the virulence of English isolates of Aph. astaci to Ast. leptodactylus; and subsequently, using histological techniques, aimed to study the location, severity and extent of individual foci of fungal infection and to relate this information to the survival time of infected animals.

METHODS.

1.Infection.

Isolates of Aph. astaci were obtained by plating out small pieces of arthrodial membrane from infected Austropotamobius pallipes from the Lea valley and the River Wey, onto a glucose peptone agar with oxolinic acid incorporated as an antibacterial agent. Spores were raised from these cultures by incubating plugs of agar plus mycelium in sterile distilled water overnight. The resultant suspension of spores was plated out onto glucose peptone agar and single mycelia removed to separate plates. In this way, single spore isolates were obtained. These isolates were then tested for their pathogenicity towards crayfish by introducing large doses of spores (approx 100 000) to 25 litre aquaria containing several crayfish. Four of the tested isolates found to be pathogenic to crayfish were tested for their virulence towards Ast. leptodactylus. Table 1 details the source of

the isolates used in this experiment.

Table 1. Source of isolates tested for virulence against Astacus leptodactylus.

Isolate No.	Source.
28-1083(4)	River Wey
1-1283(2)	River Rib
1-1283(3)	River Rib
13-0184(1)	River Beane

Ast. leptodactylus of mean weight 23.1 + 1.8g were kept in 30 litre polythene troughs in 25 litres of aerated tap water, at a density of nine animals per trough. Both male and female animals were used. Zoospore suspensions were produced by cutting a number of small pieces of agar from the colony edge and incubating them overnight in sterile distilled water at 18°C. The concentration of zoospores in the medium after incubation, was determined using an Improved Neubauer Haemocytometer. Since the zoospores are sensitive to mechanical disturbance (Svensson and Unestam 1975), tank aeration was turned off and the spores were gently introduced into the tanks with a wide bore sterile pipette, to a concentration of 4 zoospores per ml. (100,000 spores per 25 litres). As far as possible spores were evenly distributed throughout the tank. Tanks were left overnight before aeration was continued. Hides were provided in the form of sections of

PVC guttering.

Animals were maintained at 18°C, and were not fed during the experiment. Control animals were kept under the same conditions but were not challenged with Aph. astaci.

Animals were removed from the tank when moribund or dead and all were examined for signs of infection. Those animals that were moribund were fixed for later histological examination. Crayfish were considered moribund when they failed to respond to external stimuli (such as being prodded or turned over), although their scaphognathite still moved. Animals dead longer than 2 hrs. were not fixed since the fungus is believed to spread rapidly after the death of the animal (Unestam and Weiss 1970); and also to prevent the growth of saprophytes, which may be confused with A. astaci during histological examination.

2. Fixation and histology.

Immediately after examination, animals were fixed in Davidson's decalcifying fixative (Shaw and Battle 1957, appendix 5). Prior to fixation the legs and chelae were removed between the second and third articulation and the branchiostergite was cut away to allow easier decalcification and embedding. Decalcification continued until the rostrum could be easily bent with the finger, during which time the fixative was changed daily. Decalcified pieces were dehydrated in alcohol, cleared in

CNP 30 overnight and embedded, under vacuum, in wax.

Three 10um sections were cut at 750um intervals throughout the block. Head sections were cut longitudinally, as were thorax sections. Abdominal sections were cut transversely.

3.Staining procedure.

Sections were stained using Grocott's modification of Gomori's silver stain (Grocott 1955, appendix 6), which has previously been demonstrated (Southgate pers. com.) to be suitable for staining Aph. astaci in crayfish tissues. Counterstaining was carried out using Cole's haematoxylin and 0.1% eosin.

4.Slide analysis.

4.1.Infection.

Slides were examined under the light microscope for signs of infection of the cuticle and internal tissues. It was generally not possible to distinguish the number of discrete foci of infection in each membrane, thus in practice, the terms infection focus, site of infection and infected arthrodial membrane were used interchangably. The progress of invasion of each infected arthrodial membrane was scored on a 1-5 basis (Table 2.).

Tissues beneath the cuticle (epidermis, connective

Table 2. Values assigned to cuticular invasion severities

Description	Severity
No infection detected	0
One or two hyphae stained	1
Minor invasion of cuticle	2
Moderate invasion of cuticle	3
Major destruction of cuticle	4
Extreme destruction of cuticle	5

tissue, muscle, blood vessels and nerve), were scored in a similar manner. Where arthrodial membranes were close together, for example, the proximal two leg articulations (body to coxa and coxa to basis), it was often not possible to determine with which piece of cuticle the infection of the underlying tissues was associated and these underlying structures were therefore regarded as one site, whilst the sites in the two areas of soft cuticle were regarded as separate.

The final assessment for each site was then calculated as a mean of the assessments for the site on each slide i.e.:-

$$C = \frac{\sum_{n}^{1} c}{n}$$

Where C is the mean assessment for cuticular invasion.
c is the assessment for cuticular invasion on the ith slide.

n is the number of slides in which the site occurs.

This takes account of local variations in the infection severity within each arthrodial membrane.

4.2.Tissue reaction

The tissue reaction was assessed on a scale of 1-7 as indicated in table 3).

Table 3.Values assigned to haemocyte reactions to the fungus.

Description	Severity
No haemocyte reaction	0
Very few haemocytes at site	1
Moderate haemocyte infiltration	2
Major haemocyte infiltration	3
Haemocyte compaction	4
Minor melanization	5
Moderate melanization	6
Extreme melanization	7

The assessment was based on the progress and degree of haemocyte infiltration into the infected area. An efficient haemocyte response was considered to be one that prevented spread of the invasion from its original site in the cuticle, to other tissues. Accordingly, the strength

of the haemocyte response below the cuticle was assessed as a measure of the animal's immune response to the fungus.

Each site on each slide was assessed separately and the overall haemocyte response at the cuticle (H) for each site of infection was calculated as the mean of the values assigned to the site on each slide, thus :-

$$H = \frac{\sum_{n}^{1} h}{n}$$

Where h is the assessment for the haemocyte reaction on the ith slide
n is the number of slides on which the site occurs

4.3.Spread of the fungus within the crayfish

In order to determine the proportion of tissues affected by fungal hyphae, the volumes of the section and of infected tissue in that section was calculated as follows.

Each slide was projected onto a piece of A4 paper using a photographic enlarger. The outline of the section was traced and the extent of any invaded area was then added. The area of the traced sections and the area of the infection were then measured by planimetry.

The sum of the areas of the individual sections ΣAS is proportional to the volume of the animal, and likewise the sum of the individual areas infected on each slide ΣAI is propotional to the volume of the infected tissue. Therefore the following is a measure of the

percentage of the infected tissue within the host:-

$$\frac{\sum AI}{\sum AS} \times 100$$

RESULTS.

1. Virulence of Aphanomyces astaci isolates.

Figure 1 illustrates the cumulative mortality curves for each isolate of Aph. astaci. Table 4 lists the mean survival time (MST) for animals infected with each isolate of the fungus. Each isolate caused the death of all nine crayfish challenged. The last animal died 94 days after the initial challenge. Identification letters refer to figure 1.

Table 4. Comparison of MST for four isolates of A. astaci.

Isolate No.	Id. letter	MST (+ SD)
28-1083(4)	A	9 ± 3.5
1-1283(3)	B	16 ± 17.3
13-0184(1)	C	18.5 ± 13.4
1-1283(2)	D	30.3 ± 33.9

Id. letter = identification letter.

Clearly the most virulent isolate tested was that from the River Wey (28-1083(4)) since it not only caused the fastest knockdown effect but also gave the shortest mean survival time.

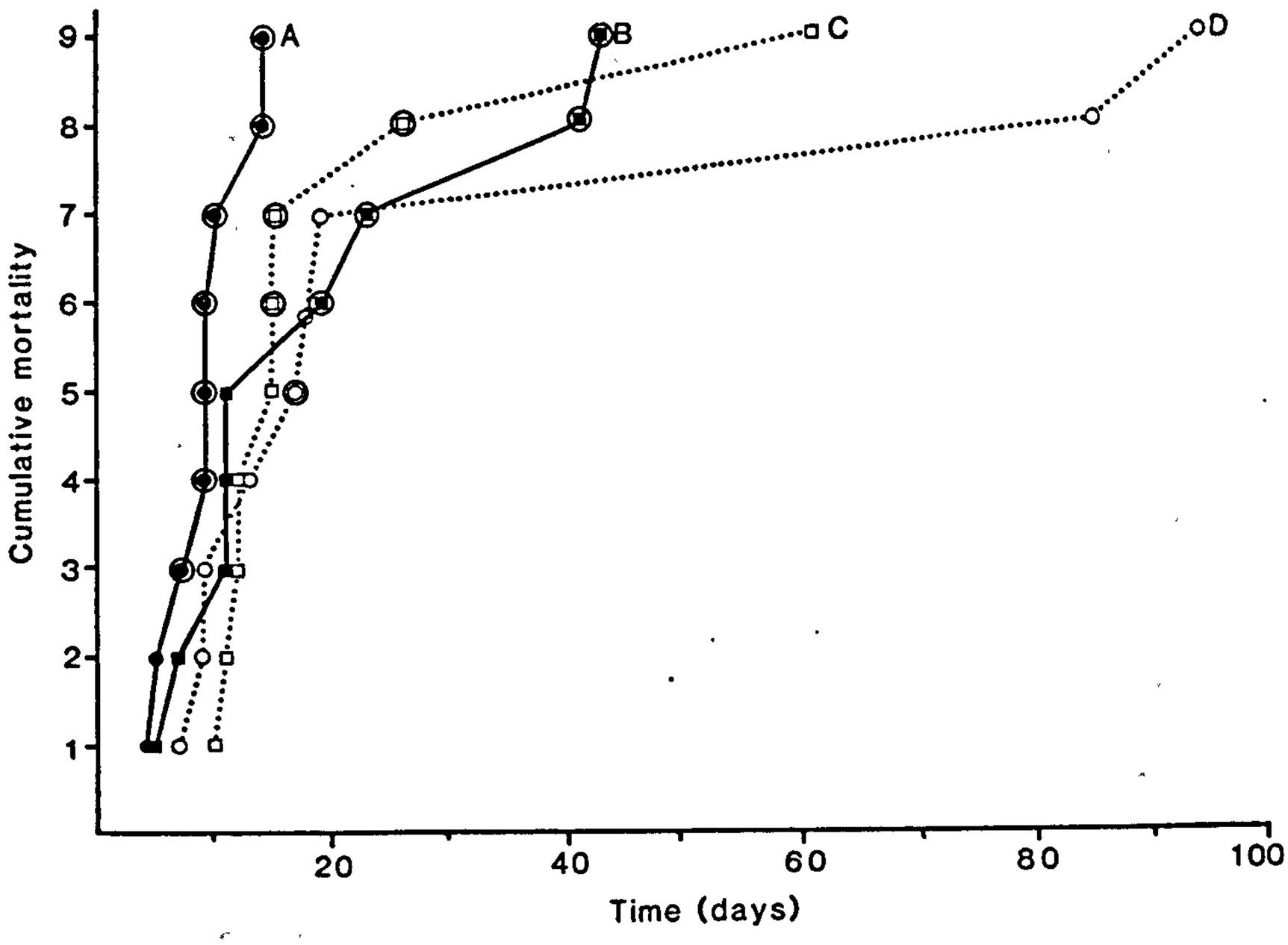
Figure 1. Time mortality curves of four groups of animals infected with Aphanomyces astaci.

Each group of 9 animals was infected with a separate isolate of Aph. astaci at a dose of 4 swimming zoospores / ml and infected animals were kept at 18°C in static aerated water.

O Denotes animals subsequently used for histopathological examination.

A - isolate 28-1083(4)
B - isolate 1-1283(3)
C - isolate 1-1283(2)
D - isolate 13-184(1)

TTD = Time to death.


A
B
C
D
Cumulative mortality
1
2
3
4
5
6
7
8
9
20
40
60
80
100
Time (days)

1.Gross external signs of infection.

Animals were examined for gross external signs of infection and the results are recorded in table 5.

Table 5. Gross external signs of Aphanomyces astaci infection.

Site of infection.	No.	Percentage
No obvious pathology	12	33.3
Chelae or walking legs	24	66.6
Subabdominal cuticle	1	2.7
Eye	1	2.7

No. = number of animals.

Sites of infection were not always easy to detect, indeed only one third of animals examined showed any visible pathology at all. The most common manifestation of Aph. astaci infection was the presence of a cream to orange discoloration of the proximal two joints of the walking legs or chelae. This is due to the infiltration of a large number of haemocytes into the infected area, and the subsequent production of melanin, in response to the fungal hyphae. Infection of the ventral abdomen was seen in only one of the 36 animals examined. This was manifested as a brown patch, appearing to radiate from the base of one pleopod, within which melanized hyphae could be seen. In one animal an infection of the eye was prominent. The entire surface of the eye appeared to be

white where hyphae had erupted through the distal portion, and ramified across its surface.

The distribution of visible foci of infection agrees largely with earlier published descriptions of the disease in European crayfish. Schikora (1905), for example, describes foci of infection "in the hip joints of the legs, the cornea of the eyes, the gills, the appendages of the abdomen and the anal region". However, the scarcity of visible infected areas in the arthrodial membranes of the ventral abdomen is noteworthy. Only four animals out of 15 had such infections and these were of a minor nature. Unestam (1972) however, suggests that this is the most common site of invasion in the susceptible European species, Ast. astacus. In P. leniusculus however, a species resistant to crayfish plague, the ventral abdomen was seldom infected, although chronic infections were found in the leg and chelae joints and in the tips of the pleopods.

2.Microscopic examination.

2.1.Gross Histopathology.

Individual infection foci vary widely in their severity. Some may consist of only a few hyphae (Figure 2), whilst in other cases the cuticle may be badly degraded (Figure 3). Although hyphae ramify throughout the soft cuticle they are most common in the exocuticle. From the cuticle the fungus spreads to the epidermis below. In severe cases the hyphae occasionally spread into areas of

Figure 2. Mildly infected cuticle.

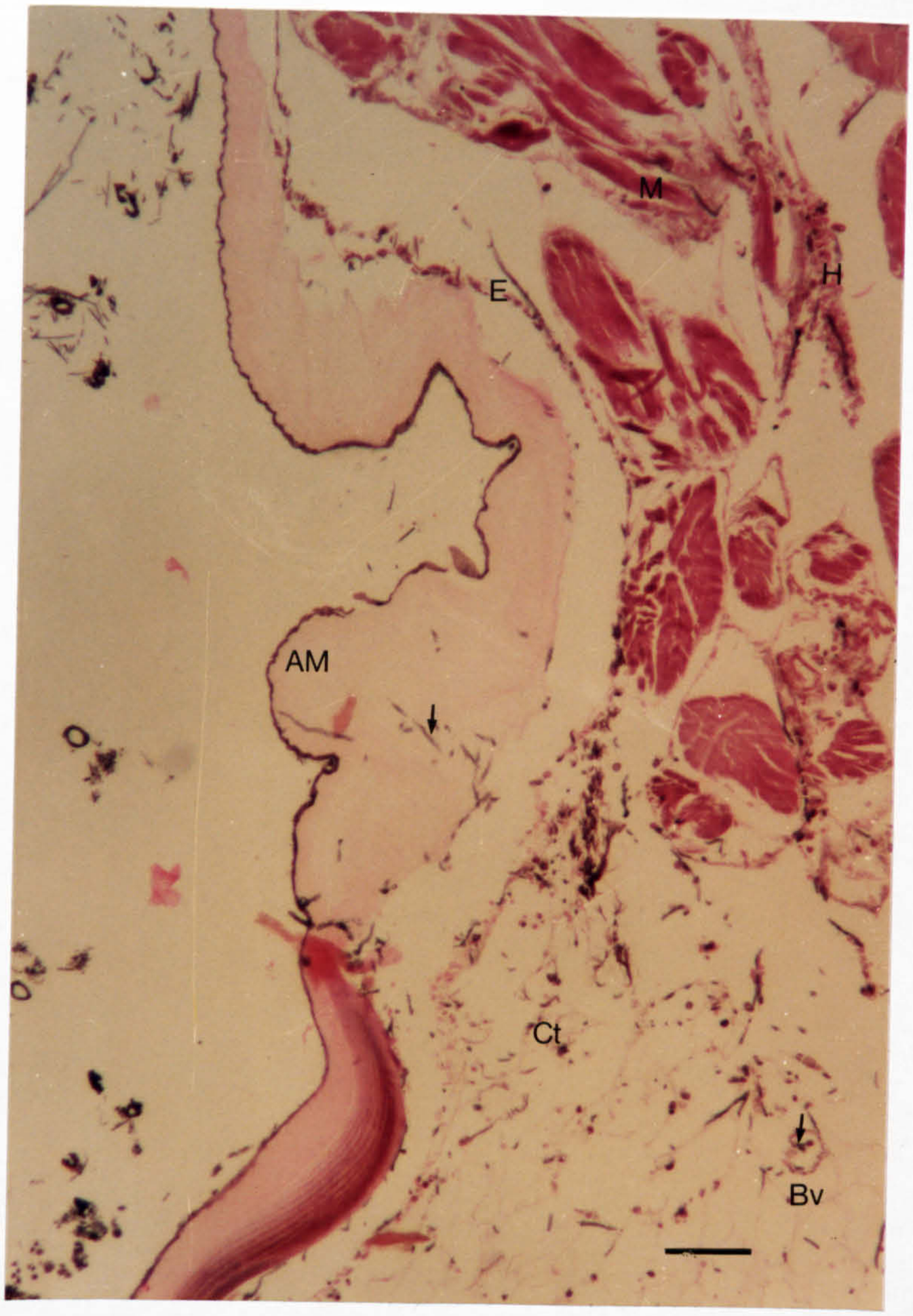
This figure shows a portion of mildly infected arthrodial membrane from the second joint of a walking leg. Haemocyte infiltration in the area immediatly below the cuticle is slight. Below the arthrodial membrane (AM), epidermis (E), connective tissue (Ct), muscle (M) and a minor blood vessel (Bv) have been invaded by fungal hyphae (arrowed). Close to the infected muscle, haemocyte encapsulation(H)of the fungal hyphae can be seen.

Scale bar = 200um.

Figure 3. Severely infected cuticle.

This figure shows a portion of arthrodial membrane from the proximal jeg joint of the rear most pair of walking legs. The cuticle has been badly degraded and the haemocyte reaction to the invading organism is well advanced.

Scale bar = 100um.


M
H
E
AM
Ct
Bv

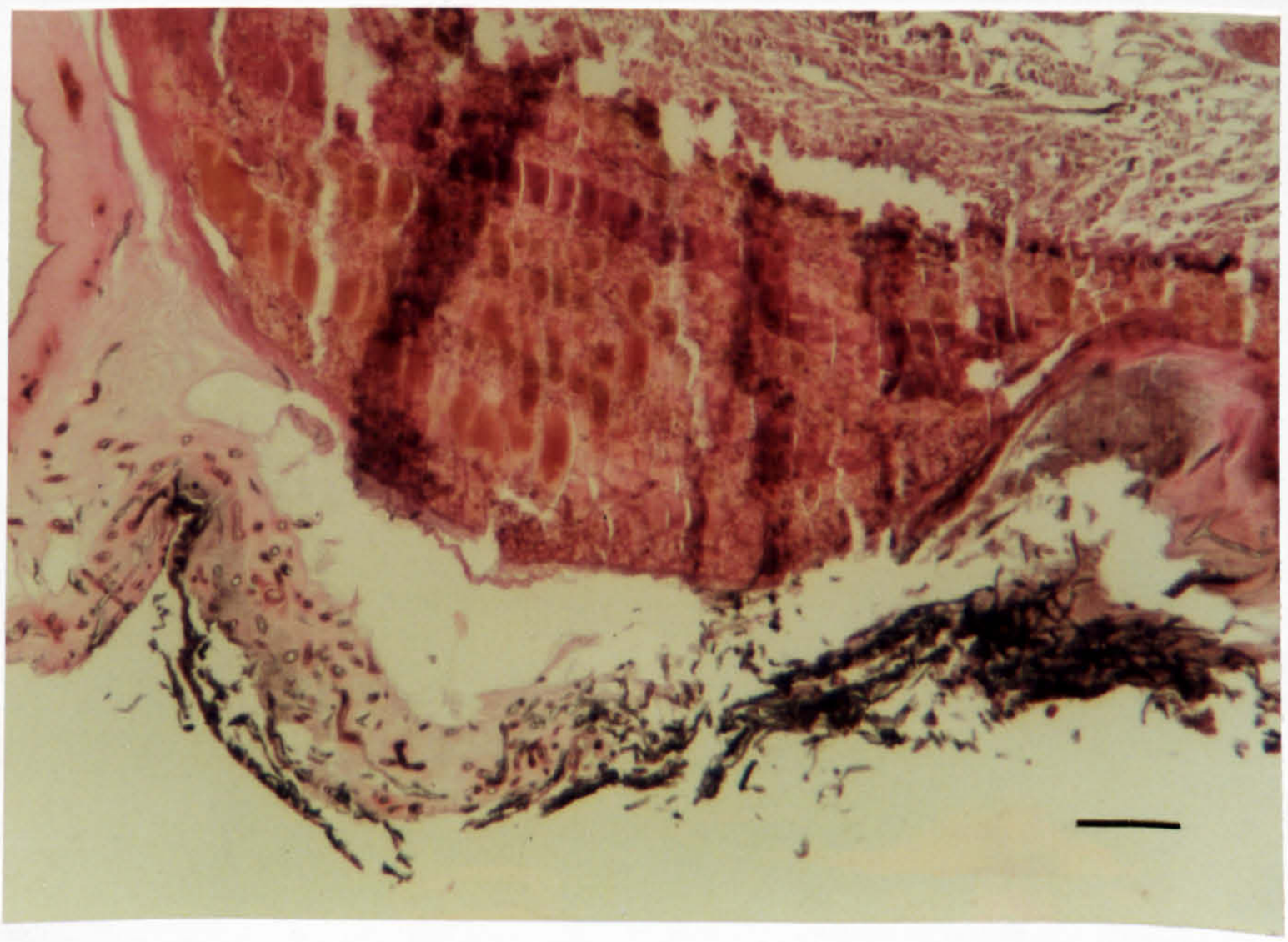
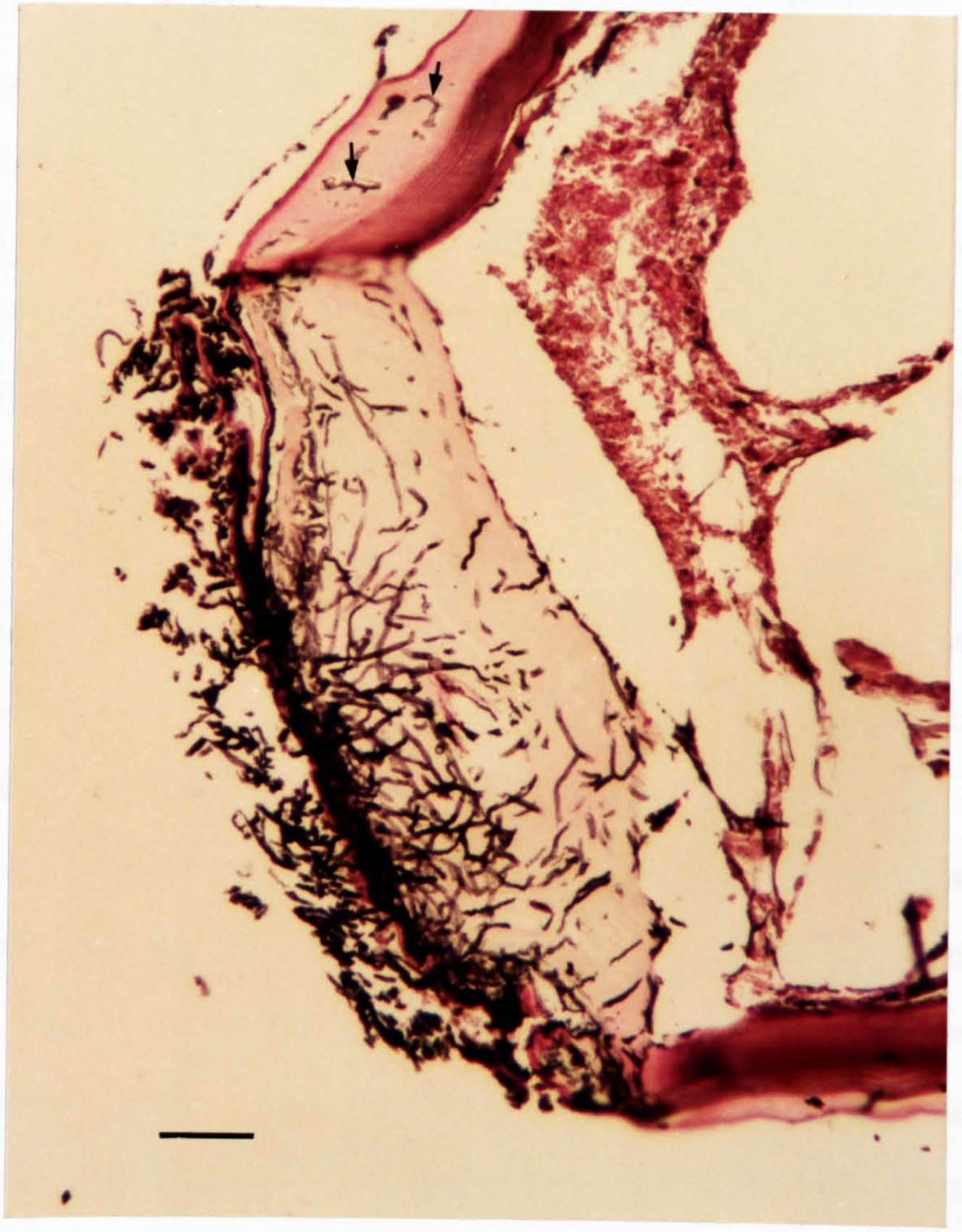


Figure 4. Severe infection with limited invasion of the hard cuticle.

This figure shows a severe invasion of one of the proximal joints of an antenna. Hyphae (arrowed) can be seen invading the hard cuticle. Note the concentration of hyphae below the epicuticle.

Scale bar = 200um.



hard cuticle (Figure 4). Often swellings were apparent in hyphae immediately below the epicuticle (Figure 5a & b). Occasionally hyphae are seen to be clustered below the epicuticle, possibly as a result of expansion of the mycelium after the sporeling penetrates the epicuticle (Figure 4). In some cases the epicuticle appears to be lifted away from the rest of the cuticle by the action of the fungal hyphae beneath it. This may be an artefact caused by the preparation of the animal for histology, however, since the effect is not seen in control animals prepared in a similar way, it must be due, at least in part, to the effect of the fungus in weakening the structure of the cuticle.

In crayfish, the epidermis is only one cell thick and hence any infection of this structure is locally severe, that is, fungal hyphae occupy a large proportion of it (Figure 6). Hyphae may pass directly through the epidermis to deeper tissues, or ramify within it, sometimes for a considerable distance beyond the main bulk of fungal hyphae.

Where haemocyte reactions failed to restrict the spread of the fungus to the cuticle or epidermis, hyphae proliferated within the connective tissue (Figure 7). In several cases the fungus was seen to spread beyond melanized areas by ramifying within the epidermis. It was in the cuticle epidermis and connective tissue that most of the fungus was found.

Infection of muscles was usually minor, with a few

Figure 5. Hyphal bulging.

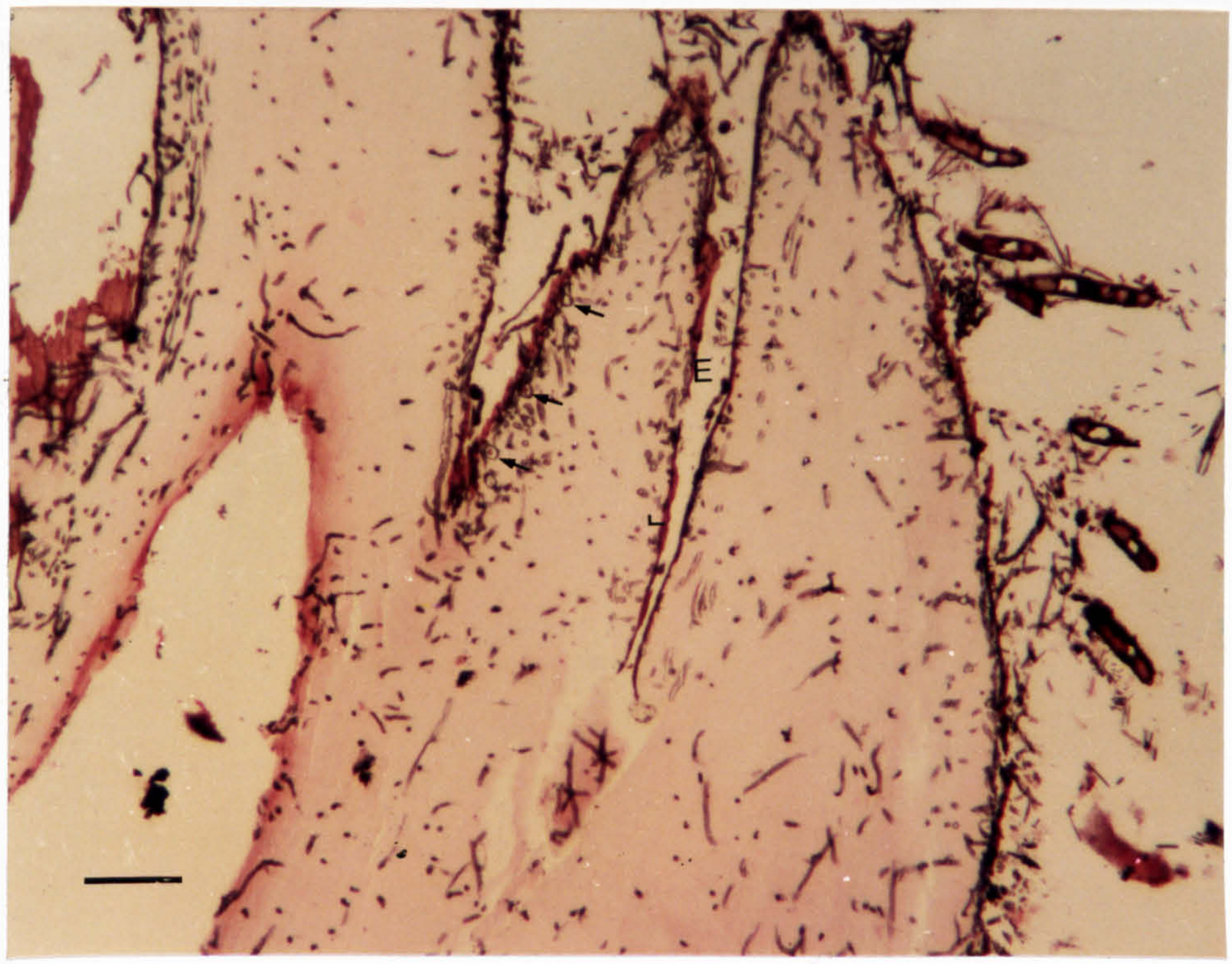
These two figures show hyphal bulging in Aph. astaci hyphae where they contact the epicuticle. Bulging may also occur on the outer surface of the epicuticle as in figure 5b.

Figure 5a scale bar = 200um.

Figure 5b scale bar = 50um.

E = Epicuticle.

a



b

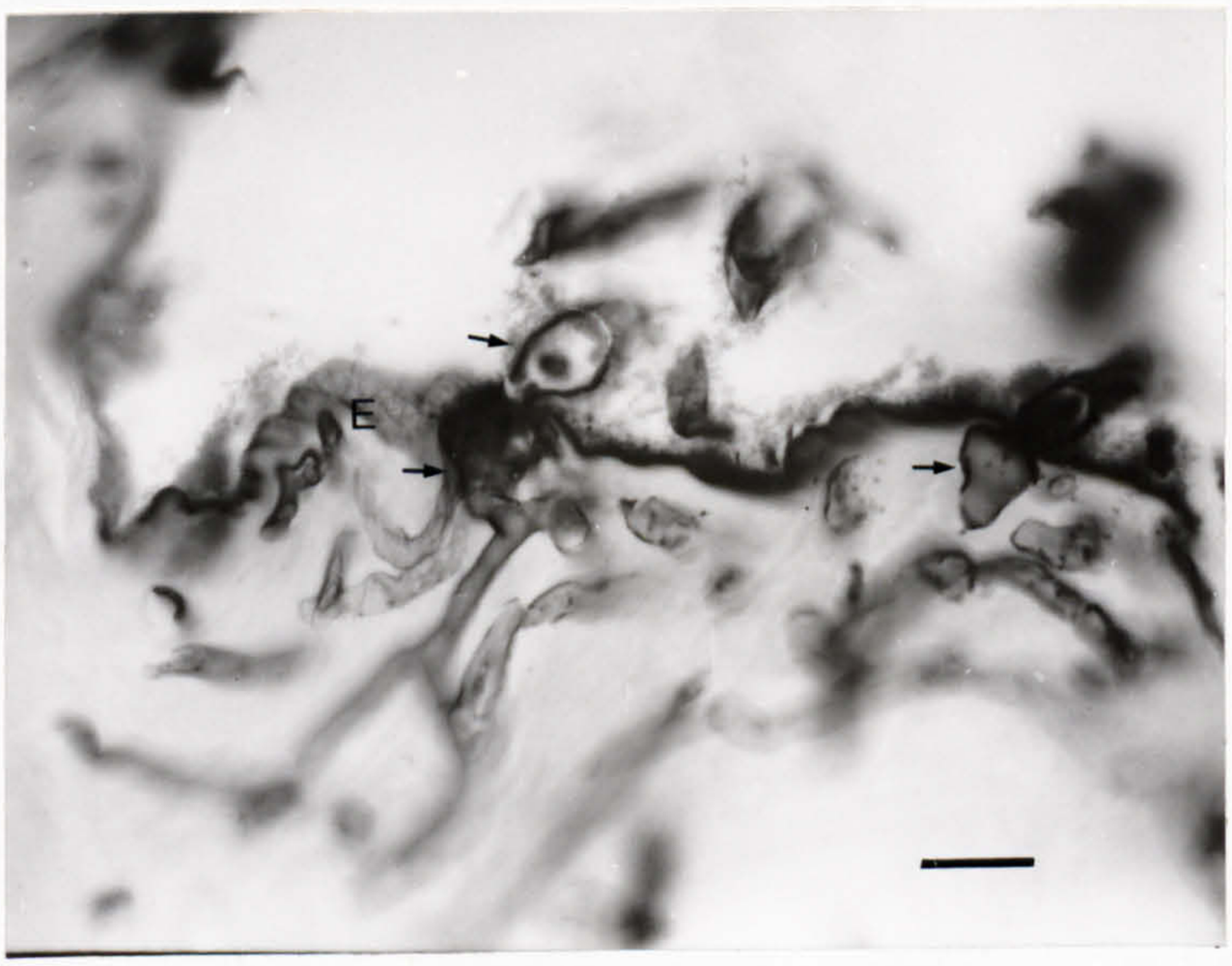


Figure 6. Fungal invasion of the epidermis.

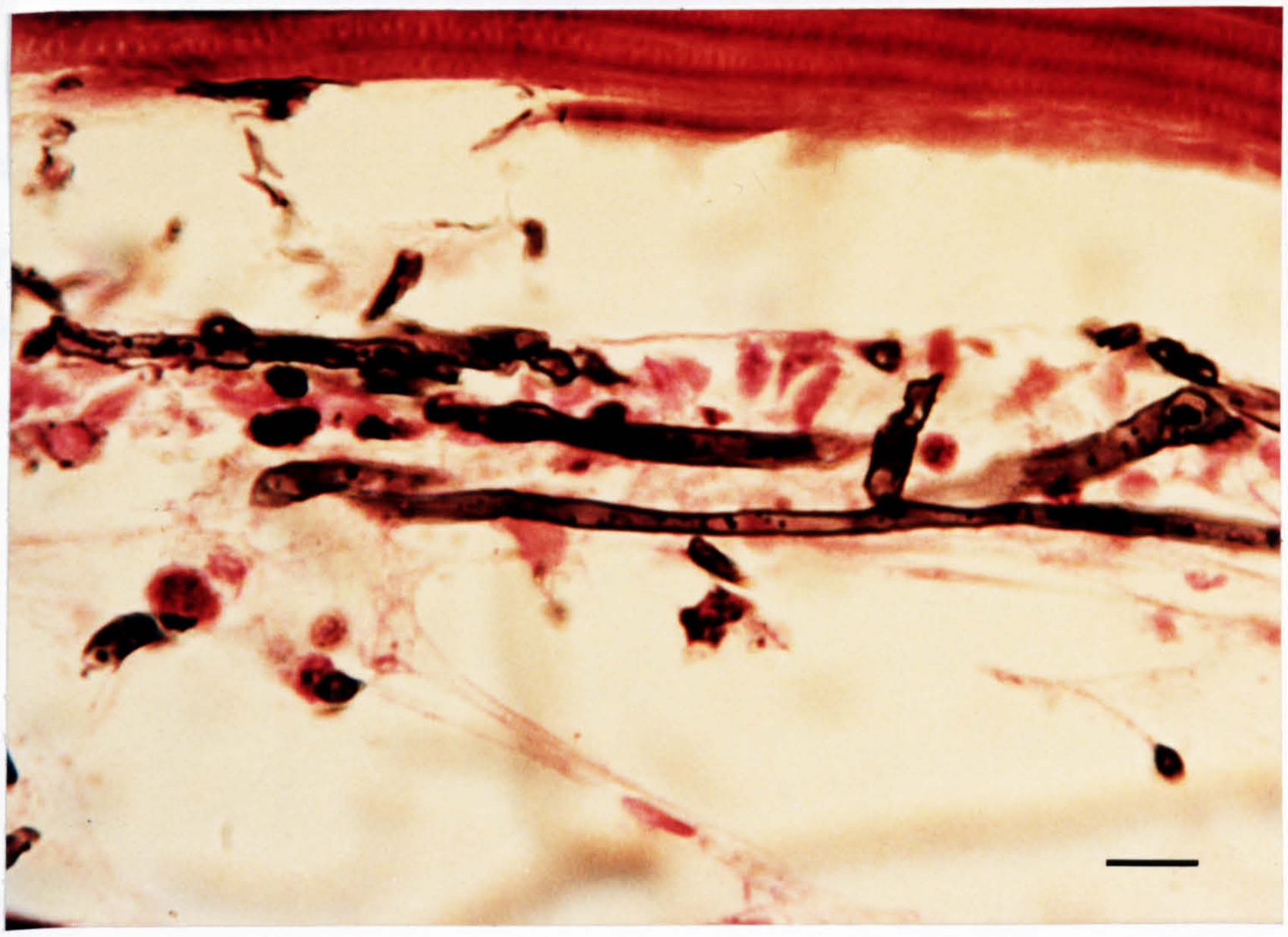
After passing through the arthrodial membrane the fungus often ramifies within the epidermis for some distance. in this case the hyphae are seen below the hard cuticle of the dorsal abdomen, where they occupy a large proportion of the tissue.

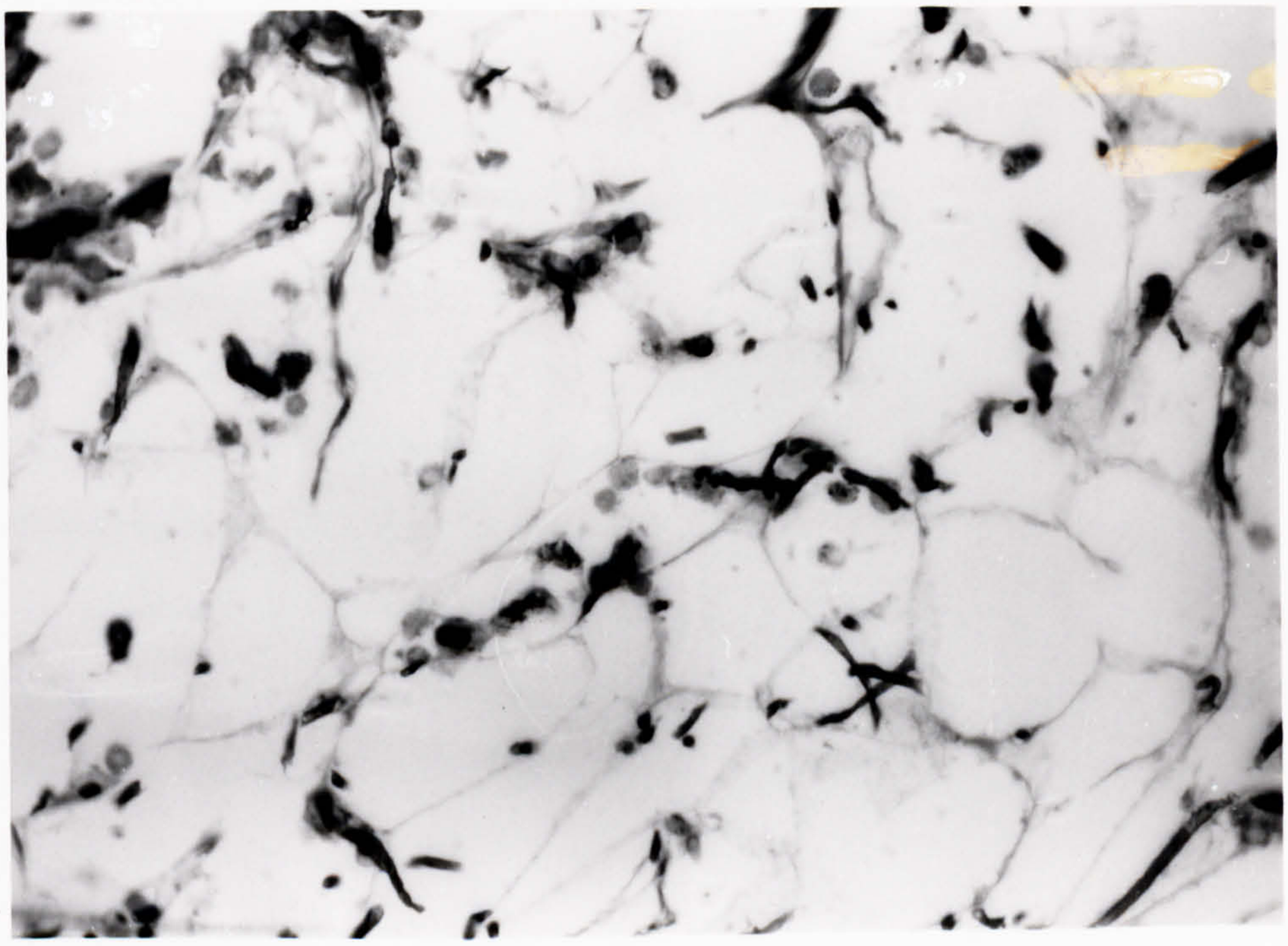
Scale bar = 20um.

Figure 7. Aphanomyces astaci hyphae in the conective tissue.

This figure shows hyphae ramifying through the connective tissue with little haemocyte response.

Scale bar = 200um





hyphae being seen between and occasionally within the fibres (Figure 8a & b). Infection was usually confined to those blocks in the vicinity of the cuticular lesions. Thus leg muscles, abdominal flexor muscles and occasionally eye articulation, and uropod and abdominal extensor muscles are involved. Muscle infection is only severe when hyphal proliferation in the surrounding connective tissue is strong, and such severe infections are generally local in nature.

Invasion of blood passages was common, and both major and minor sinuses and vessels were affected. Hyphae ramified not only through the vessel walls, but also within the lumen, and whilst vessels were seldom blocked by the hyphae themselves, the haemocyte response to the fungus occasionally occluded them (Figure 9a, b & c). Thus, in a number of animals the haemolymph supply to certain areas of the body may have been restricted to a degree by hyphal invasion of blood passages. Since hyphae were occasionally observed in blood vessels several millimeters distant from the main body of hyphae, it seems reasonable to suppose that infection may spread along these vessels to other parts of the host's body.

Invasion of the nervous system was as common as invasion of the blood passages. Although both major and peripheral nerves were infected, the most commonly infected nerves were the motor nerves of the abdominal flexor muscles and the smaller muscles of the dorsal

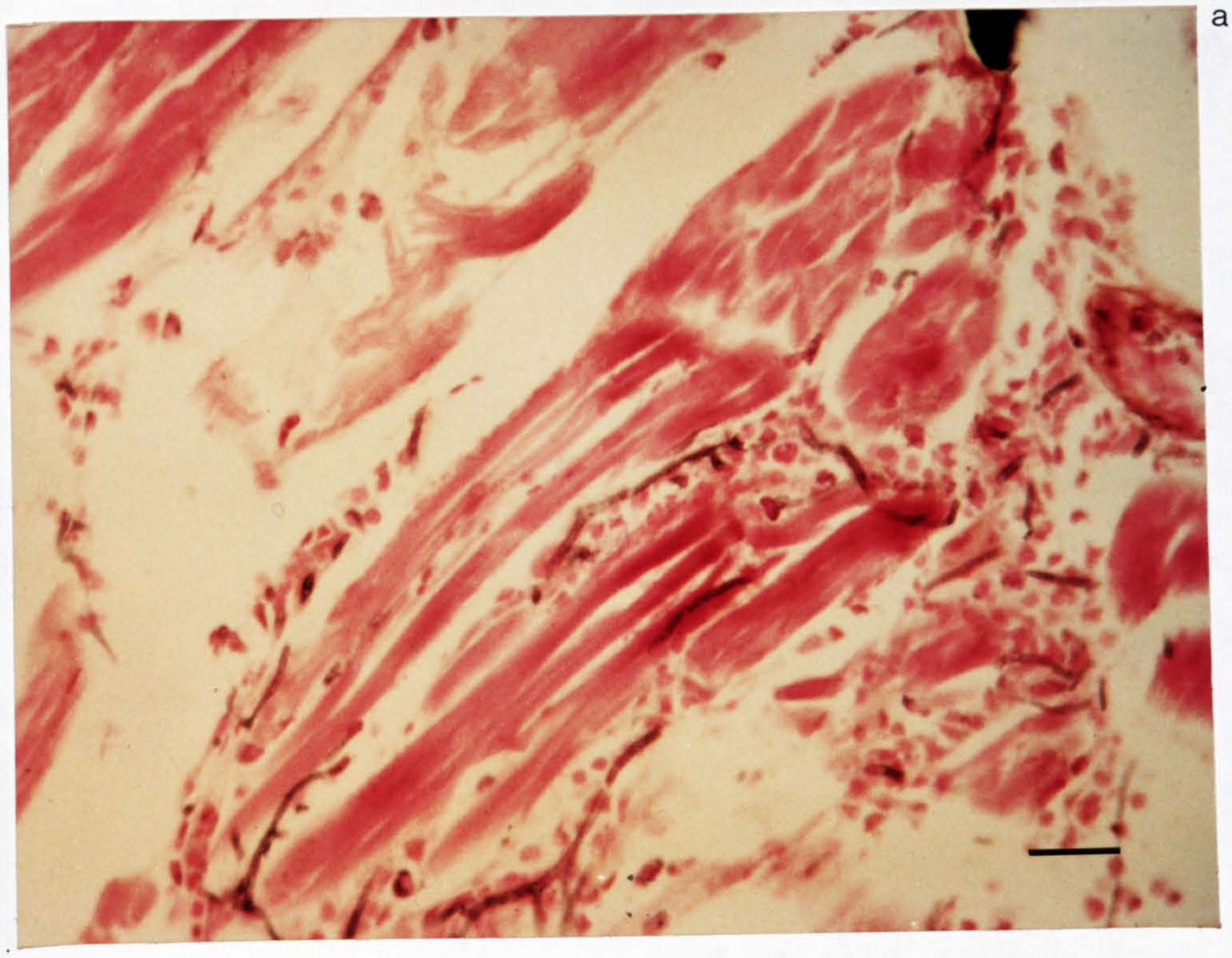
Figure 8. Aphanomyces astaci hyphae in muscle.

Both figure 8a and 8b show Aph. astaci hyphae in the muscle. Figure 8a shows hyphae between the fibres of a leg muscle. Haemocytic encapsulation of the fungus is taking place.

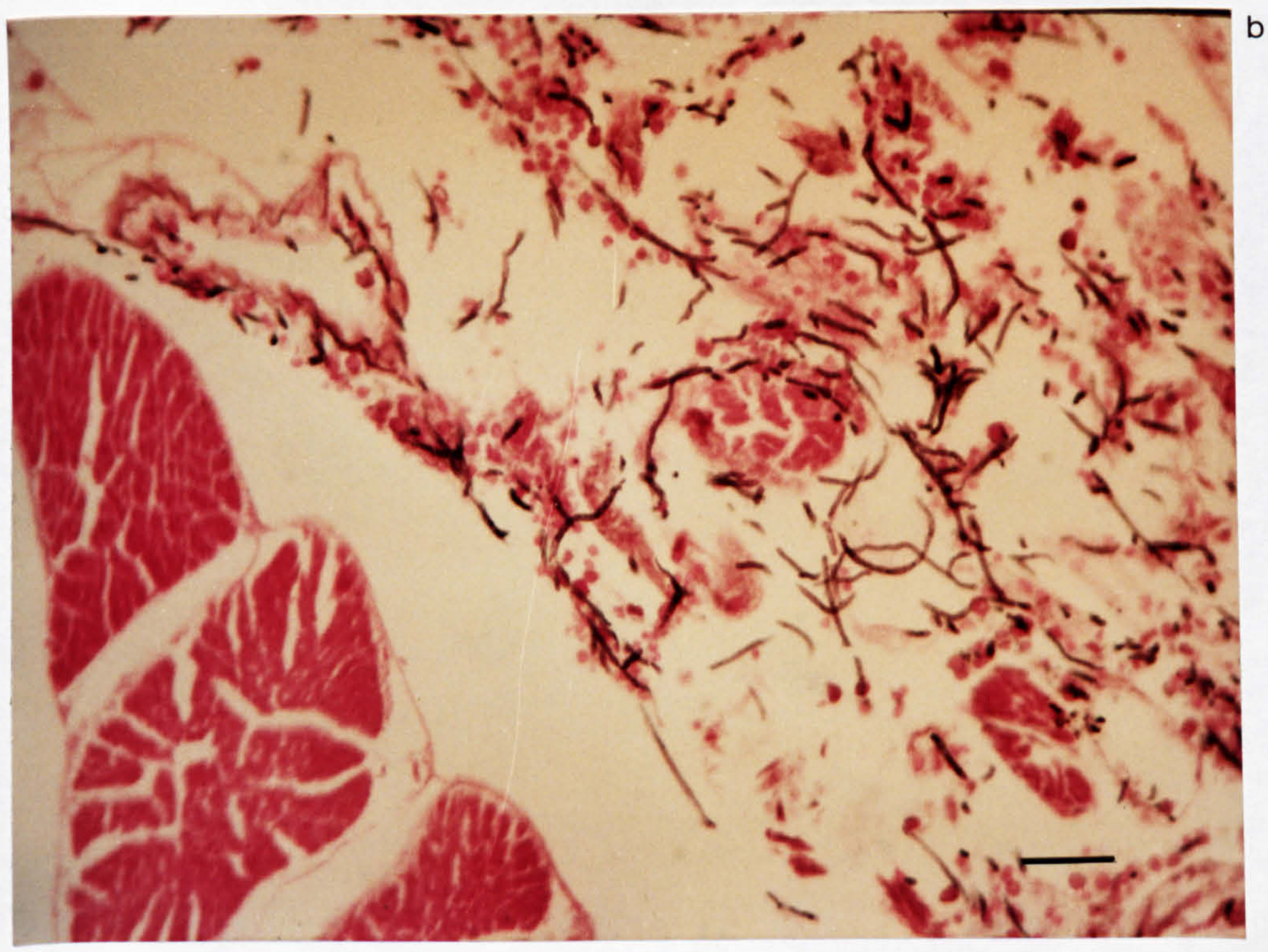
Scale bar = 200um.

In figure 8b, a smaller muscle block is involved, again hyphae are mainly ramifying between the fibres of the muscle and again a haemocyte reaction to the fungus is taking place. Note also the infection of two small blood vesssels in the upper right and lower left of the figure.

Scale bar = 100um.



a



b

Figure 9. Aphanomyces astaci hyphae in blood vessels.

Figure 9a shows the dorsal abdominal blood vessel invaded by fungal hyphae. A haemocyte reaction has taken place to hyphae both inside and outside the vessel. The lateral blood vessels are also infected.

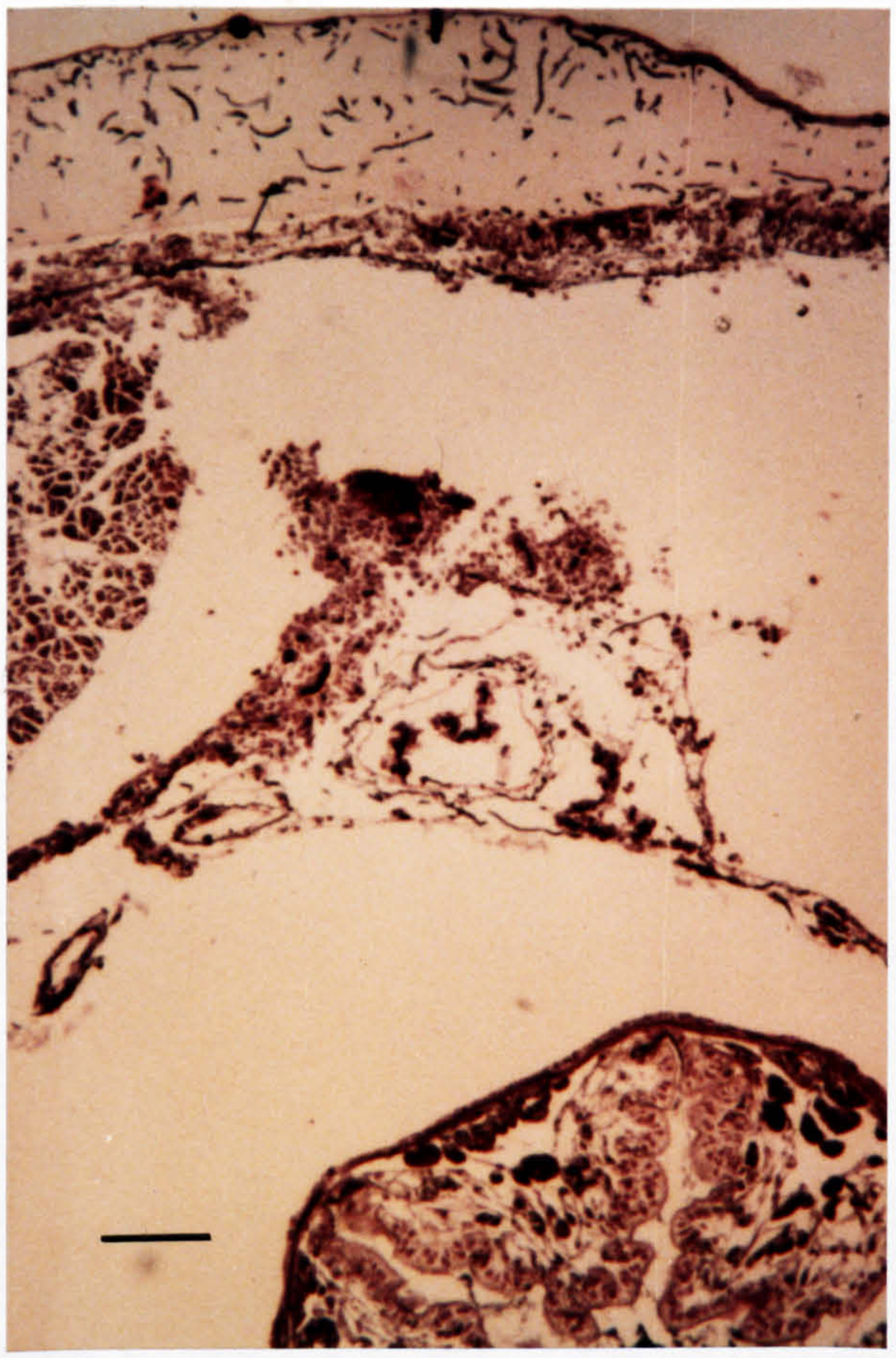
Scale bar = 400um.

Figure 9b shows fungal hyphae in a superficial blood vessel of the leg. Note that hyphae ramify both through the thin walls of the vessel and within the lumen. See also figure 8.

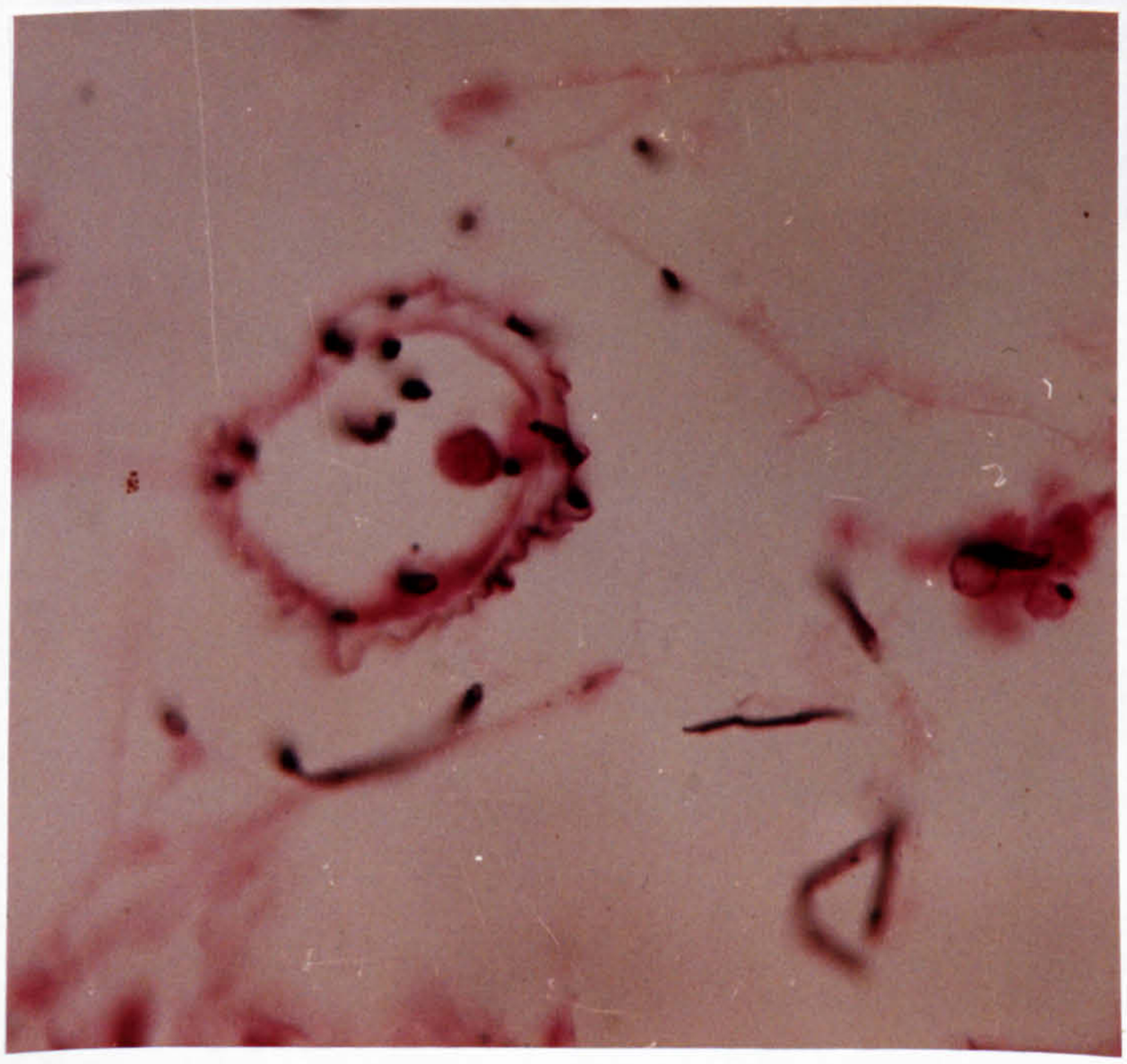
Scale bar = 100um.

Figure 9c (over leaf) shows hyphae in a larger blood vessel of the leg. A strong haemocyte reaction to the fungus has occurred and this has almost blocked the vessel.

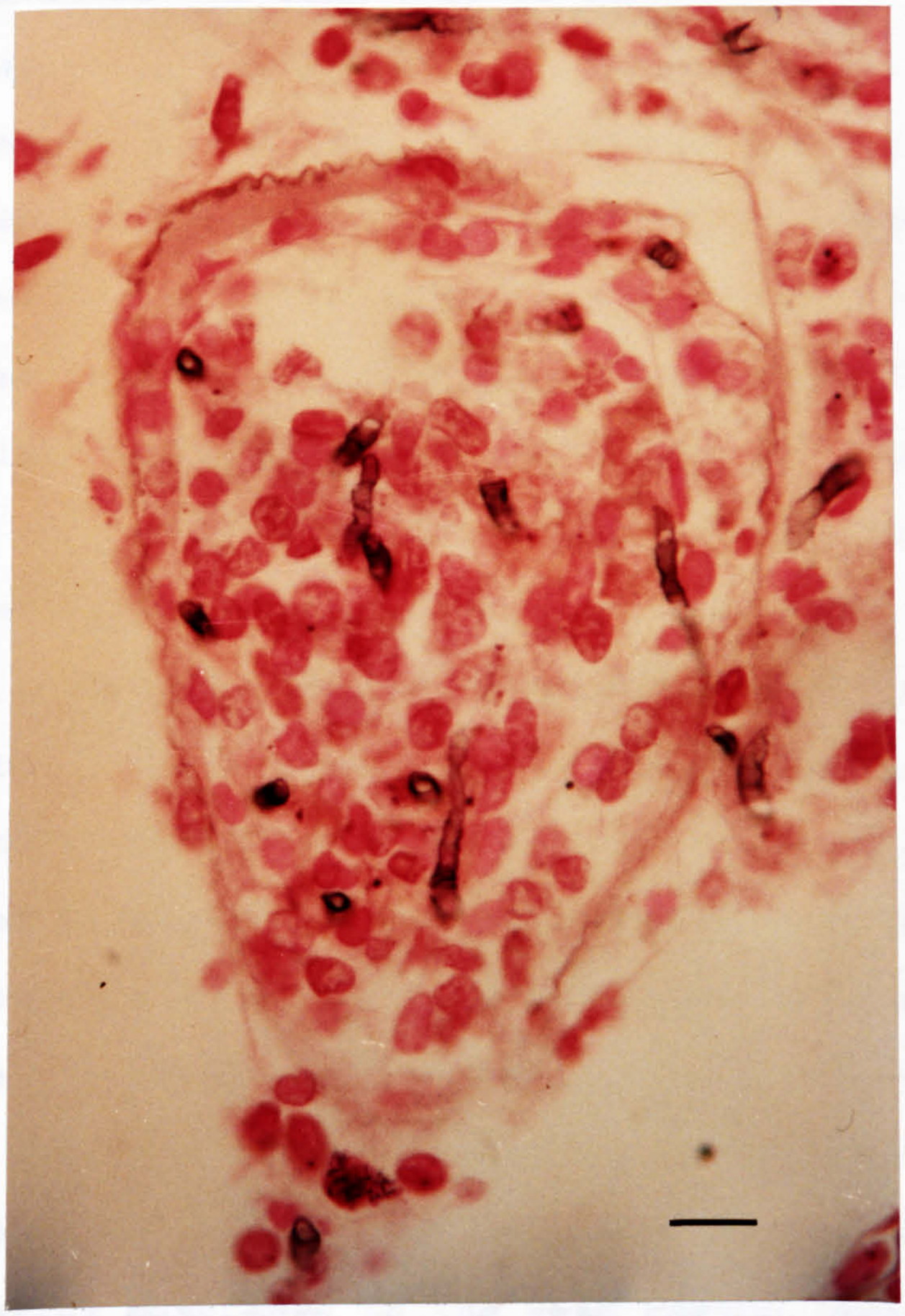
Scale bar = 50um.



a



C



abdomen and the leg muscles. The most commonly infected major nerves were the distal portions of the lateral nerves serving the legs (Figure 10a).

Although there was no evidence that the fungus was neurotrophic, where fungal hyphae were seen in the nerves infection was occasionally severe, to the extent that the nerve bundle was barely discernable. However, on several occasions large numbers of fungal hyphae could be seen in the cuticle and connective tissue very close to nerves, and yet no sign of invasion of these structures was seen. Invasion of the animals brain was not seen in the crayfish studied, although connective tissue immediately adjacent to the brain was heavily infected. A single hypha was seen in one circumoesophageal commissure in one case . In these instances it appeared that the membrane surrounding the brain acted as a barrier to the fungus. There was no evidence to suggest that the fungus used the ventral nerve cord as a pathway to the brain, since any infection of the brain appeared to occur as a direct result of invasions of articulating membranes in the head region, namely those of the eye or antennae. In fact the nerve cord was only found to be infected on one occasion (Figure 10b). Fungal hyphae were also found in the lateral nerves of the thorax and abdomen.

Infections of the eye occurred in two of the animals studied (bilaterally in on case), although only one of these was visible on gross external examination (Table 5). Since these invasions occurred in the absence

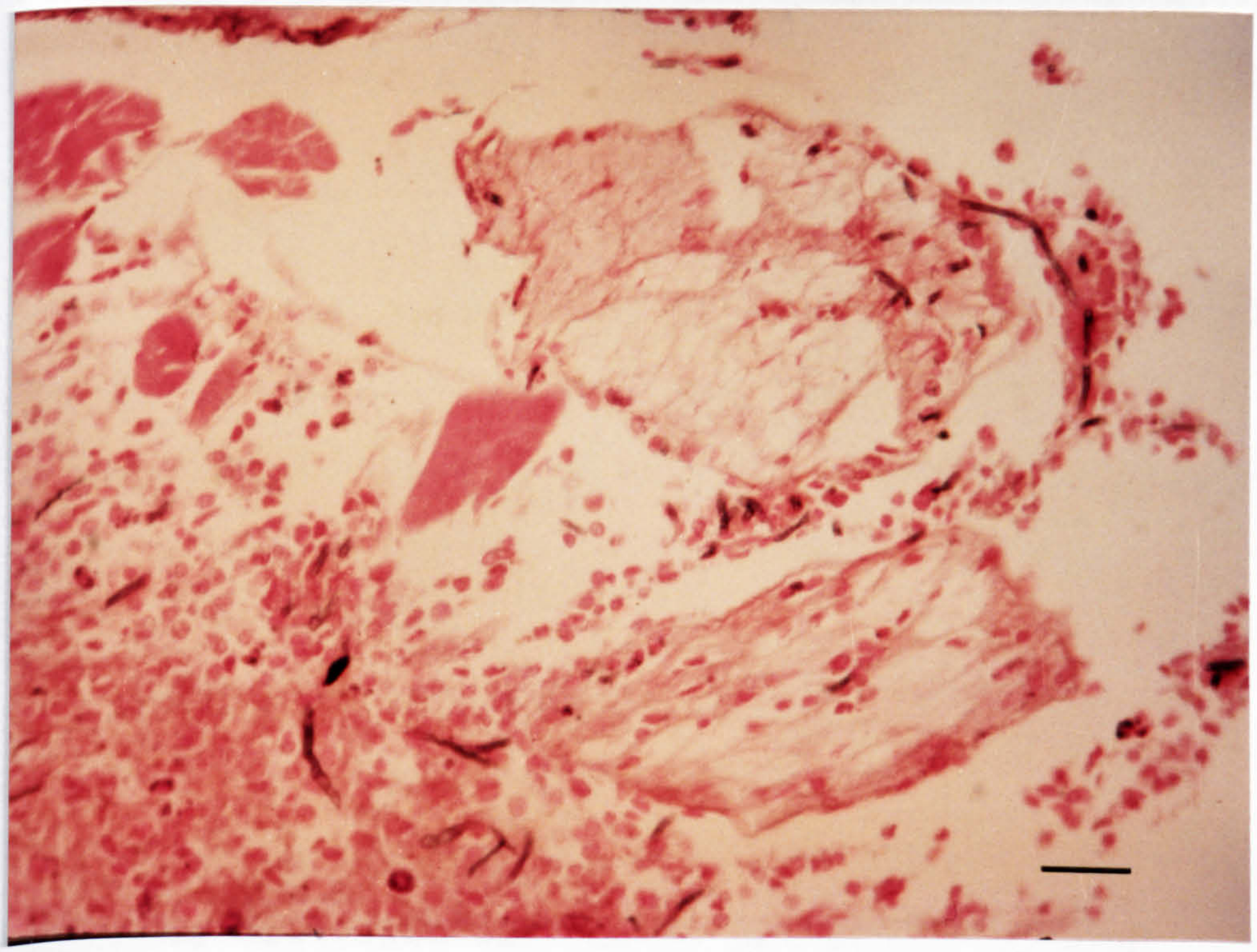
Figure 10. Hyphal invasion of nerve tissue.

Figure 10a shows minor hyphal invasion of a leg nerve. In the bottom left of the photograph is a strong haemocyte reaction to a near-by group of fungal hyphae.

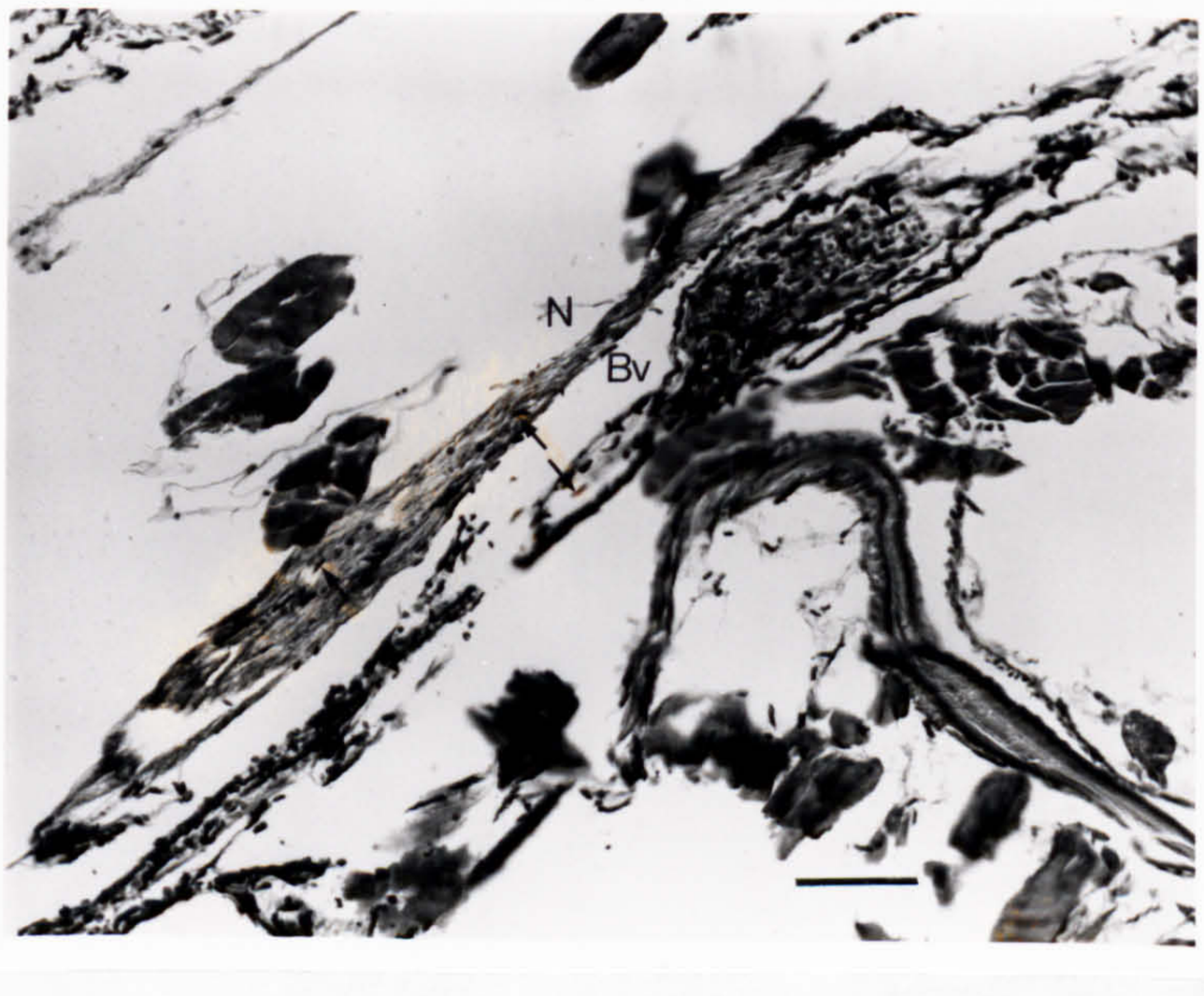
Scale bar = 100um.

Figure 10b shows hyphal invasion of the nerve chord (N) and also of the main ventral blood vessel (Bv) immediately below it, which has become blocked by the haemocyte reaction to the fungus.

Scale bar = 200um.



a



b

of infection of the brain, and the articulations of the eyes in question were severely infected, it is probable that these eye infections arose either as a result of fungal hyphae spreading from the articulating membranes or by direct infection of the cornea, rather than from infections of the optic nerve.

Hyphae were found ramifying through most of the eye including the cornea (Figure 11), where they also covered the surface. In one case a severe local infection of the optic nerve also occurred, but hyphae did not penetrate the brain.

Gill infection (Figure 12a) was found only in animals dying between 7 and 10 days post infection. However, where gill infection did occur, it often either completely destroyed the fabric of the gill or the haemocyte response to the invasion caused blockage of the blood passages of the gill lamellae (Figure 12b). In either case, the gill was probably rendered useless. In cases where gill infection was seen, no more than four lamellae were involved, although the infection was usually strong.

2.1. Number of foci of infection.

The number of infected arthrodial membranes was noted in each animal. The number of such infected areas of soft cuticle varied from only one, in animal 9, to 23 in animal 2. The relationship between survival time (time to death or TTD) and the number of infected arthrodial

Figure 11. Infection of the eye.

This figure shows a strong invasion of the distal portion of an eye. Hyphae are seen (arrow) ramifying immediately beneath the cornea and also in the lower layers.

Scale bar = 200um.

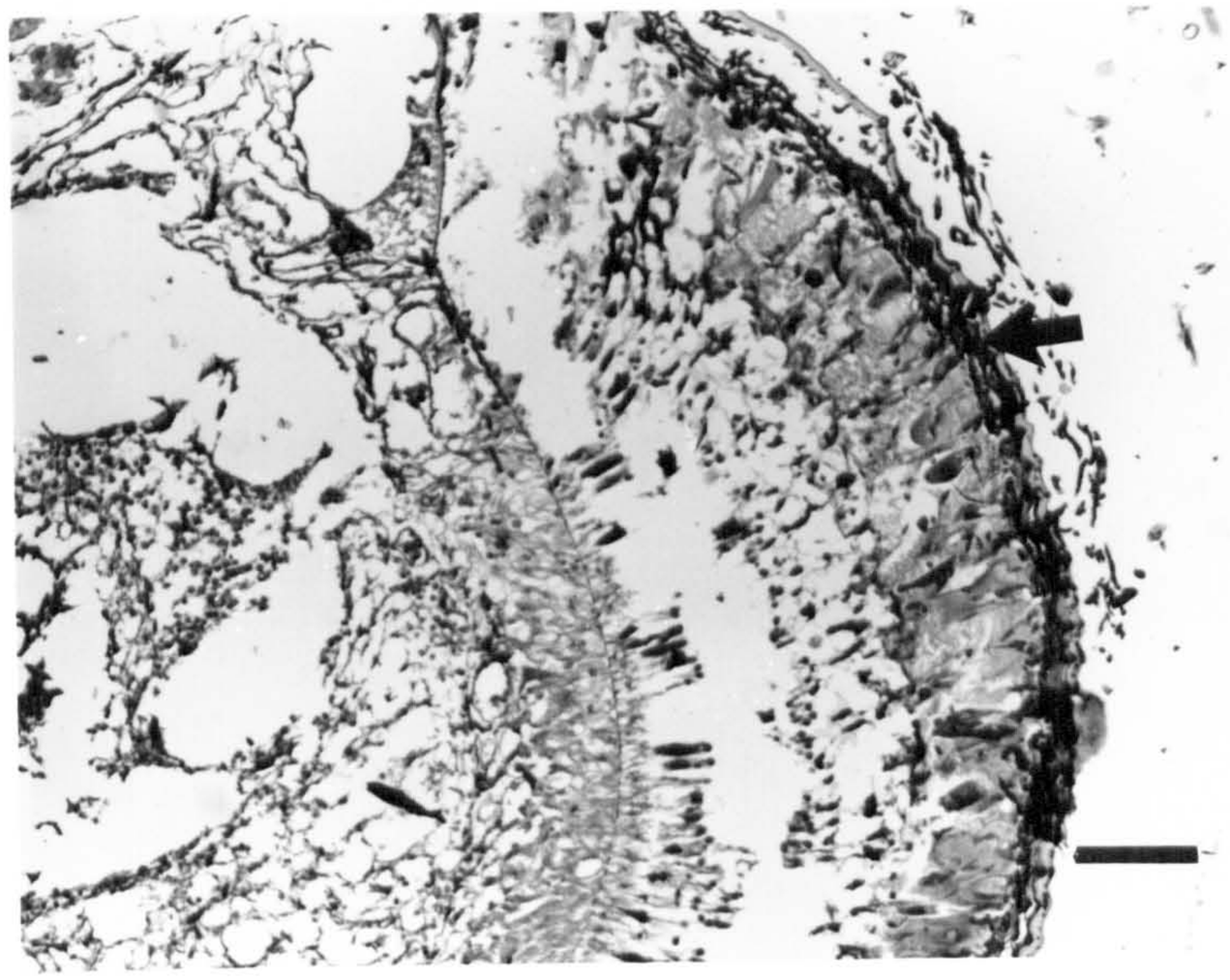


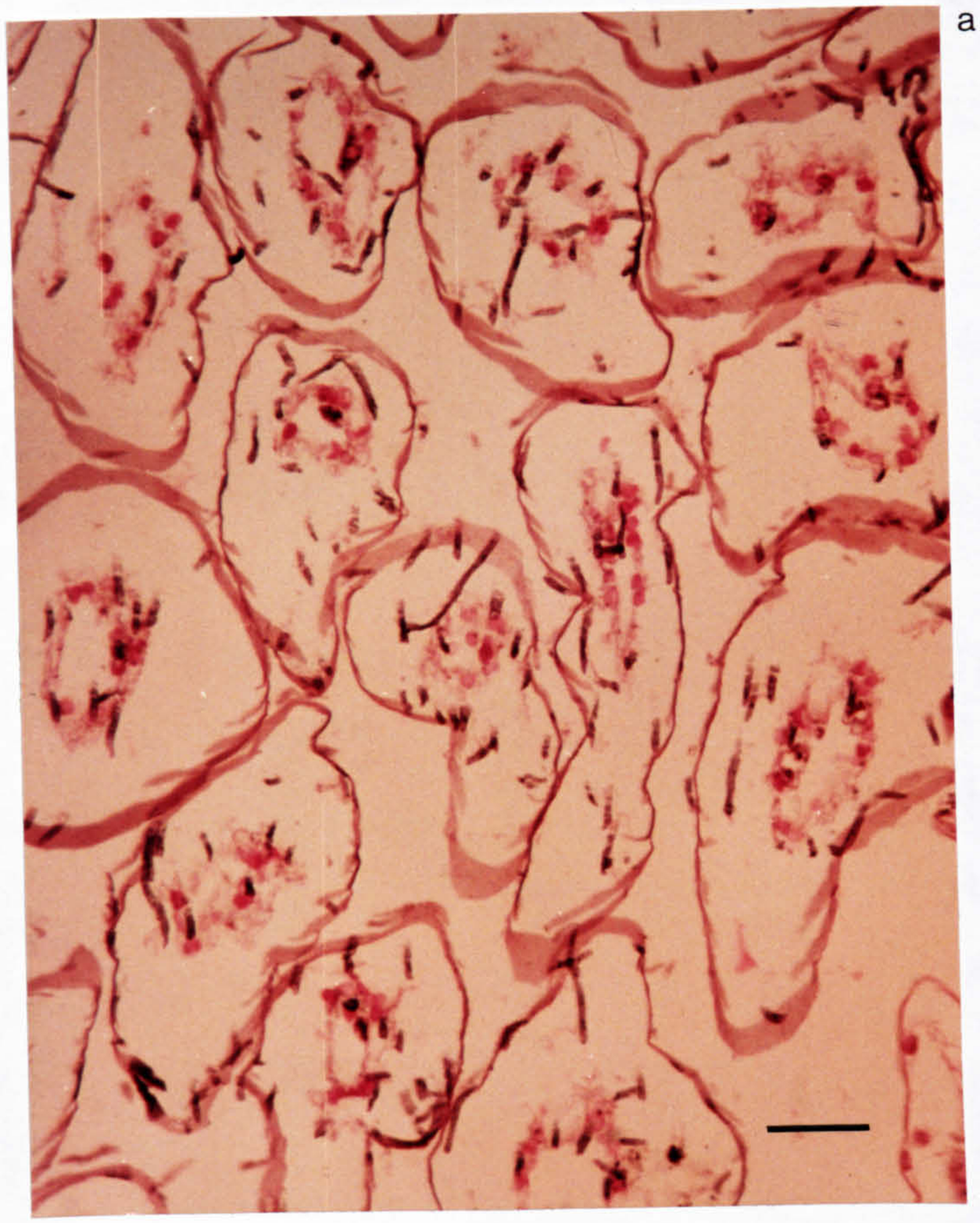
Figure 12. Infection of the gills.

Figure 12a shows an infection of the gills in which the haemocyte reaction is relatively minor (lamellae sectioned transversely). However in figure 12b (sectioned longitudinally) the haemocyte reaction is stronger and has blocked off the blood vessel supplying the gill.

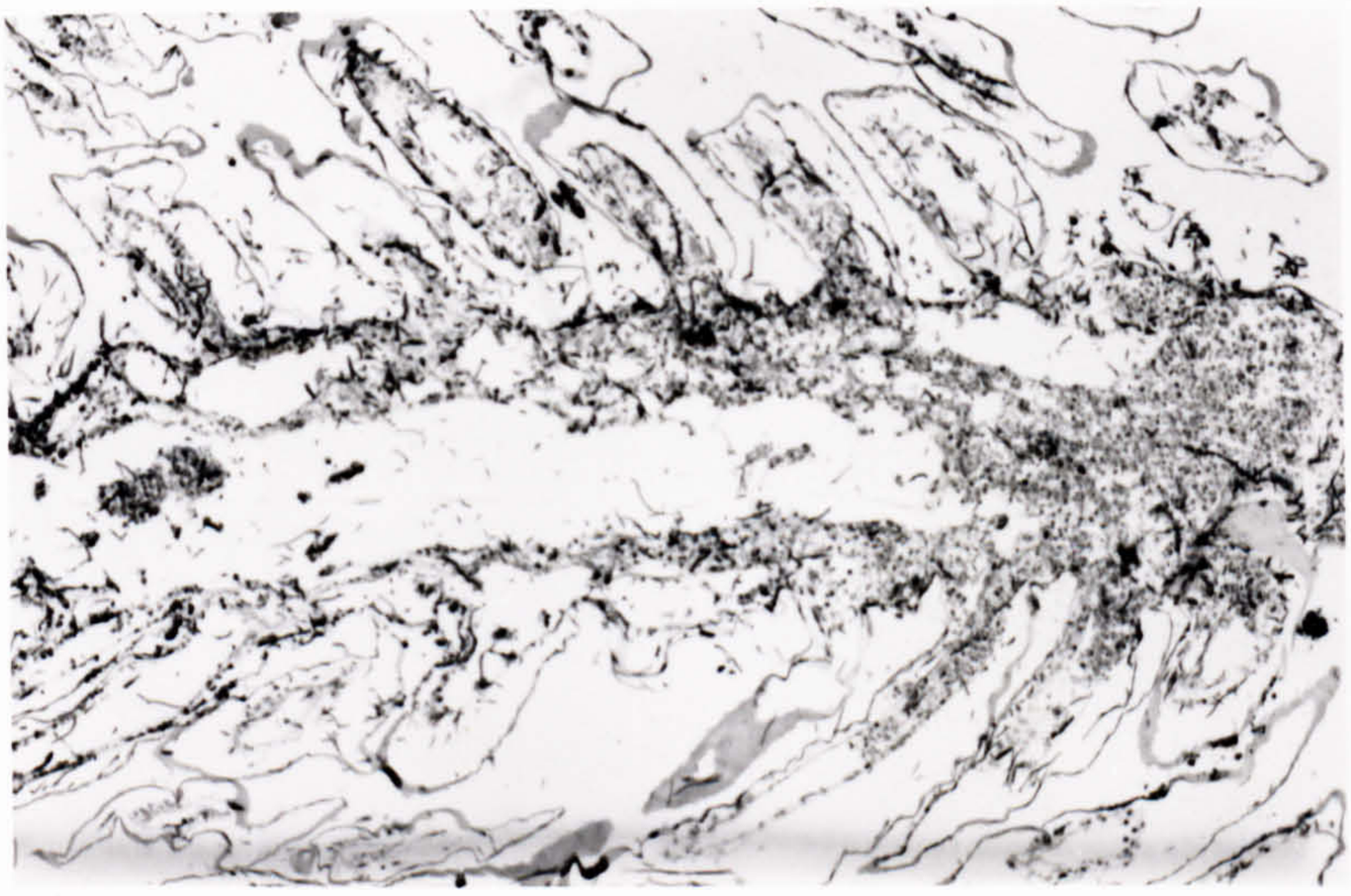
Figure 12a scale bar = 100um.

Figure 12b scale bar = 400um.

a



b



membranes is illustrated in figure 13.

Clearly, the animals that died early in the experiment (7 & 9 days) showed large numbers of invasion sites (10-23), whilst those that died between 10 and 17 days showed far fewer (1-6). Between 7 and 14 days post infection, the time to death is closely correlated (R=-0.77) to the number of foci of infection. However, animals that died after 17 days showed a reversal of this trend, having between 7 and 16 areas of infection.

It is interesting to note that animals dying in the early stages of the experiment showed a marked tendancy towards infections of articulations in the head region, and of one or more gill lamellae. Three of the five animals dying between seven and ten days post infection had infections of the eye articulations, the antennae or the mouthparts, whilst four out of five had infections of the gill lamellae. Animals dying very late in the experiment (41 & 43 days) also had infections in the head region, although no gill involvement was evident.

Figure 13 takes no account of the severity of the invasion at each site involved, thus, an area having a few hyphae only, is represented in the same way as one where the invasion is so severe that the cuticle is locally almost completely destroyed. In figure 14, histograms representing the range of severity (C) of the cuticular infection foci are presented.

In general each animal shows a range of cuticular

Figure 13. Relationship between time to death and number of infected arthrodial membranes.

This graph illustrates the relationship between the number of infected arthrodial membranes visualized histologically, and the survival time (or time to death) of infected animals. Over the first 17 days the relationship is best expressed by a straight line with the formula

Y = 26.4 - 1.5x. The associated correlation coefficient (R) is 0.77.

O denotes animals with infections of the head region.
□ denotes animals with infections of the gills.

TTD = Time to death.

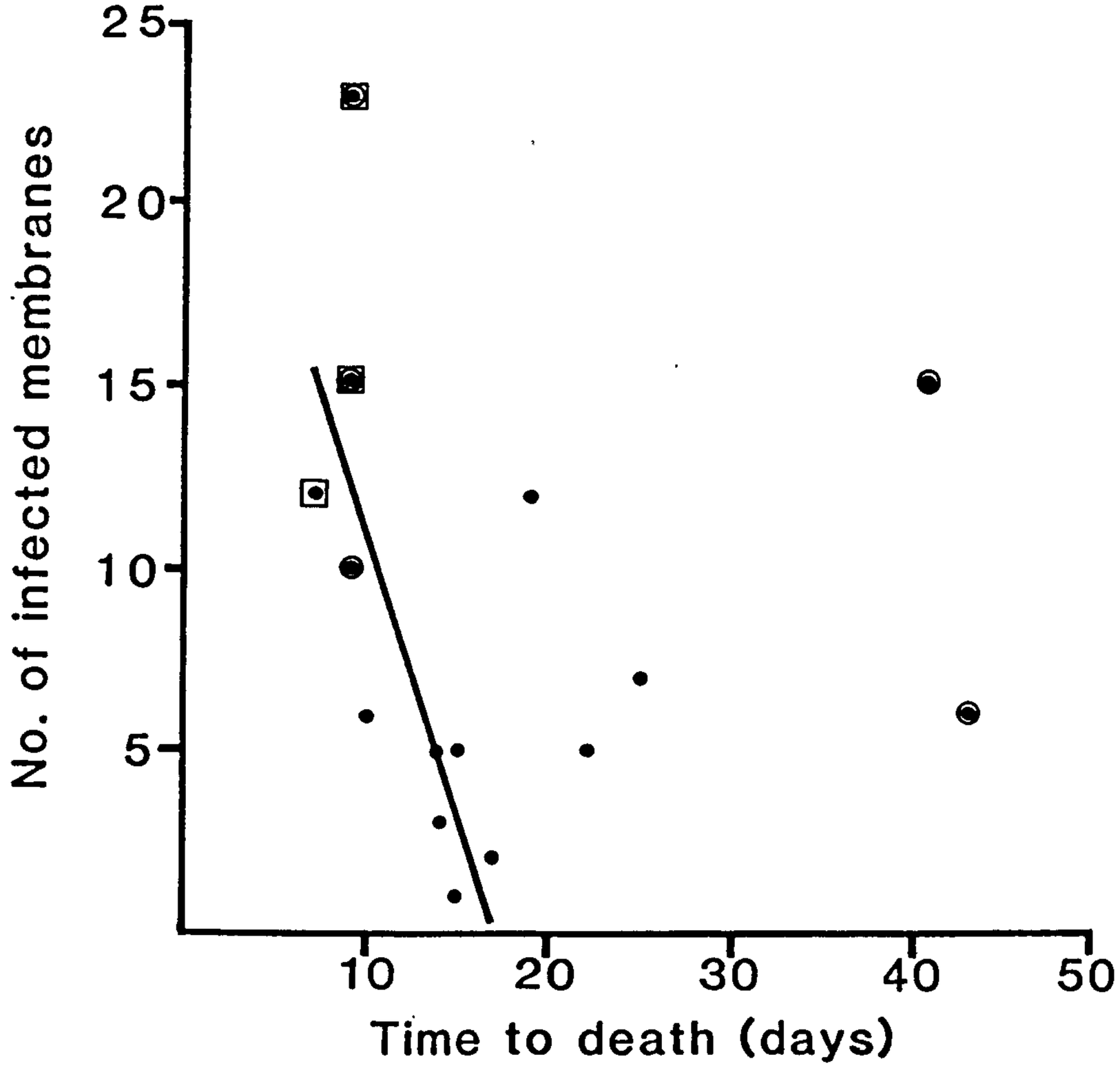
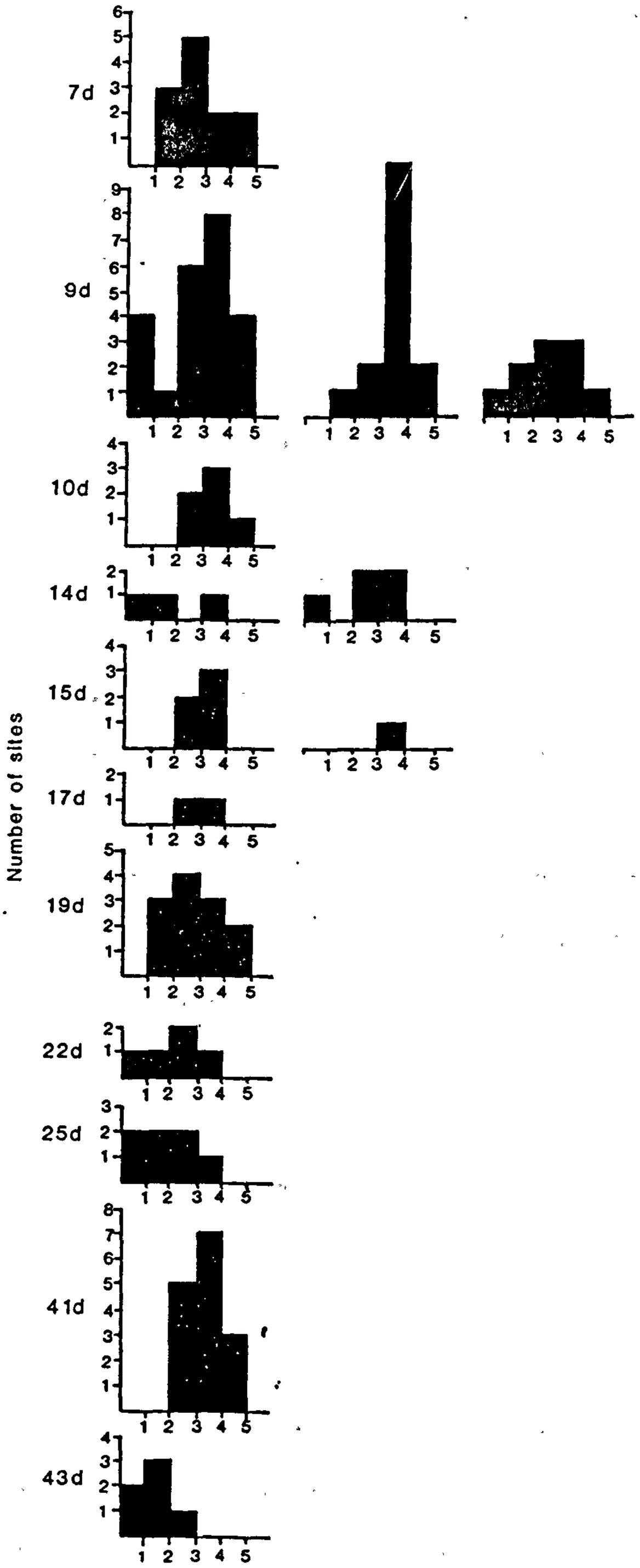

25
20
15
10
5
No. of infected membranes
10
20
30
40
50
Time to death (days)

Figure 14. Histograms representing the range of cuticular invasion severities in examined animals.

The severity of each focus of infection was calculated on a semi-quantitative scale (methods 4.1.) and the C values obtained were grouped 0-1, 1.1-2 etc. before being compiled into a series of histograms. The individual histograms are shown to the right of the time to death for that animal or group of animals.


7d
9d
10d
14d
15d
17d
19d
22d
25d
41d
43d
Number of sites
Invasion severity (C)

invasion severities, although certain C values predominate in each case. In the animal dying after seven days, infection foci are generally not as strong as in those dying after nine or ten days. In animals surviving for between 10 and 17 days the small number of foci present are of the same order of magnitude as in animals dying earlier, having C values between 2.1 and 4. Those animals that survived longer than 17 days often had weaker cuticular infections than did animals dying earlier.

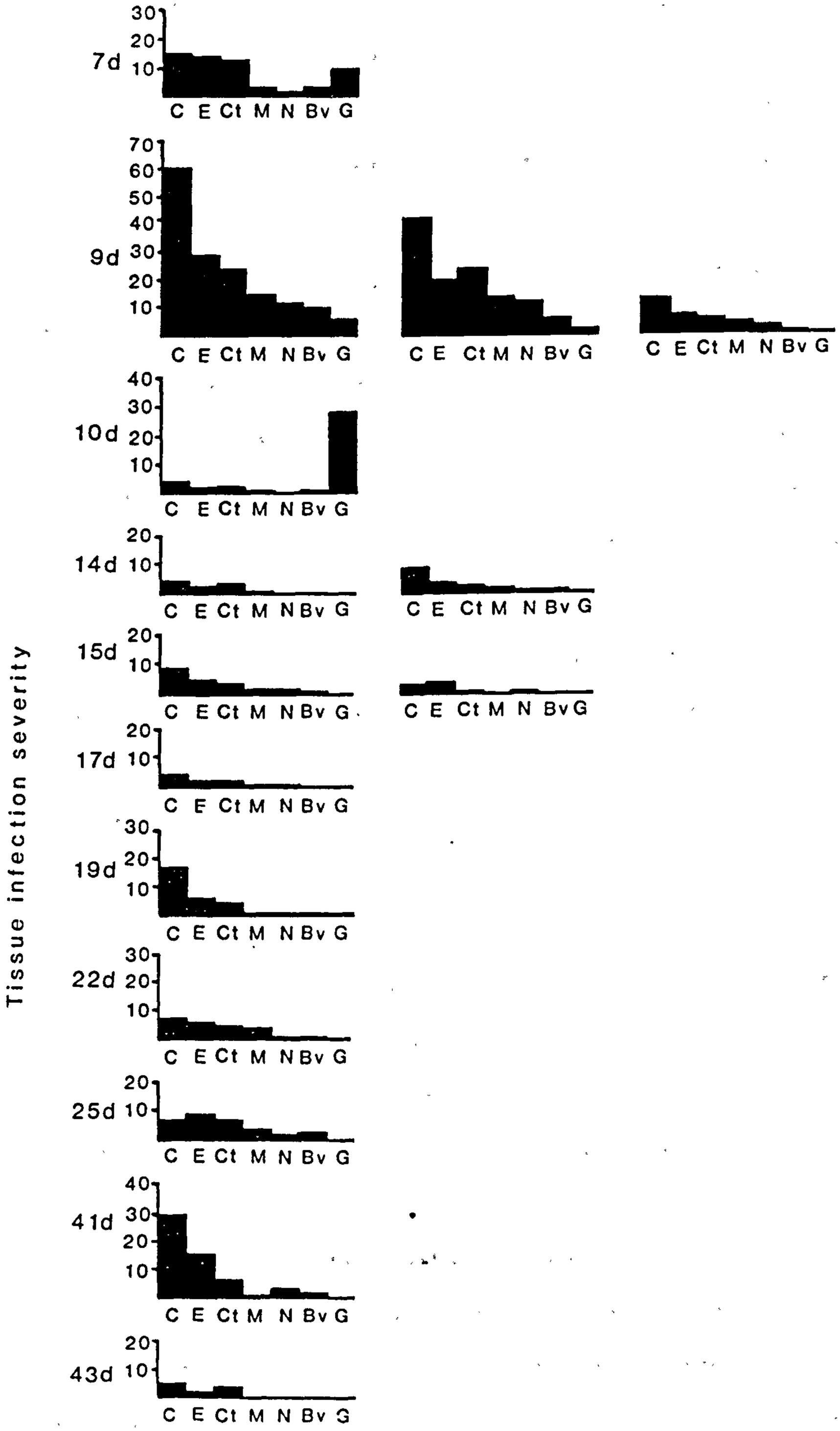
2.2.Extent of involvement of other tissues.

Figures 13 and 14 above, demonstrate the state of the cuticle of infected animals at the time of death. However, the ramifications of the fungus are not limited to the soft cuticle and deeper tissues are often involved. In order to illustrate the progress of fungal invasion of deeper tissues asociated with the foci of cuticular invasion, at the time the animal succumbed to the infection, individual indicies of invasion severity were calculated for the deeper tissues. Thus E, Ct, M, Bv, N, and G were the values assigned to infections of epidermis, conective tissue, muscle nerve, blood vessels and gills at each site of invasion, and were calculated in a similar manner to C (section 4.1 of the methods.); and ΣC, ΣE, ΣCt, ΣM, ΣBv, ΣN and ΣG, were the sums of values for the particular type of tissue, at each focus of invasion, in individual animals. Figure 15 presents the data in the form of a histogram for each animal.

Figure 15. Histograms representing the severity of infection of seven tissue types around the infected arthrodial membranes.

The severity of infection of each tissue type was calculated on a semi-quantitative scale (results 2.2.) and compiled into a series of histograms. The individual histograms are shown to the right of the time to death for that animal or group of animals.

C represents the overall severity of infection of cuticle.
E represents that of epidermal tissue,
CT that of conective tissue,
M that of muscle,
Bv that of blood vessels,
N that of nervous tissue,
and G that of gill tissue.


7d
9d
10d
14d
15d
17d
19d
22d
25d
41d
43d
C E Ct M N Bv G
Tissue infection severity
Tissue type

In this representation, cuticle is generally the most severely infected tissue, followed by epidermis and connective tissue. Blood vessels and nerve are generally also infected although infection of the gills is confined to animals dying before 14 days. It is clear the the pattern of invasion in animals dying early in the experiment with large numbers of foci of infection, is simi lar to that in animals dying later on with fewer foci of infection and also to those animals that survived for longer than 17 days with intermediate numbers of foci of infection. However, in the animal that died after seven days (animal 1), the fungus appears to be confined mainly to the cuticle, epidermis and conective tissue. Infection of the muscles, blood vessels and nerves in this animal, are minor, presumably since infection has not had time to spread this far.

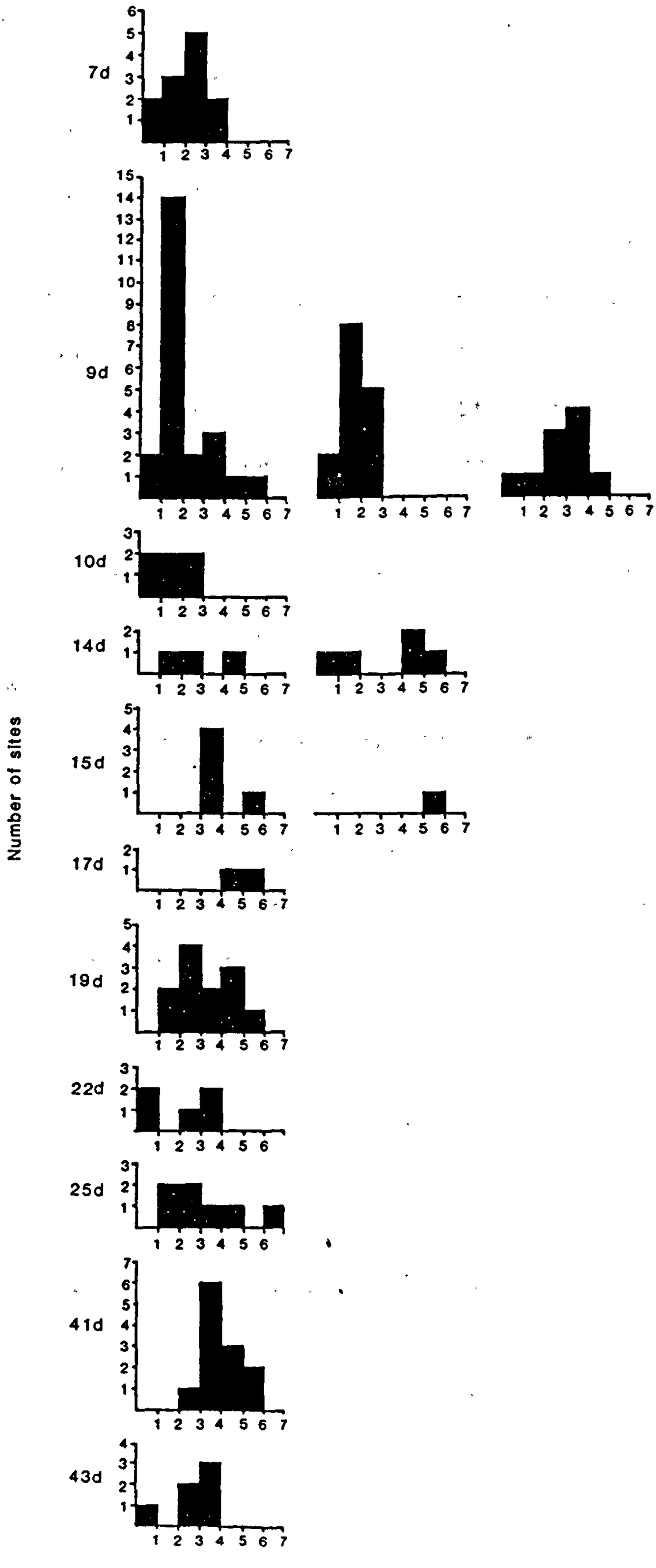
2.3.Haemocyte response.

For each animal studied, the haemocyte reaction, H, at each site of infection was calculated. These values were placed into one of seven groups (0-1, 1.1-2 etc.) and the results displayed as a series of histograms (Figure 16).

In the animals that died between 7 and 17 days there is a tendency for the haemocyte response to be greater in animals dying later. Thus in general the haemocyte responses in animals that died before 14 days had H values below 3, whilst in those that died between 14

Figure 16. Histograms representing the range of strengths of haemocyte reaction to individual foci of fungal infection.

An index of the strength of the haemocyte reaction (H) to individual foci of fungal infection was calculated on a semi-quantitative basis (methods 4.2.) and the values obtained were grouped 0-1, 1.1-2 etc. before being compiled into a series of histograms. The individual histograms are shown to the right of the time to death for that animal or group of animals.


7d
9d
10d
14d
15d
17d
19d
22d
25d
41d
43d
Number of sites
Haemocyte response (H)

and 17 days H values tended to be greater than 3. Animals that died after 24 days had a wider range of haemocyte responses. The trend is more apparent if mean haemocyte responses are plotted against TTD (Figure 17).

H values between 5 and 7 represent advanced encapsulation and melanization of the invading fungus by crayfish haemocytes. Such high H values are not common in animals that died before 14 days, although in animal 2 one site of invasion had been strongly melanized by the time of death. It is apparent therefore that the first signs of strong melanization of the affected portions of the body do not occur in these animals until after 9 to 14 days post infection.

For each animal studied, the haemocyte response, H, to each site of infection was plotted against the cuticle invasion severity, C, to produce a series of graphs, showing the range of haemocyte responses to the individual cuticular invasions (figure 18). The haemocyte response to a given severity of invasion varied considerably, not only between animals, but also, in cases where animals have more than one cuticular infection of the same degree of severity, within animals as well. For example, animal 2 dying after 9 days of infection, had several infected arthrodial membranes with a C value of 4. Haemocyte response values associated with these infected areas varied between 0.7 and 3.2. Animal 14, dying after 41 days also had several sites with a C value of 4, however the associated H values varied between 4 and 6, demonstrating

Figure 17. The relationship between mean haemocyte response and Time to death.

A mean of the H values associated with each cuticular focus of infection was calculated for each animal and plotted against the time to death of that animal.

TTD = Time to death in days.

MHR = The mean haemocyte response value.

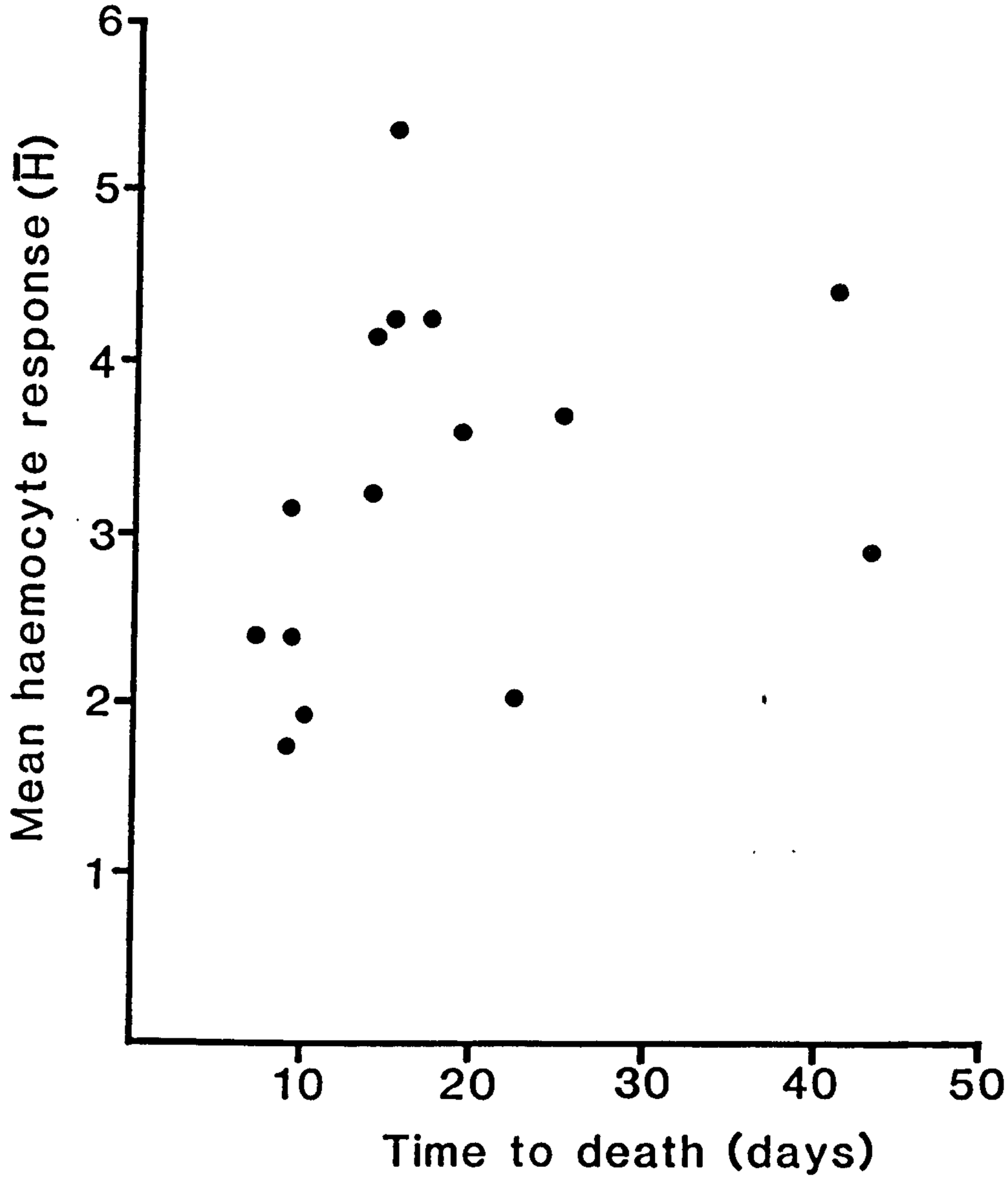
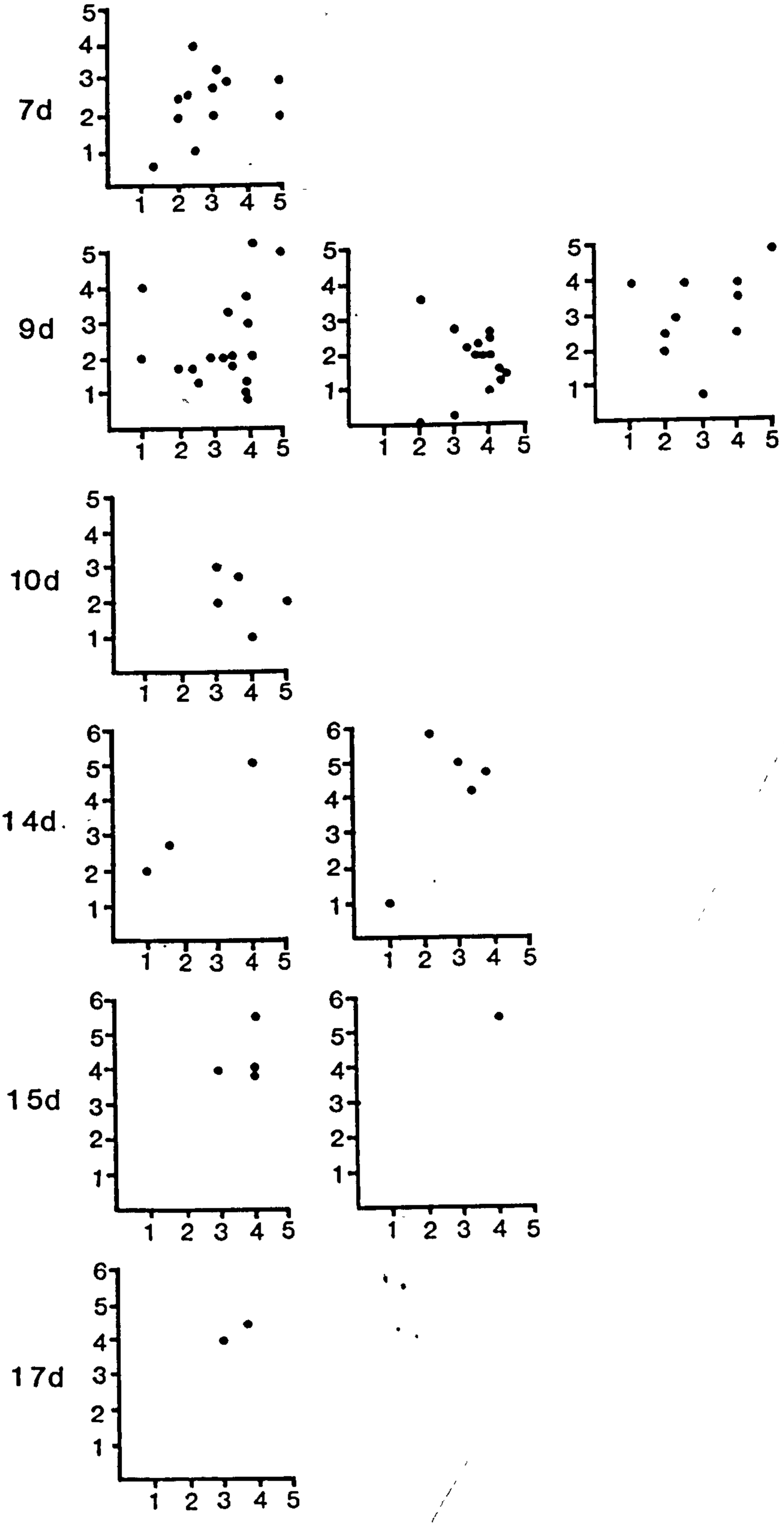
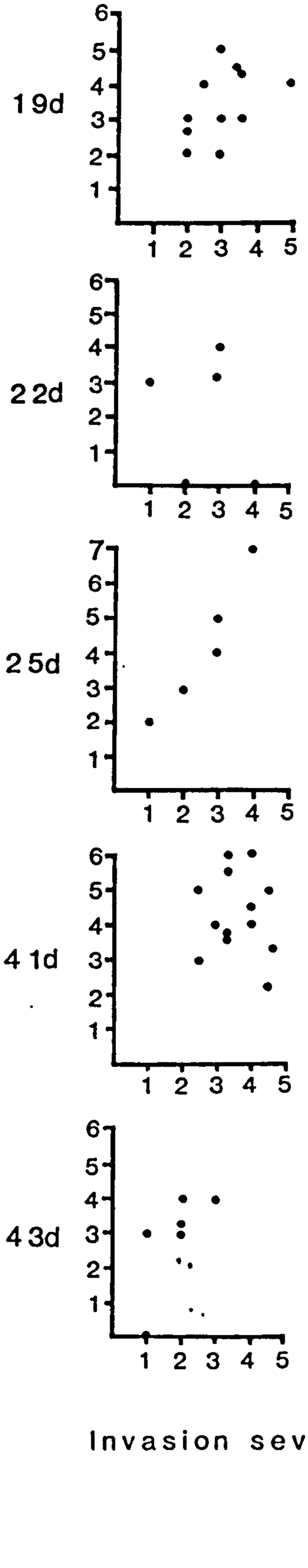

Mean haemocyte response (H̄)
1
2
3
4
5
6
10
20
30
40
50
Time to death (days)

Figure 18. Relationship between the severity of infection, C, and the strength of the haemocyte reaction, H, to it.

The severity of invasion of the cuticle at each focus of invasion is plotted against the strength of the haemocyte reaction to it. The number of points on these graphs does not necessarily coincide with the number of infected arthrodial membranes in figure 2 since several foci of infection may have the same C and H values.To the left of each graph of group of graphs is shown the time to death for that animal or group of animals.

see over leaf.

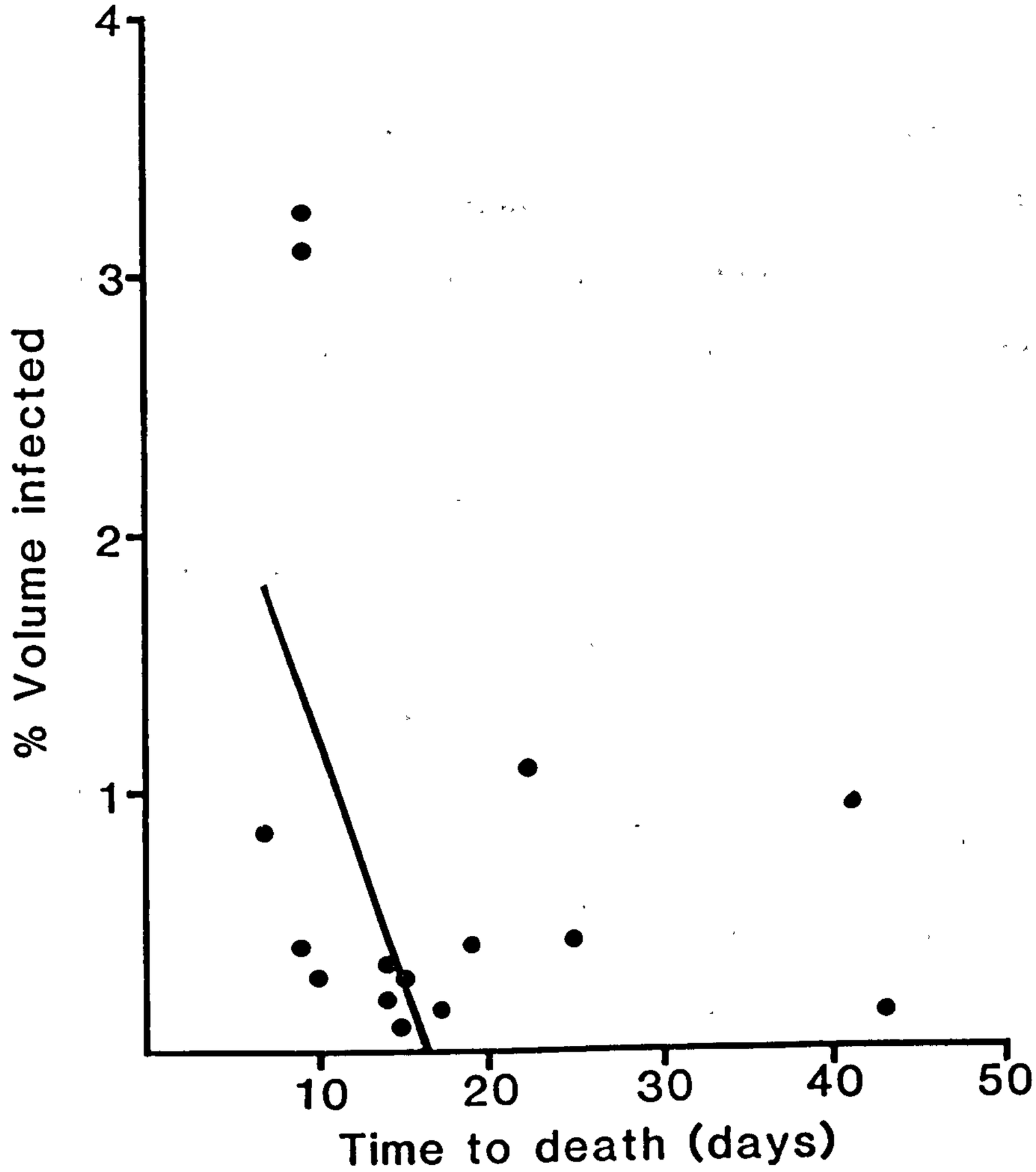

7d
9d
10d
14d
15d
17d
Haemocyte response (H)
Invasion severity (C)



Invasion severity (C)

Figure 19. The relationship between the percentage volume of infected tissue and the time to death.

The % volume of infected tissue was calculated as detailed in methods 4.3.. The relationship between this value and time to death for the period upto and including 17 days, is best expressed as a straight line with the formula Y = 3.3 - 0.2x. The associated correlation coefficient (R) is 0.56.


% Volume infected
4
3
2
1
10
20
30
40
50
Time to death (days)

a much more advanced response to the fungus.

Animals dying early in the experiment (7-10 days) had a large number of relatively severe infections, the haemocyte reaction to these infections is generally weak, probably because it has not had time to develop before the animal died from the infection. In animals dying later on the number of infected arthrodial membranes was smaller but the severity of the individual infecion foci was similar, however the haemocyte reaction to the fungus was stronger.

In some cases (ie animals 1, 6, 11, 13 and 15) the strength of the haemocyte reaction appears to be related to the severity of the cuticular invasion severity. However, if the haemocyte reaction to each focus of fungal infection progresses to maturity, that is encapsulation and melanization of the fungal hyphae, then it is likely that the apparent relationship is due to the progression of both the fungal invasion and the haemocyte reaction with time.

2.4.Estimate of the percentage of infected tissue.

Figure 19 shows the relationship between the percentage of infected tissue and time to death in the animals studied. The percentage of infected tissue is small and varies between 0.09% and 3.25%.

In animals that sucumbed to infection between 7 and 10 days post infection, between 0.3% and 3.25% of the tissues were infected with Aph. astaci. In those animals

that died between 10 and 17 days post infection, a smaller proportion of the body was infected, between 0.09% and 0.35%. The survival times of animals dying between 7 and 17 days post infection could be correlated with the percentage of tissue that was infected with Aph. astaci, however the correlation (R=-0.563) was not as strong as that between TTD and the number of foci of infection (R=0.77). In animals that died after 17 days, the proportion of of the body infected was generally greater than those dying between 10 and 17 days, between 0.4% and 0.97%.

2.5.Spread of infection within the host.

Figure 20 shows the extent of the infection in each animal at the time of death. In animals with infections of the head region, the eye articulations and cornea were affected as were mouth parts and antennae. Infections of the thorax were found in the leg joints and gills. Infections of the leg joints were local in nature and did not spread to other areas of the thorax. In the abdomen, in the dorsal intersegmental membranes and often coalesced below the cuticle infecting a large part of the sub-cuticular tissue. Infections were also found in the perianal area, and in the articulating membranes of the uropods, as well as in the pleopods and in the ventral abdominal cuticle.

Except for animals with (relatively mild) gill infections and animals 2 and 3 with infections close to

Figure 20. The extent of infection at death.

Figures 20 (i) to 20 (xv) show the spread of infection within each animal studied. The sex of the animal, as well as the number assigned to it, time to death and number of sites of infection are also given.

x = a site where infection has occurred in the joint between the body and coxa. These sites are arrowed to avoid confusion with infection of the conective tissue around the nerve cord.

P = Perianal soft cuticle.

g = the position of infected gills.

Figure 20 (i)

Sex Male

Number 1

TTD 7 days

No. of sites of infection 12

Other details No obvious gross symptoms of infection except that the animal was moribund.

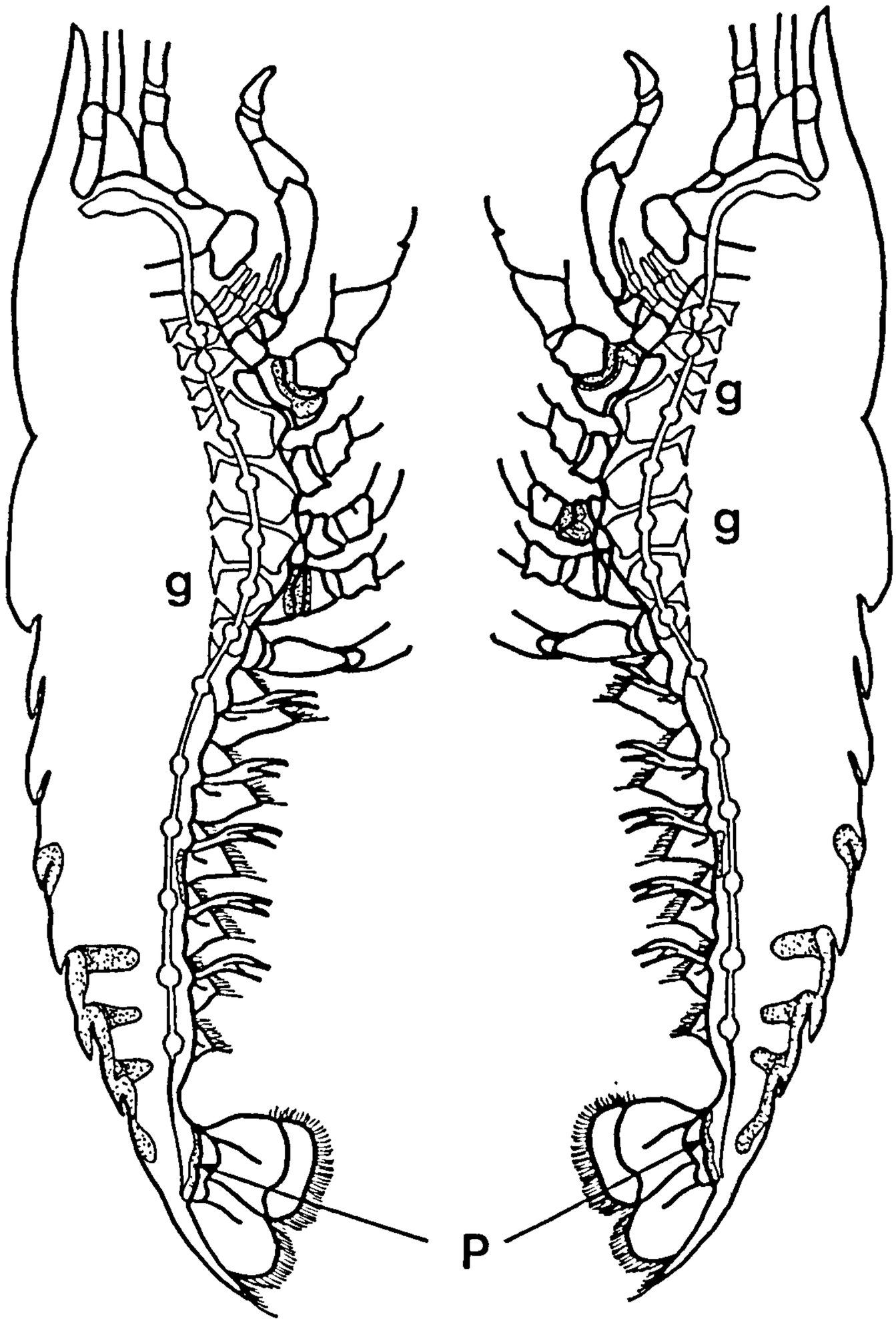

g
g
g
P

Figure 20 (ii)

Sex Female

Number 2

TTD 9 days

No. of sites of infection 23

Other details Proximal joint of one chela and of two walking legs, discoloured. Animal moribund.

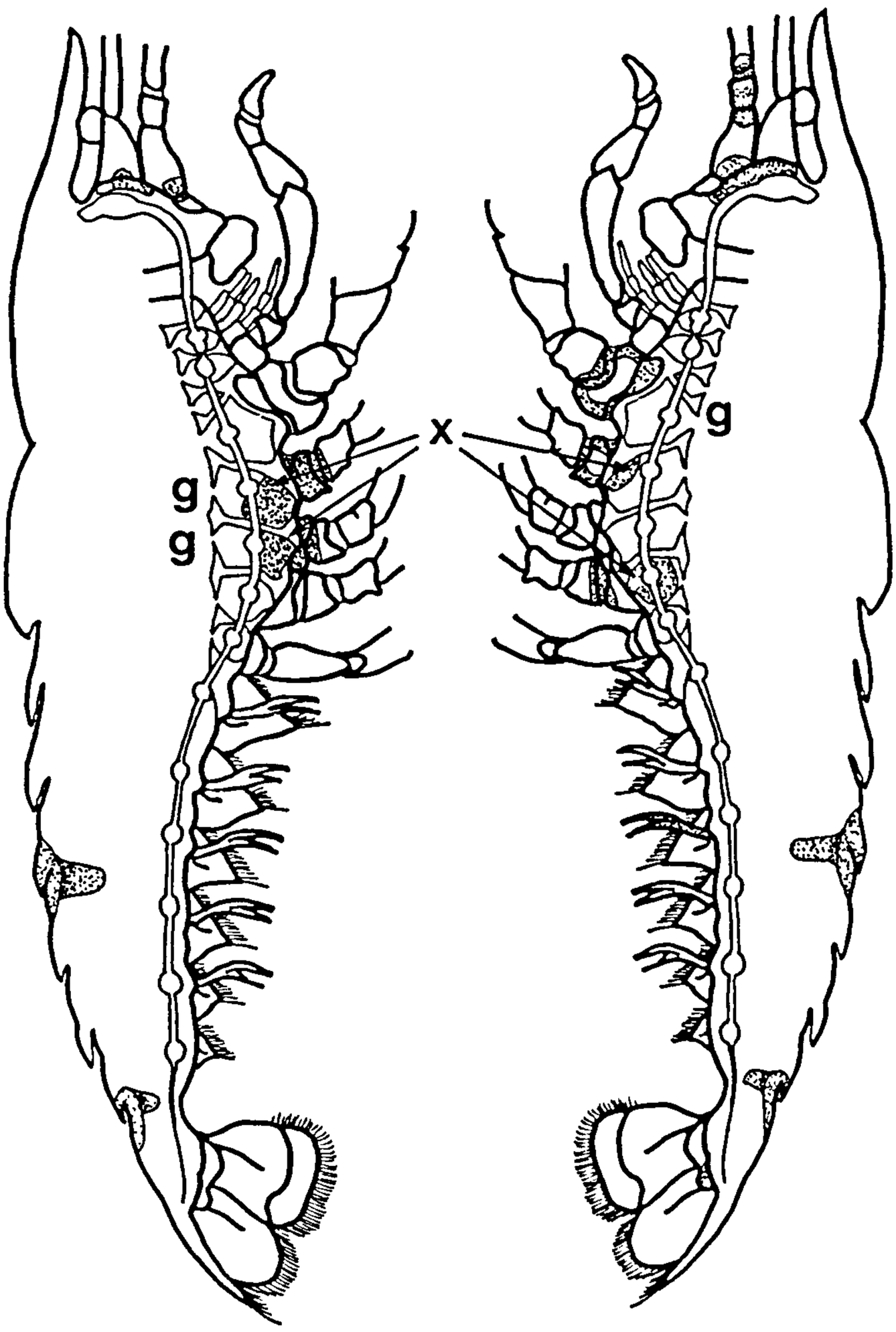

g
g
x
g

Figure 20 (iii)

Sex Female

Number 3

TTD 9 days

No. of sites of infection 15

Other details Animal dead less than 1 hr.

Proximal joints of two non-chelate walking

legs discoloured

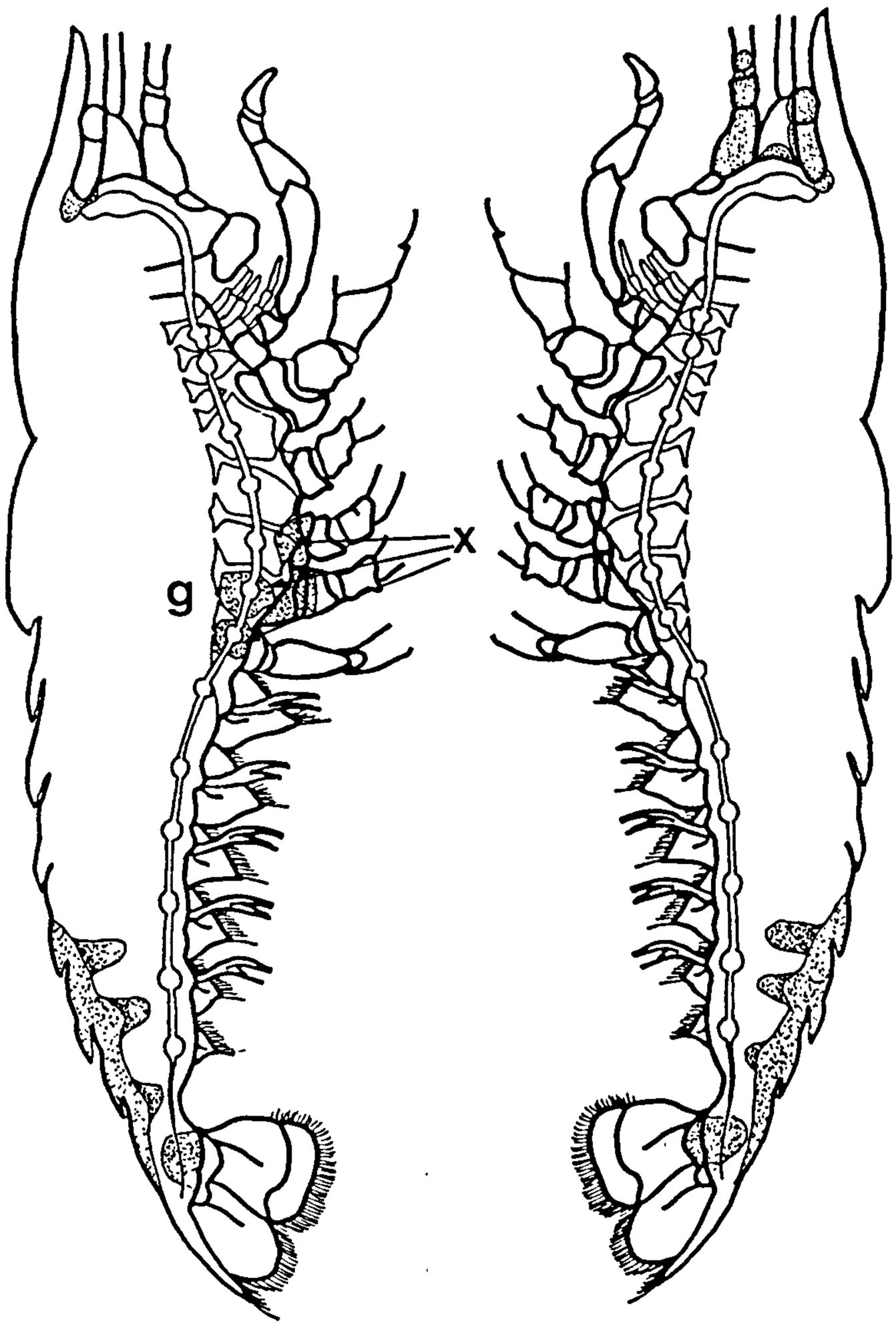

x
g

Figure 20 (iv)

Sex female

Number 4

TTD 9 days

No. of sites of infection 10

Other details Animal moribund. Discolouration of proximal joints of the first pair of non-chelate walking legs.

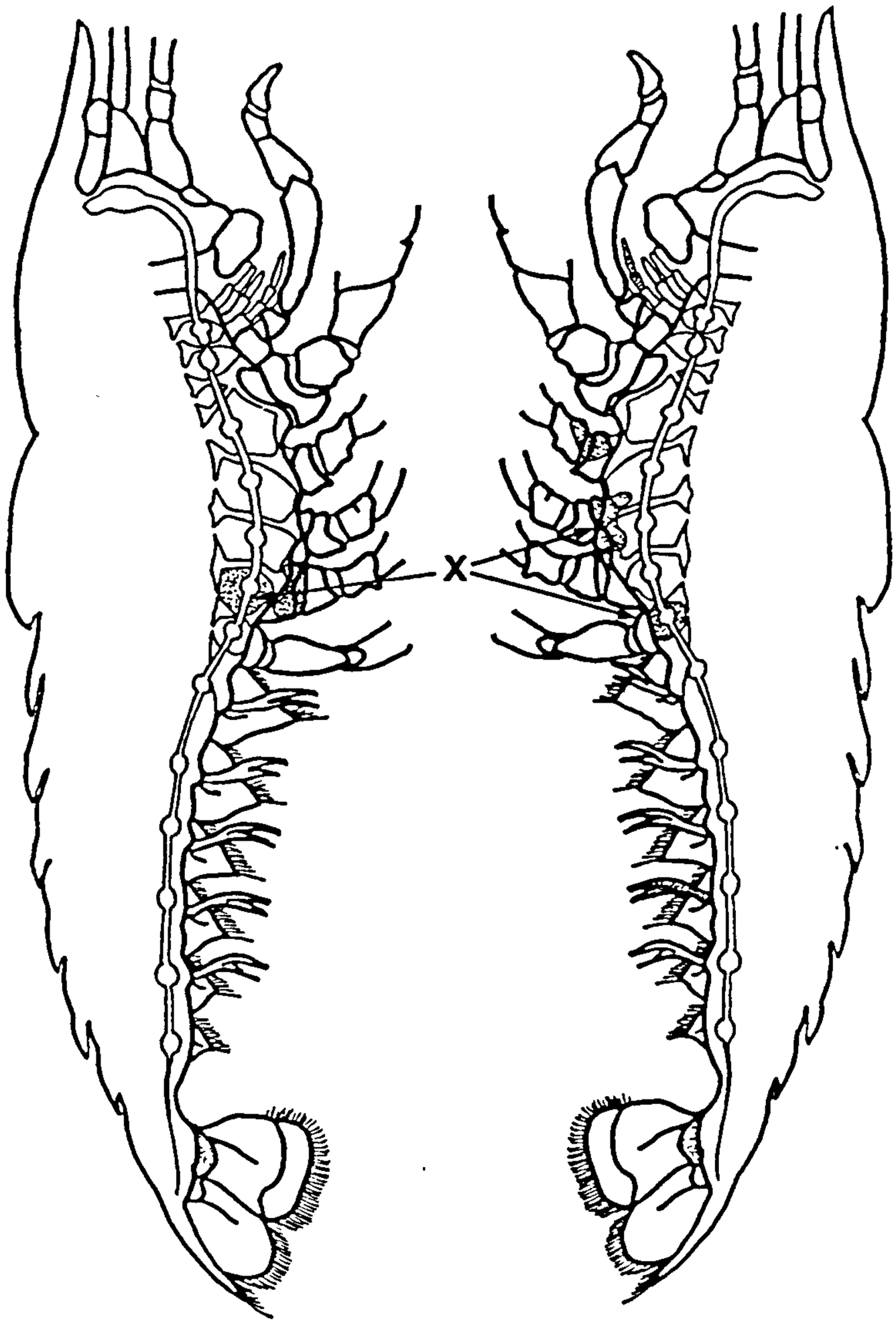

X

Figure 20 (v)

Sex Male

Number 5

TTD 10 days

No. of sites of infection 6

Other details Animal moribund. Discolouration of proximal joints of walking legs.

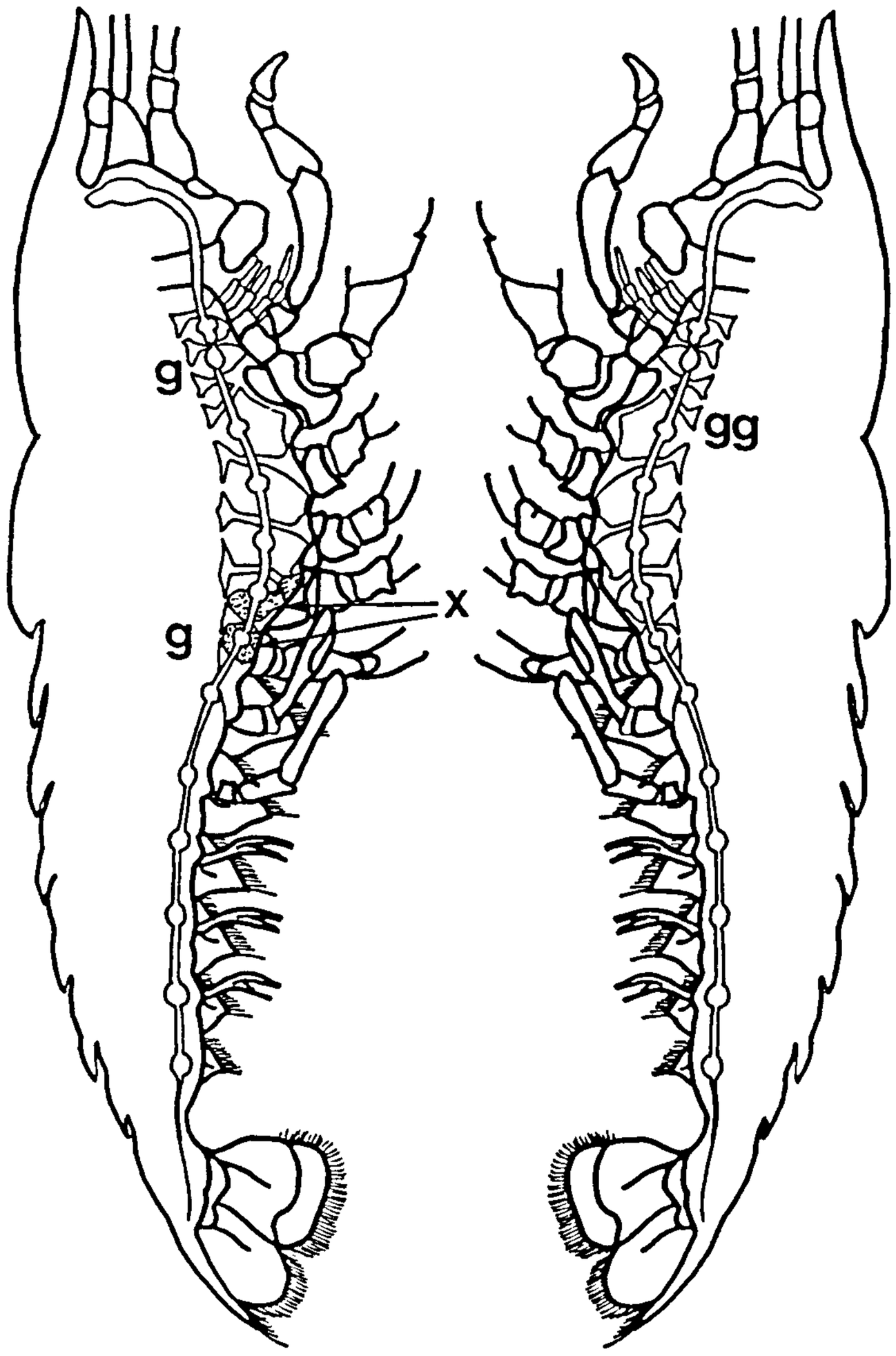

g
gg
g
x

Figure 20 (vi)

Sex Female

Number 6

TTD 14 days

No. of sites of infection 3

Other details Only gross sign of infection was the infection of the proximal joint of one walking leg.

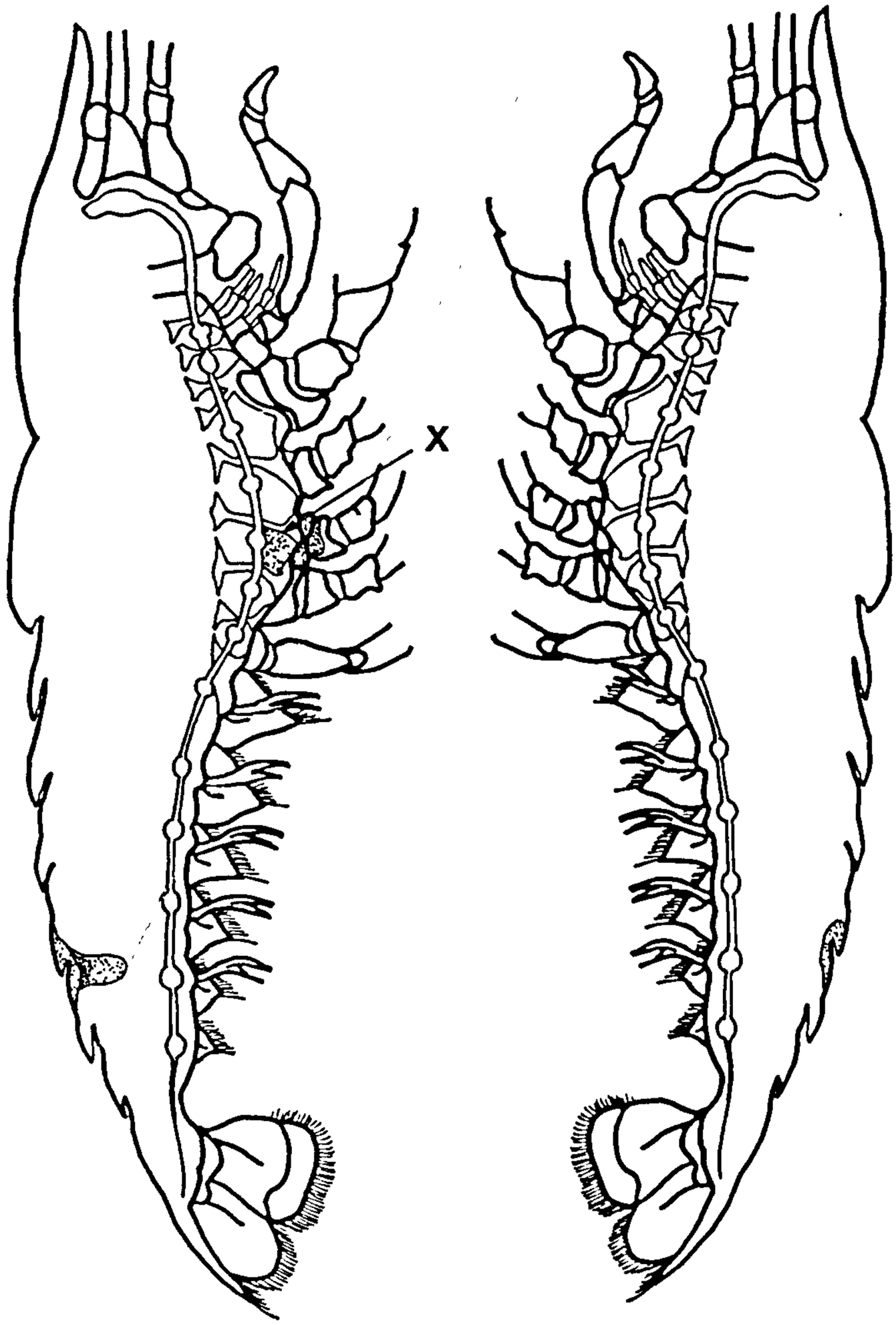


Figure 20 (vii)

Sex Female

Number 7

TTD 14 days

No. of sites of infection 5

Other details Animal moribund. Infection apparent in proximal joints of 2 walking legs.

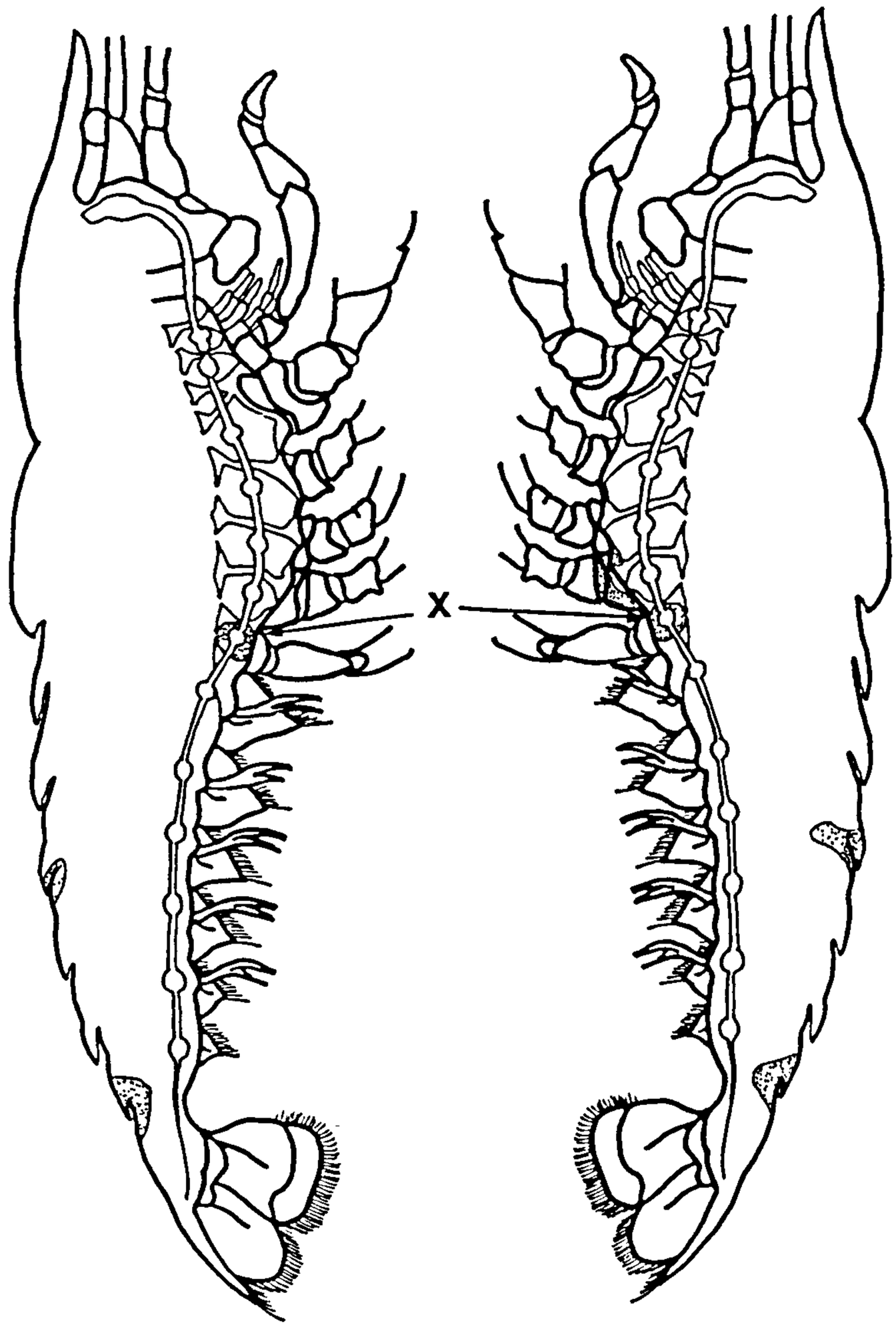

X

Figure 20 (viii)

Sex Male

Number 8

TTD 15 days

No. of sites of infection 5

Other details Animal moribund. Infection seen in proximal joints of both pairs of non-chelate walking legs.

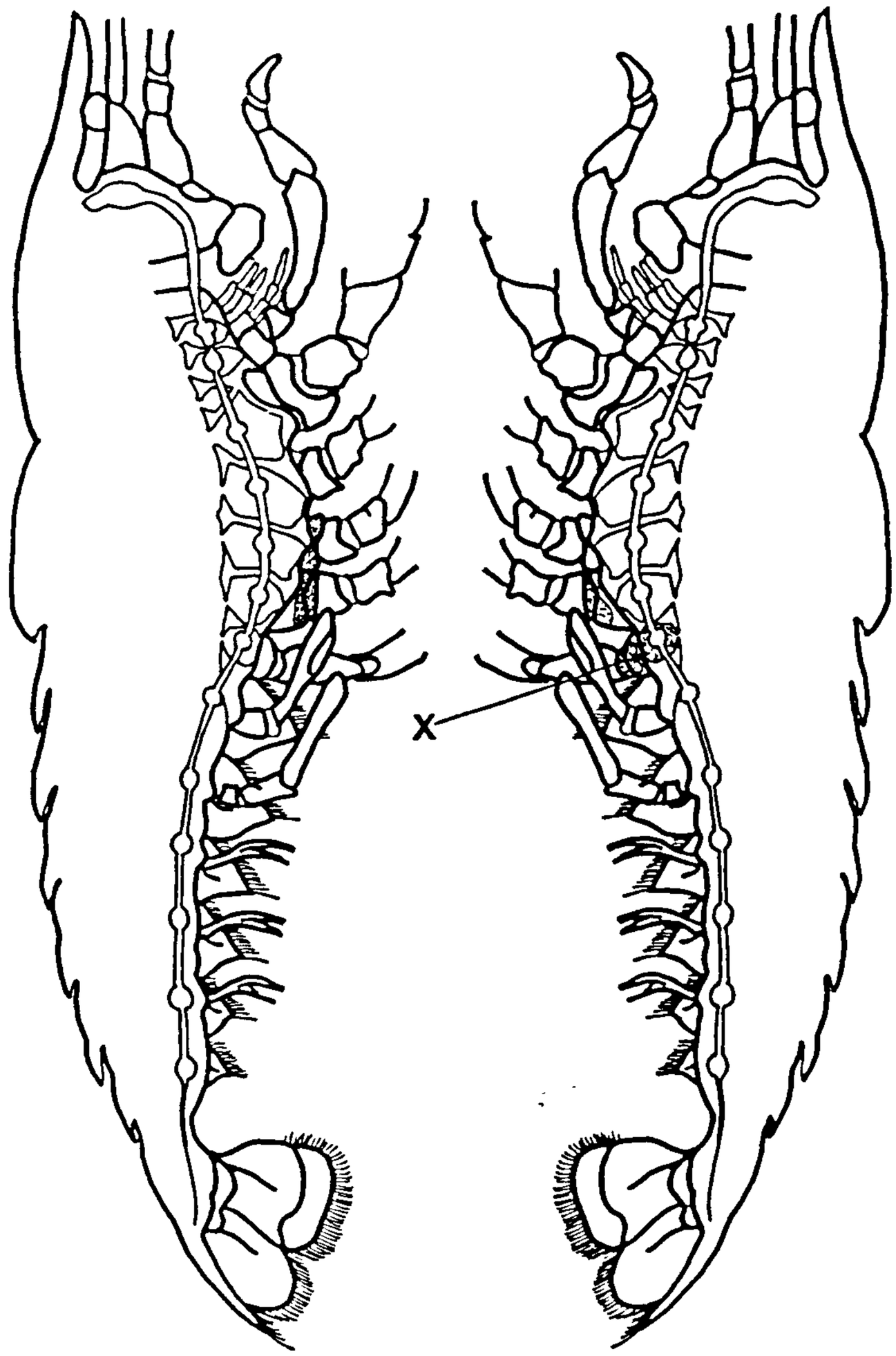

X

Figure 20 (ix)

Sex Female

Number 9

TTD 15 days

No. of sites of infection 1

Other details Animal moribund. No gross signs of infection.

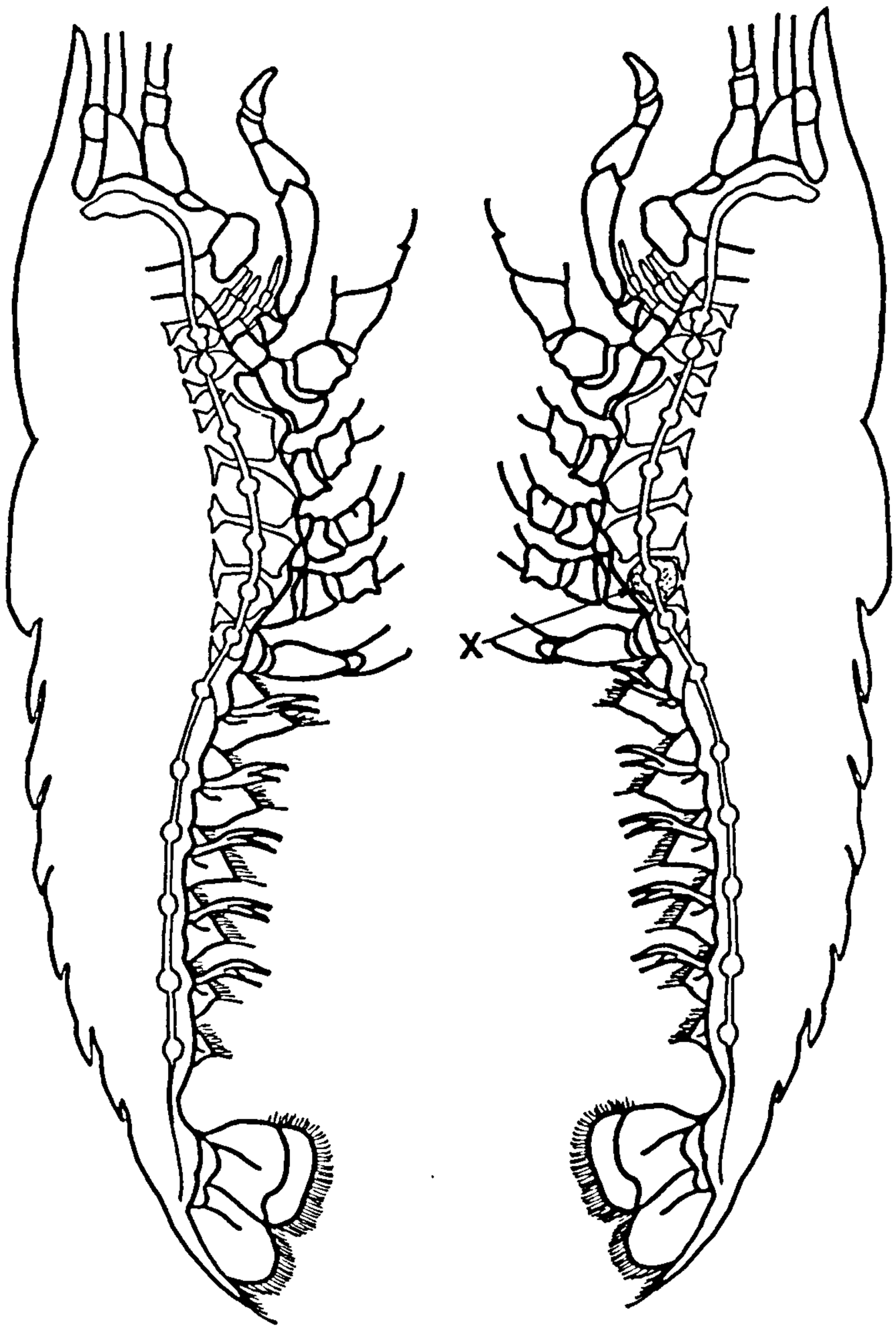

X

Figure 20 (x)

Sex Female

Number 10

TTD 17 days

No. of sites of infection 2

Other details Animal moribund. Infection visible in the distal end of one chelate walking leg and in the proximal joints of two non chelate walking legs were melanization was apparent.

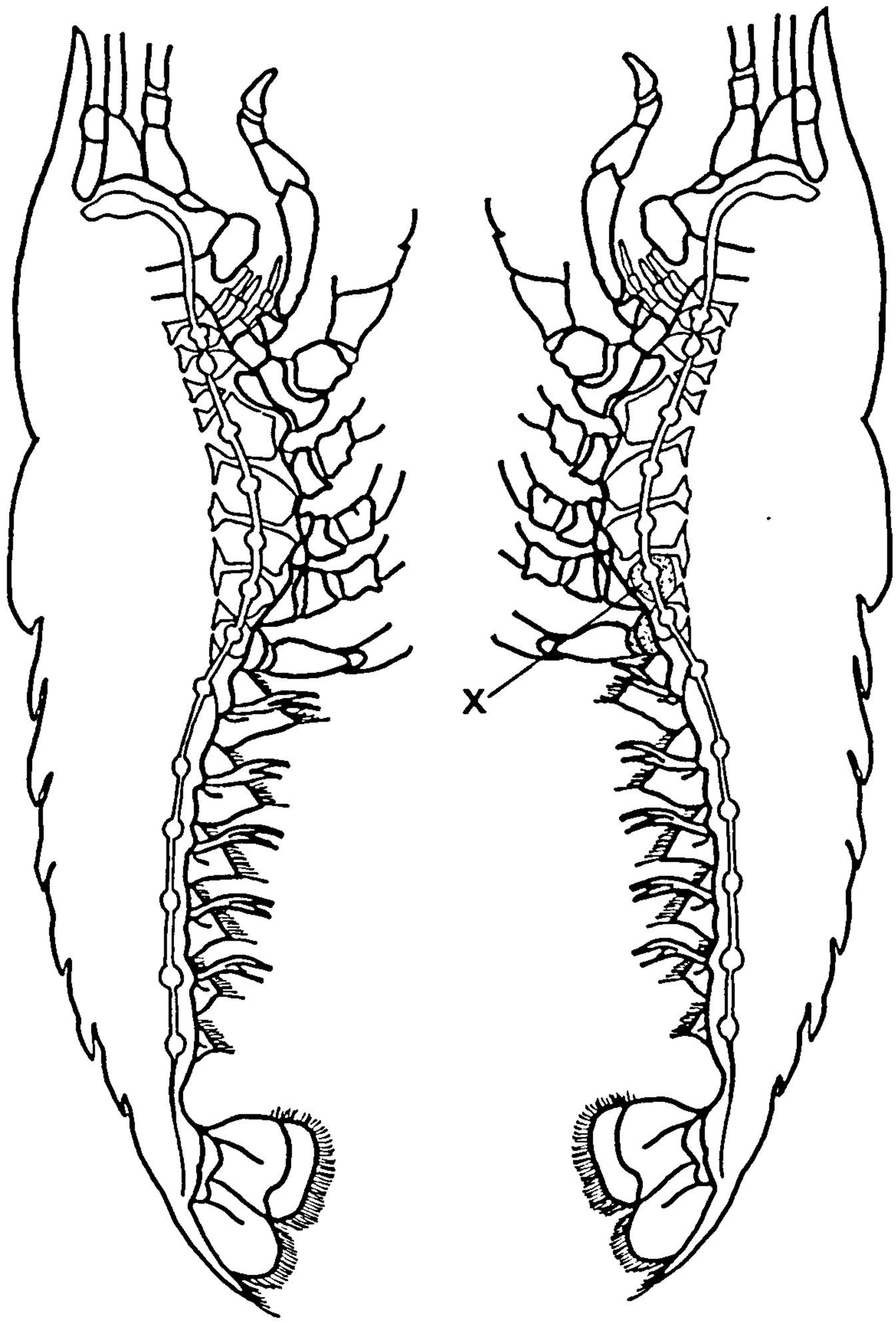

X

Figure 20 (xi)

Sex Female

Number 11

TTD 19 days

No. of sites of infection 12

Other details Animal moribund. Both chelae lost. Infections visible in the proximal joints of 4 walking legs which appeared to be heavily melanized. The ends of all but one leg were also necrotic. One membrane of the ventral abdomen had brown marks.

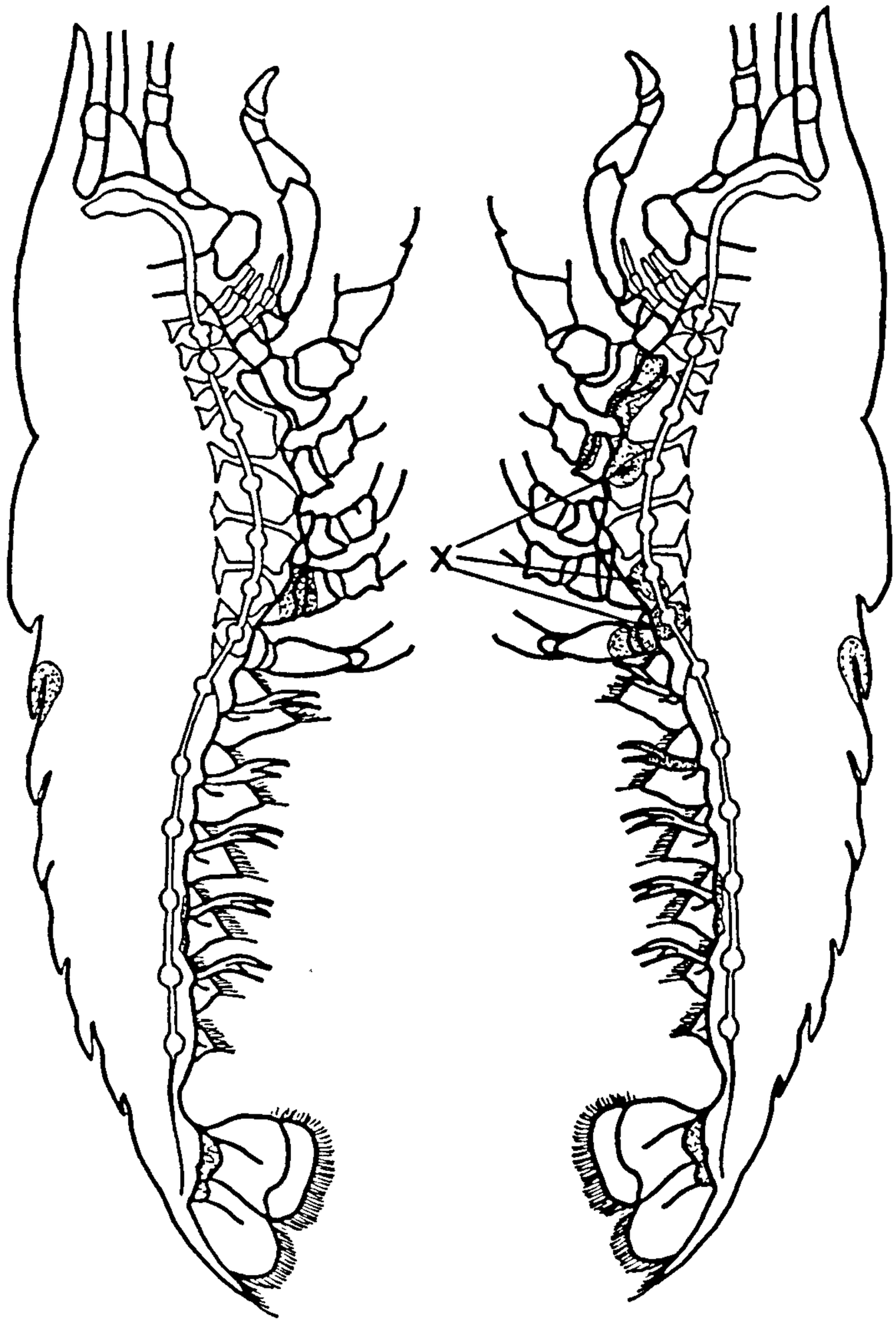


Figure 20 (x11)

Sex Female

Number 12

TTD 22 days

No. of sites of infection 5

Other details Animal dead less than 1 hr.

Had developed tendancy to carry telson curled under the abdomen. Brown patch on back covering the fifth and sixth abdominal segment.

No other signs of infection.

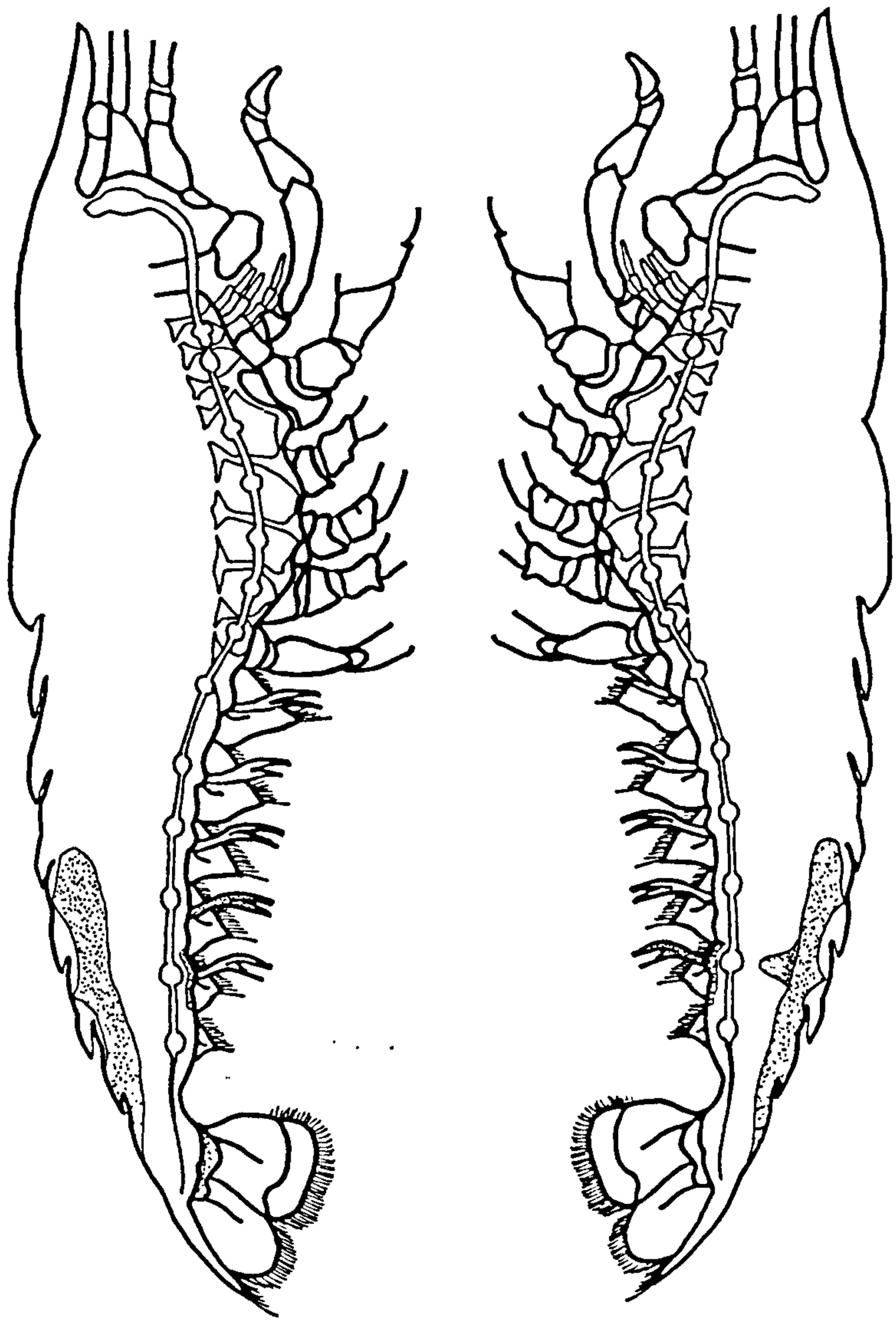


Figure 20 (xiii)

Sex Male

Number 13

TTD 25 days

No. of sites of infection 7

Other details Animal dead less than 2 hours.

Only gross sign of infection was infection of one walking leg.

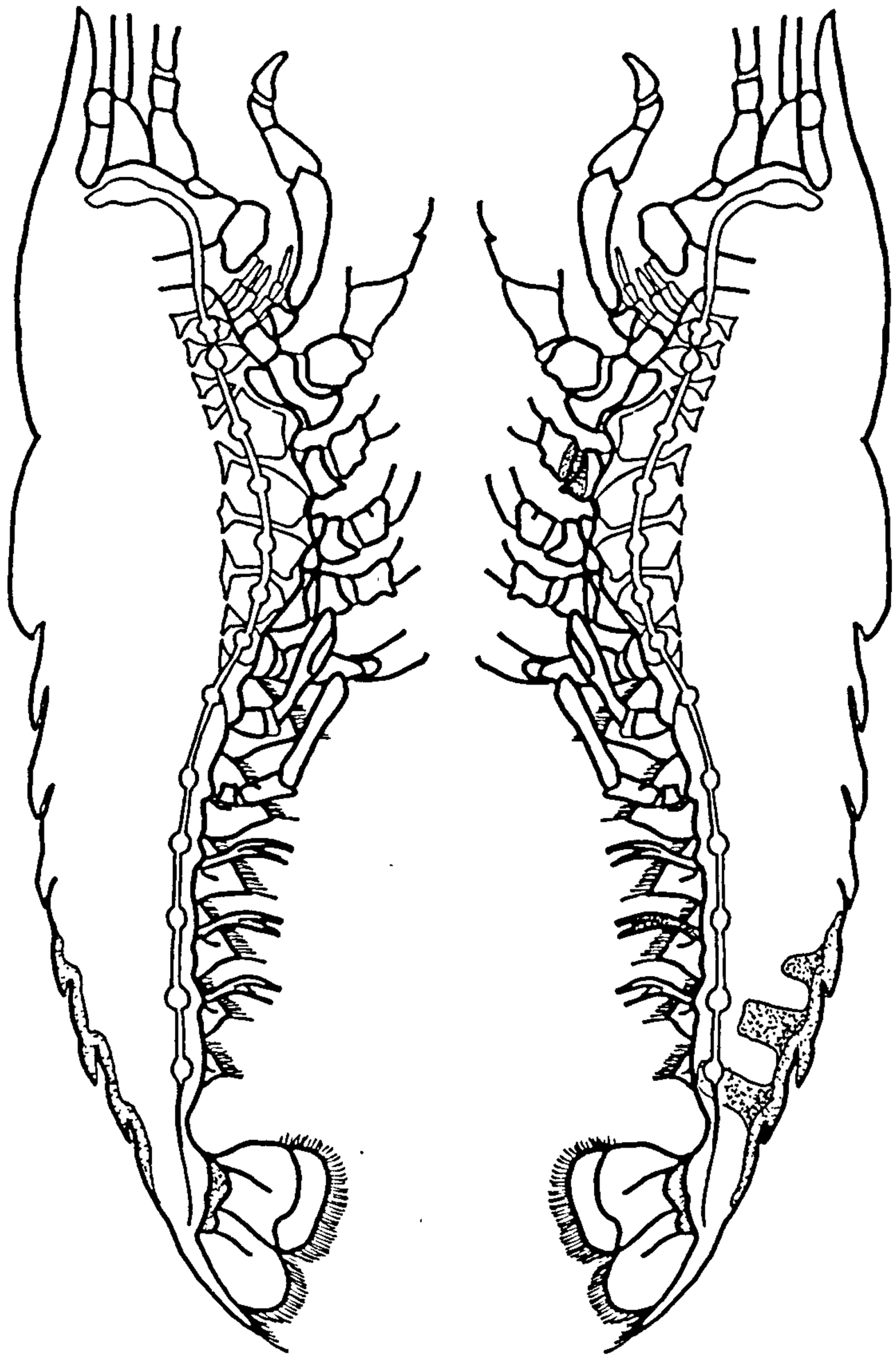


Figure 20 (xiv)

Sex Male

Number 14

TTD 41 days

No. of sites of infection 15

Other details Animal moribund. Signs of infection visible in proximal joints of four walking legs.

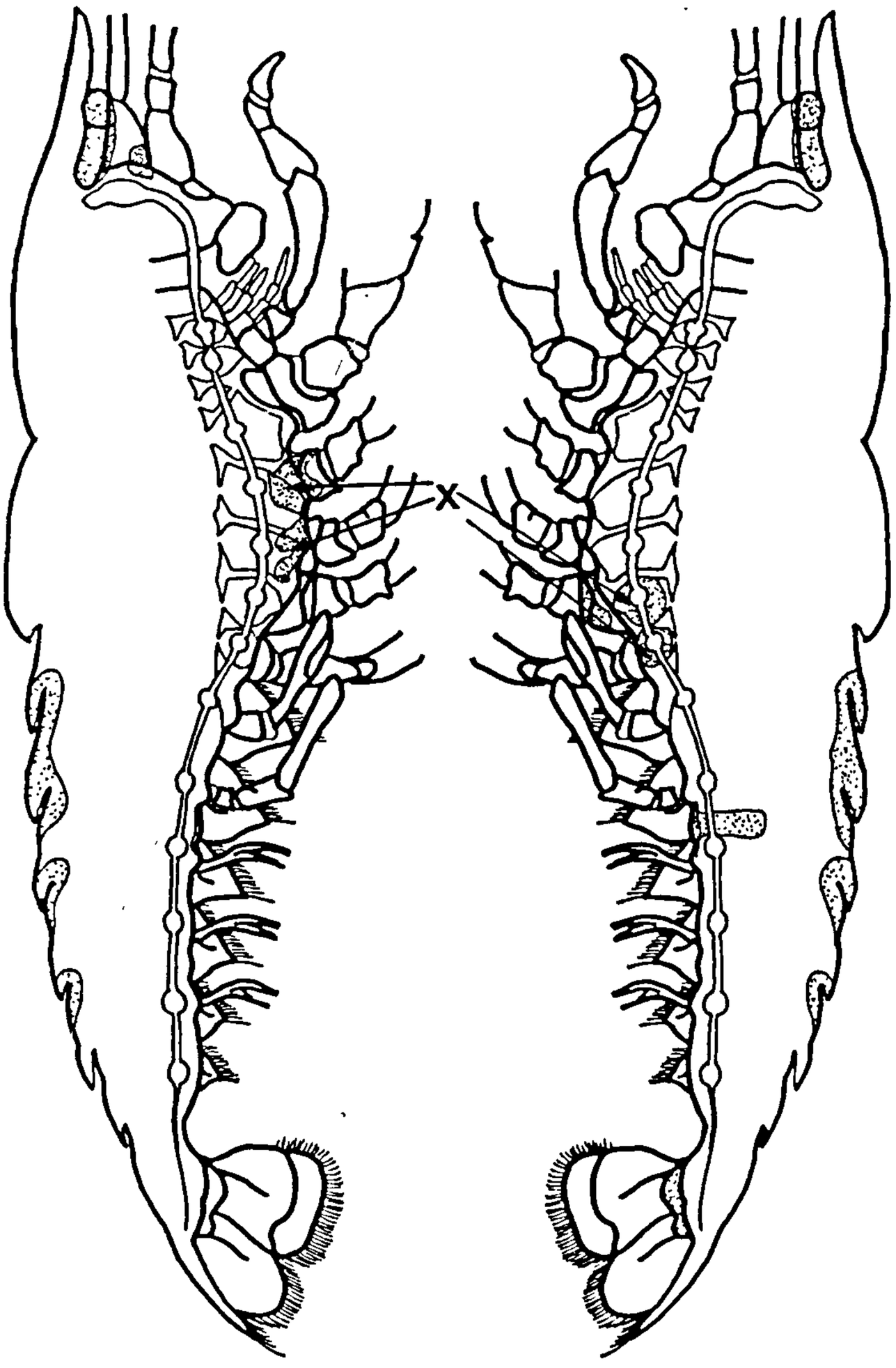


Figure 20 (xv)

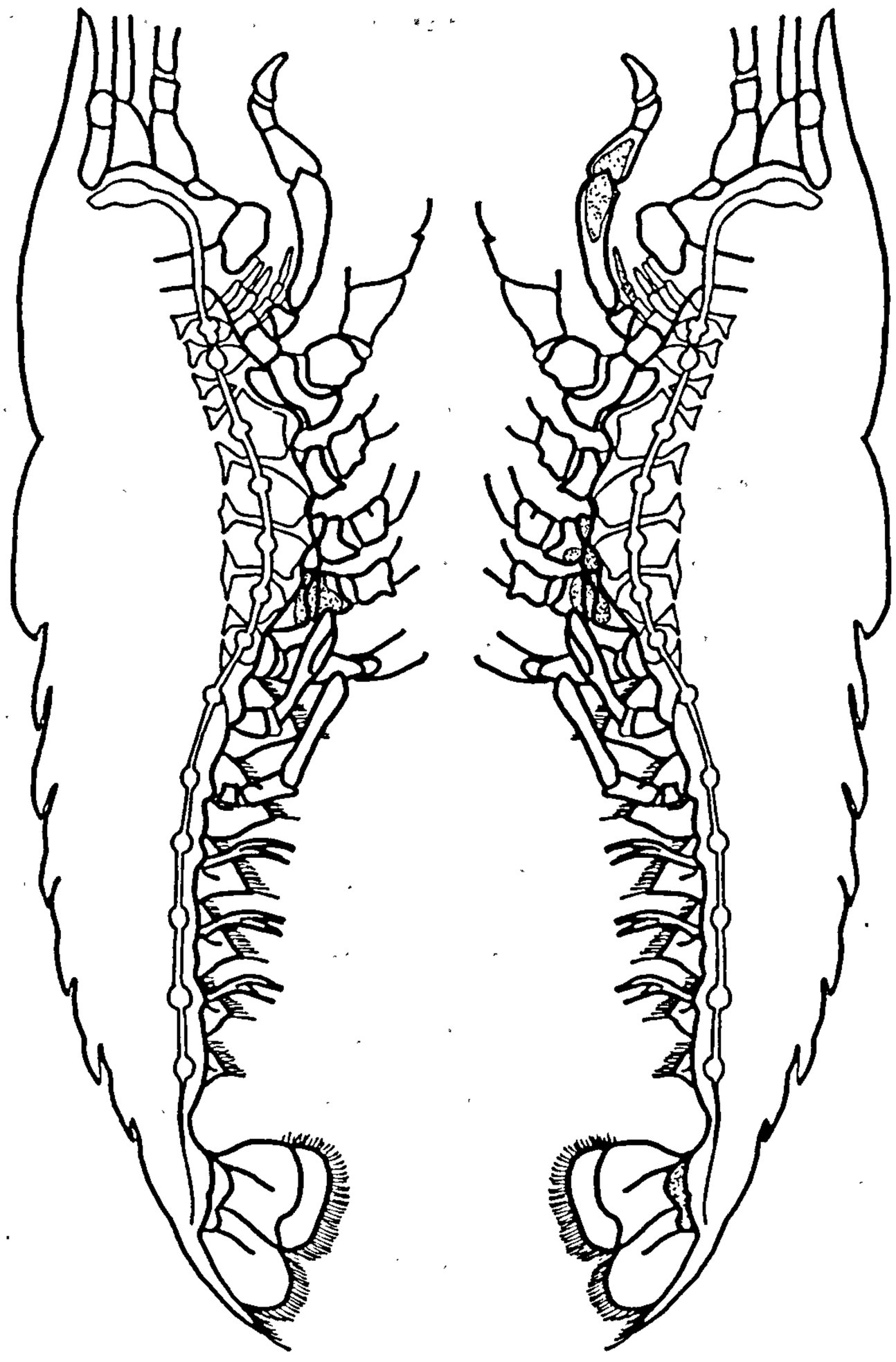
Sex Male

Number 15

TTD 43 days

No. of sites of infection 6

Other details Animal dead. No obvious gross signs of infection.



the brain, foci of infection were relatively remote from vital organs.

DISCUSSION.

On the basis of his work using a Swedish isolate (D1) of Aph. astaci, Unestam (1969b) suggested that Ast. leptodactylus was not fully susceptible to Aph. astaci. That is, only three out of five animals exposed to zoospores of Aph. astaci died within 17 days of infection. Previously, Schikora (1906 and 1916) found that this species of crayfish was easily infected. On the basis of these results it was suggested that resistance to the disease in Ast. leptodactylus may have inceased since the earlier experiments (Unestam 1969b).

In the present study, using crayfish imported from Turkey, the isolates used, killed all the animals tested despite the fact that the spore dose used (four zoospores / ml.) was 2,500 times smaller than that used in the previous work and seven and a half times smaller than the calculated LD 50 for Ast. astacus (Unestam and Weiss 1970). In the present study, 27 out of 36 animals died before 17 days. The last animals died after relatively chronic infections (up to 94 days). The animals infected with isolate D1 died between 14 and 17 days post infection.

From the results of this study, it appears that strains of Aph. astaci recently isolated from English

crayfish are at least as virulent as strains infecting crayfish in Germany in the early part of this century. There was no evidence of the moderate resistance to Aph. astaci thought to be shown by Ast. leptodactylus in earlier work (Unestam 1969c). Whether this is because the isolates used in this study were more virulent than those used earlier, or whether the crayfish showed less resistance is unclear.

In this study infected animals have been characterised by the number of foci of infection in the arthrodial membranes, of variable severity, associated with which are infections of deeper tissues. These may be limited to the epidermis or may include connective tissue, muscles, blood vessels, nervous tissues and gills. The extent of any infection may be limited by a host defence reaction.

The survival time of animals dying before 17 days was correlated to the number of foci of cuticular infection (R = 0.77). This agrees with the results obtained by Alderman et al (1987) who showed that, in Ast. leptodactylus, the survival time of animals challenged with Aph. astaci zoospores was largely a function of challenge size. Survival time was also correlated to the percentage of infected tissue, but not as strongly (R = 0.56).

It is apparent that a number of animals survived longer than 17 days and yet had a large number of infected arthrodial membranes. The existance of a number of weak

(C<2) foci of infection in these animals suggests that reinfection may have occurred. Further, data fom figures 17 and 18 indicates that the haemocyte reaction in these animals is not as advanced as might be expected, given the length of time the reaction has had to develop.

Thus, it appears likely that the animals in this experiment that survived for longer than 17 days, did so because they originally had few or no foci of infection but were infected later by spores released from other animals.

An interesting pattern emerges from figure 14. That is, animals that succumb rapidly to infection not only have large numbers of infection sites but also tend to have infections of the head region or of the gills or both. Animals dying later on do not have gill infections and head infections only occurredin two other animals.

Infections of the head region occurring in the eyes or antennae spread to the conective tissue in the vicinity of the brain, but, contrary to the findings of Nyblin (1931) and Schaperclaus (1954a), infections of the brain were limited to only one or two hyphae and only two animals were involved. However, the effects of fungal enzymes and metabolites on the brain of these animals may have played a major role in their death (see below).

The occurance of invasions of gill tissue is in agreement with the findings of Schikora (1906). Four such animals were seen, all of which succumbed rapidly to the disease. Two of these also had invasions of the head

region.

It was shown that the gills of the mud crab Scylla serrata not only serve a respiratory function, but also exclude foreign molecules from the haemolymph (Mullainadham et al 1984). When Aph. astaci invaded the gill it often either destroyed the lamella, or the haemocyte reaction to the invasion resulted in blockage of the blood passages. This would have reduced oxygen uptake and also reduced the area over which toxic fungal metabolites may be excluded. However, since the maximum number of infected gill lamellae in any animal was four, it is unlikely that invasion of gill structures caused a reduction in oxygen uptake likely to cause the death of the animal. However, in animals already weakened by fungal attack, the reduction in respiratory efficiency coupled with a reduction in the capacity to remove toxic fungal metabolites may well have hastened the animal's demise.

Several authors have suggested that the involvement of the nervous system in animals infected with Aph. astaci ultimately leads to their death (Schaperclaus 1927 & 1954, Rennerfelt 1935 and Nyblin 1931) and Nyblin (1931) has demonstrated fungal infections of the brain in a large proportion of the animals studied. In this study, any neural involvement was usually confined to leg nerves and minor abdominal motor nerves, and was of a localized nature. Whilst infections of the brain were seen in two out of fifteen animals, they were minor in nature and did not appear to have occurred as a result of spread of the

fungus from the nerve cord, but from infection of eye or antenneal soft cuticle. Thus, except in two cases, this study has been unable to demonstrate neural involvement of a degree likely to cause death of the animal.

The possibility that invasion of the blood vessels and possibly the heart may contribute to the death of crayfish infected with Aph. astaci has been suggested (Alderman pers. com.). In this study the invasion of blood vessels was seen in the legs and the gills as well as in the superior abdominal aorta, although hyphae were never found in the heart. When hyphae were found in the blood vessels they seldom caused blockage, however the haemocyte response to the fungus did occasionally block the vessels. This may have lead to reduced oxygen supply to tissues as well as to a reduction in the supply of haemocytes to other infected areas. However, because infections often occurred at sites such as the leg joints, that were remote from vital organs, it is unlikely that, in this experiment, infection of blood vessels lead directly to the death of infected animals.

Nyblin (1931) and Schaperclaus (1927) have both postulated that secondary invasion of bacteria plays a role in the death of animals suffering from aphanomycosis, and Schaperclaus (1927) showed bacteria to be present in the hepatopancreas of infected animals. This study has shown that, in some cases, the cuticle can be severely degraded by Aph. astaci, and it is possible that, in such cases, bacteria could enter the animal and multiply.

However there was no further evidence in this study, for the invasion of the animals by opportunistic bacteria.

Animals dying from Aph. astaci infection during the course of this experiment, did so with only a small proportion of their bodies occupied by fungal hyphae. These results concur with those of Unestam and Weiss (1970) who found that in animals infected with a small number of zoospores fungal involvement was often limited to the arthrodial membrane of a single leg or pleopod.

The limited extent of the spread of Aph. astaci within its crayfish host is in contrast to some mycoses of other crustaceans. In the case of Lagenidium sp. infections in larvae of the shrimp Penaeus setiferus for example the fungus invaded and replaced nearly all the larval tissues before death of the shrimp (Lightner and Fontaine 1973). In larvae of the shrimp Palaemonectes kadiakensis, infections with the fungus Saprolegnia parasitica killed the shrimp within 24 hrs., infecting all the tissues (Hubschmann Schmitt 1969).

The ascomycete fungus Trichomaris invadens that infects the Tanner crab Chionoecetes bairdi enters the host via the hard cuticle and subsequently spreads throughout the epidermis and subepidermal tissues which are virtually completely replaced. The infection then spreads to deep muscles, connective tissue, nerves, blood vessels, haemopoietic tissue, gastrointestinal tract, heart, eyestalks and gills. It is suggested that the disease eventually kills the animal (Sparks 1982). Thus

the types of tissue involved in this infection are similar to those involved in the Aph. astaci infection of crayfish, although the spread of the fungus is more extensive than in the present study, and tissues are more severely infiltrated.

Thus in some cases of fungal infection in crustaceans the invading organism may infect a large part of the host before the host is killed. In this experiment, foci of infection were not only small but were often at some distance from major organs, usually in the walking legs. Infection of tissues such as nerve or blood vessels in these areas were of a somewhat local nature, and probably only had a debilitating effect, since blockage of a blood vessel in a leg, would only reduce haemolymph supply to that one leg. Likewise, the infection of a nerve would only impair function in the leg affected. It seems likely, therefore, that the presence of the fungus itself within the animal, is not the cause of the animal's death. Rather, death is likely to be due to a secondary consequence of fungal invasion, such as fungal enzymes, metabolites or waste products, or secondary bacterial invasion.

The production of proteases by the fungus Metarhizium anisopliae that are toxic to Galleria mellonella has been demonstrated (Kucera 1981 and 1982) and mycotoxin production has been demonstrated in Entomophthora egressa (Dunphy and Nolan 1982). Aph. astaci has been shown to produce proteases, chitinases,

hyaluronidases, and, during germination, lipases (Soderhall and Unestam 1975, Soderhall 1978). Further, Unestam and Weiss (1970) suggested the possible involvement of neurotoxins in the death of crayfish infected with Aph. astaci. The production of extracellular proteases and other enzymes, by Aph. astaci in vivo may well cause damage to tissues at sites remote from the site of infection, and may thus lead to the animal's death. However, when the gill rachis of infected animals were examined for signs of haemocyte accumulation, such as that demonstrated during dye clearance in the Crab S. serrata (Mullainadham et al 1984) no such accumulations could be demonstrated.

The epicuticle of aquatic arthropods is the major barrier to osmotic influx of water in a fresh water environment. If this barrier is broken down, for example by fungal hyphae, the animal may well take up water from it's surroundings, especially in cases of multiple severe invasions, and eventually die from dilution of its body fluids. Several of the animals studied showed large areas over which the cuticle was badly degraded, and in these cases body fluid dilution may have played a role in the death of the animal (Chapter 6.).

The behavioural symptoms of aphanomycosis have been described many times in the literature (eg. Unestam 1964a Mazylis and Sestukas 1968). These include walking on outstretched legs so that the animal appears to be "walking on stilts", assuming an unnatural posture often

with the tail held underneath the body, lying on their backs, often with rhytmical twiching of limbs or hanging limply when picked up. Infected animals it is often said, become disorientated and may be seen on the river bed during daylight (Hofer 1900). The loss of claws or legs is also a commonly quoted symptom of the disease, but Schikora (1905) points out that this may be caused by a variety of bacterial infections.

A number of these symptoms may be explained by the histopathology of the disease. Thus, walking on outstretched limbs may well be the result of infection of nerves or muscles in the legs, as may abnormal postures, and infections of the abdominal flexor and extensor muscles may explain the weakness of the tail flick response in some infected animals. Infections of leg muscles combined with blockage of blood passages and infection of leg nerves may lead eventually to tissue starvation and limb autotomy. The heavy infection of the dorsal abdomen seen in some animals may explain why infected animals have a tendency towards unnatural postures of the abdomen. Infection of the antenae, eyes or optic nerves may lead to the animal becomming blind and hense disorientated which may in turn lead to it walking about during the day; whilst infection of nervous tissue may lead to nervous disorders such as uncoordinated limb movements and twitches.

This study has shown that English isolates of Aph. astaci are probably comparable in virulence with those

from Europe since they are capable of killing crayfish in a matter of weeks even at low dose rates. However it is possible that some of the crayfish tested under the conditions of the experiment did not become infected by the initial dose of spores, but succumbed to infection by spores liberated by other animals in the tank. It is suggested that the longevity of animals infected with Aph. astaci was dependant upon the number of foci of infection and to some extent on the volume of tissue infected with the fungus.

The overall histopathology of the disease in Ast. leptodactylus was similar to that described by earlier workers in Ast. astacus although there was no evidence for neurotrophy. The size of individual infections was limited and often confined to areas remote from vital organs. For these reasons it was suggested that death in these animals was due to a secondary consequence of fungal invasion such as body fluid dilution, secondary bacterial invasion or the production of toxic fungal products, although the invasion of structures such as nerves, blood vessels and gills may have contributed to the animals' demise.

THE EFFECT OF INFECTION WITH APHANOMYCES ASTACI ON URINE PRODUCTION IN ASTACUS LEPTODACTYLUS.

INTRODUCTION.

In infection experiments, the articulating membrane between the dorsal thorax and abdomen, was swollen in a small number of animals, shortly before death. It was suspected that the swelling was due to an increase in haemolymph volume, however, the conection between this phenomenon and Aph. astaci infection was unclear. An increase in the haemolymph volume may have come about either by an increase in the water influx, over and above that which the animals' regulatory mechanisms could handle; or by a breakdown in the clearance of water flowing into the animal.

Histological studies of crayfish with Aph. astaci infections revealed heavy infections in some areas of soft cuticle. In some cases the cuticle was very badly degraded (Chapter 5). The cuticle of the crayfish is a barrier against the influx of water and loss of ions in the hypo-osmotic freshwater environment, thus physical and enzymatic disruption of the cuticle by Aph. astaci (Nyhlen and Unestam 1975, Soderhall and Unestam 1975, Unestam 1978) might cause a breakdown of cuticular impermeability leading to an influx of water.

In the short study described below here, it was

intended to examine the effects of Aph. astaci infection on the water balance of the crayfish Astacus leptodactylus. It was assumed that a pathological increase in the influx of water would lead to an increase in the rate of urine production in order to maintain the volume and concentration of the haemolymph. In the experiments reported here, attempts were made to develop a procedure that would allow urine flow to be measured over an extended period of time. This technique was then used to measure the rate of urine production in infected and uninfected animals over several weeks.

METHODS.

Animals were immobilized on perspex frames and collecting tubes were attached over the openings of the antennal glands in the following manner.

Sections of rubber tube were placed over each chela immobilizing them and preventing the animals removing the urine collecting apparatus. The animals were then clamped to a plastic box, ventral surface uppermost, by passing strong elastic bands around the abdomen and the outstretched chelae. The bases of the antennae were dried with tissue paper and gently cleaned with 70% alcohol taking care not to touch the membrane of the antennal gland papillae as this often caused the release of urine. Of a number of tube attachment regimes, the following was found to be the most effective.

A short (approximately 20mm) length of plastic tubing, cut on the diagonal, was passed over each papilla and held firmly in place with forceps, longest side to the front, whilst it was cemented. A number of types of tubing and adhesive were tested for this purpose. Tubing from the No. 25 Butterfly catheter manufactured by Abbott Ireland limited, Eire was found most suitable since it was of the correct diameter to pass over the papillae and could be cemented strongly in place using Permabond 910 cyanoacylate adhesive. The adhesive was applied with a drawn Pasteur pipette and set rapidly with a small amount of methyl methacrylate, which was applied with a wooden toothpick. Cement was built up around the base of the tube to prevent movement of the proximal segments of the antennae and to aid adhesion.

Once cemented in place the tubes were cut to approximately 5mm in length. This short length of tubing was joined to a length of Portex tubing by a 30 mm length of rubber tubing. The collecting apparatus was tested for leaks by gently blowing down the Portex collecting tube. Attempts to canulate the antennal gland through the nephropore with a length of drawn Portex tubing always proved fatal.

Once fitted with collecting tubes, the animals were immobilized on a perspex frame (figure 1) and suspended 20-40 mm below the surface of a small tank of aerated tap water (figure 2.). The water temperature was maintained at 16-18°C and the tank was cleaned every other day. The ends

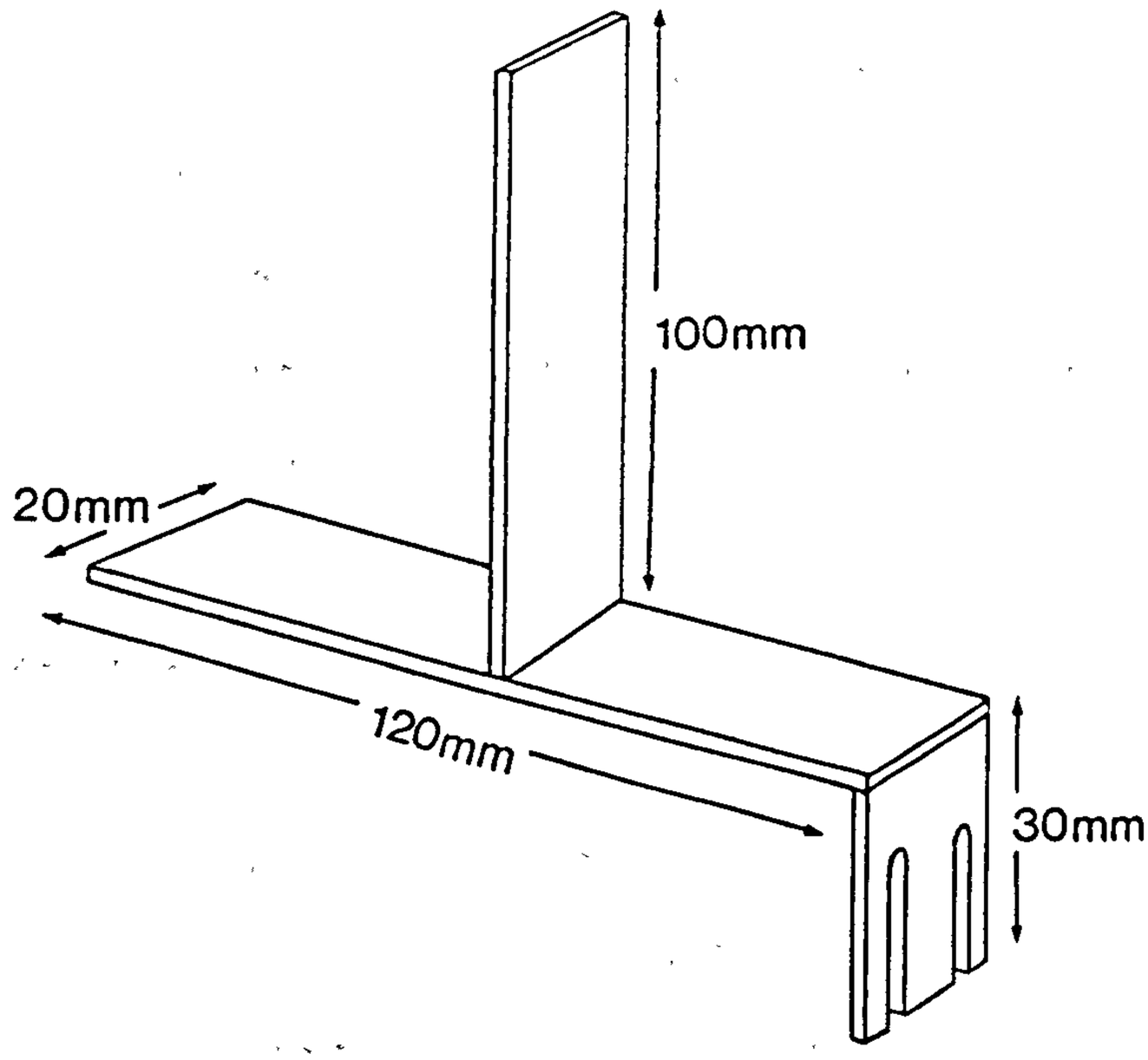
Figure 1. The perspex frames used to restrain crayfish during urine collection.

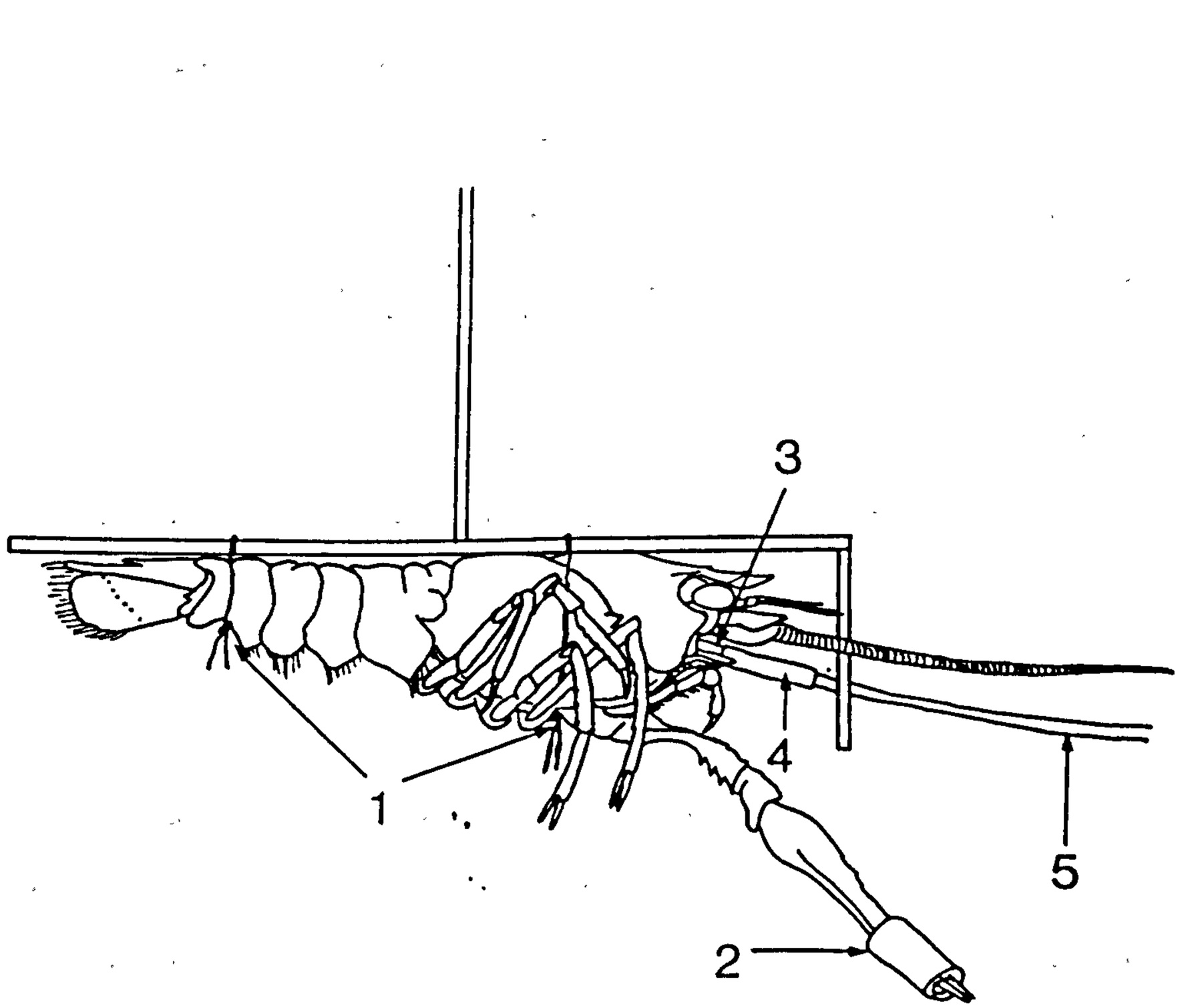
Figure 1 shows the perspex harness used to restrain crayfish during urine collection. The two slots on the front plate are guides for the portex collecting tubes - see figure 2.

Figure 2. Apparatus for crayfish urine collection.

This diagram shows the way in which the frame in figure 1. was used to restrain crayfish whilst urine was collected.

1. Cotton ties used to attach the animal to the apparatus.
2. Section of rubber tubing used to restrain the chelae.
3. Section of plastic tubing cemented to the urinary papilae.
4. Length of rubber tubing.
5. Length of Portex tubing leading to a collecting vessel.


100mm
20mm
120mm
30mm


3
4
5
1
2

of the collecting tubes were placed in separate test tubes, keeping them at the same level as the papillae to avoid syphoning effects.

Urine was collected daily and the total volume produced from the right and left ducts was measured. Urine osmolality was measured using a Roebling osmometer (Camlab).

After several days acclimatization, a final measure was made of urine produced and the animals were released from the frame and the collecting tubes were detached at the rubber tube. Zoospores of Aph. astaci (isolate 28-1083(4)) were then introduced into the water to a concentration of approximately 90 per ml. The tanks were then allowed to stand unaerated for a period of 24 hrs. The animals were then re-attached to the frame, the rubber tubing was re-attached and urine collection was continued until death. Control animals were detached from the apparatus but were not infected. They were then reconected after 24 hrs and urine collection was continued.

RESULTS.

Urine was readily collected for up to a week using this method, however, animals did not generally survive the experimental regime for long periods. Death appeared to be caused by blockage of the collecting tubes near the urinary papillae with a concretion of some kind. This probably prevented or restricted the passage of urine,

causing the animals to swell at the joints and die. Other problems included melanization of the urinary pore, possibly due to bacterial infection, or reduction of the urine flow to almost zero, in either or both collecting tubes (often at much increased osmolality) for no apparent reason. Animals with such reduced urine flow generally died within one to two days. It did not prove possible to prevent these mortalities by daily flushing of the tubes with distilled water.

The adverse reaction of the animals to urine collection using the technique described above meant that long term data was collected from a very limited number of animals. In all, ten animals survived for extended periods, six control and four infected with Aph. astaci. The results obtained from these animals are illustrated in figures 3, 4 and 5.

Figure 3. shows the results of replicate experiments in which urine flow and urine osmolality was measured in two uninfected animals over an extended period. Animal B died after 23 days and the experiment was terminated after 25 days. Daily urine production in both animals fluctuated between 200 ul. and 2.2 mls. Urine osmolality in animal A ranged from 48 to 162 mOsm / kg and that of animal B ranged from 30 to 120 mOsm / kg. Neither the rate of urine production, nor the urine osmolality remained stable for long periods.

Figure 4. shows the results of an experiment in which the rate of urine production and urine osmolality

Figure 3. Urine volume and osmolality:normal animals.

This figure shows the volume and osmolality of urine collected from two non infected animals over a period of 25 days.

——— Urine volume

..... Urine Osmolality

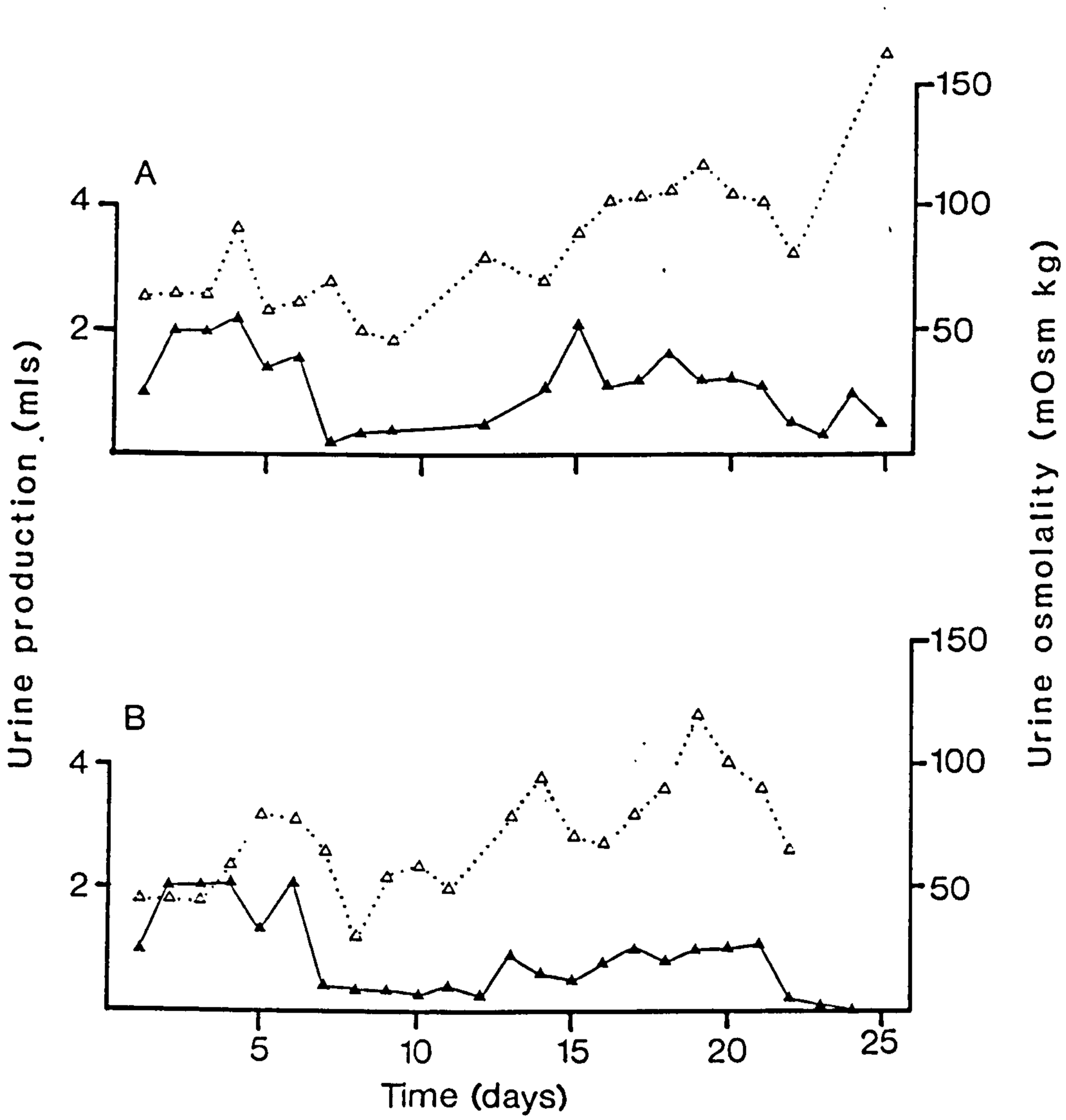

A
B
4
2
150
100
50
5
10
15
20
25
Time (days)
Urine production (mls)
Urine osmolality (mOsm kg)

Figure 4. Urine volume and osmolality in infected and control animals over a period of 28 days.

This figure shows the volume and osmolality of urine collected from two infected and two control animals (A & B and C & D respectively) over a period of 28 days. The first part of the graph in each case represents a 6 day preinfection control period. The gap in each graph represents the period of infection during which no urine was collected (see text).

———— Urine volume

..... Urine osmolality

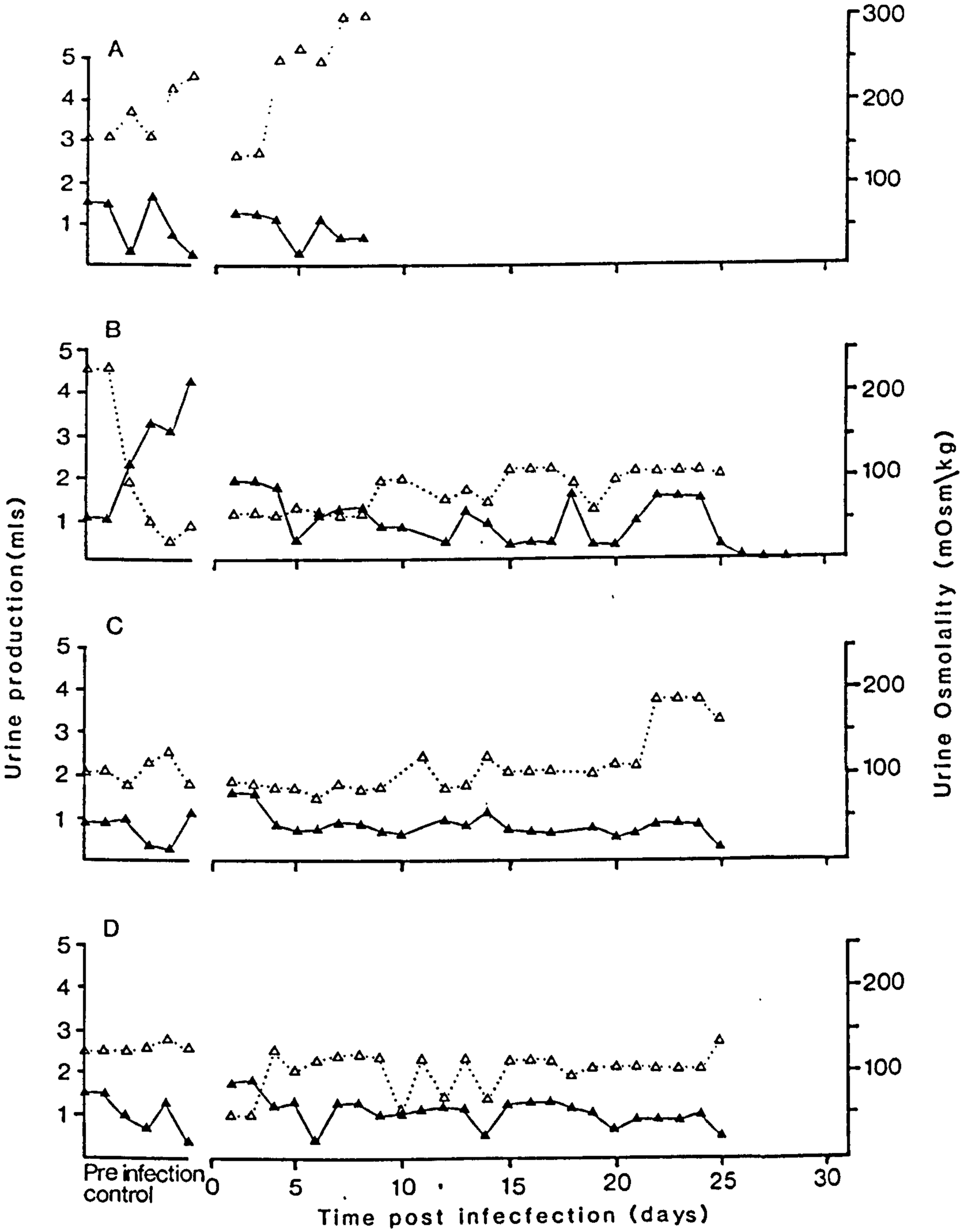
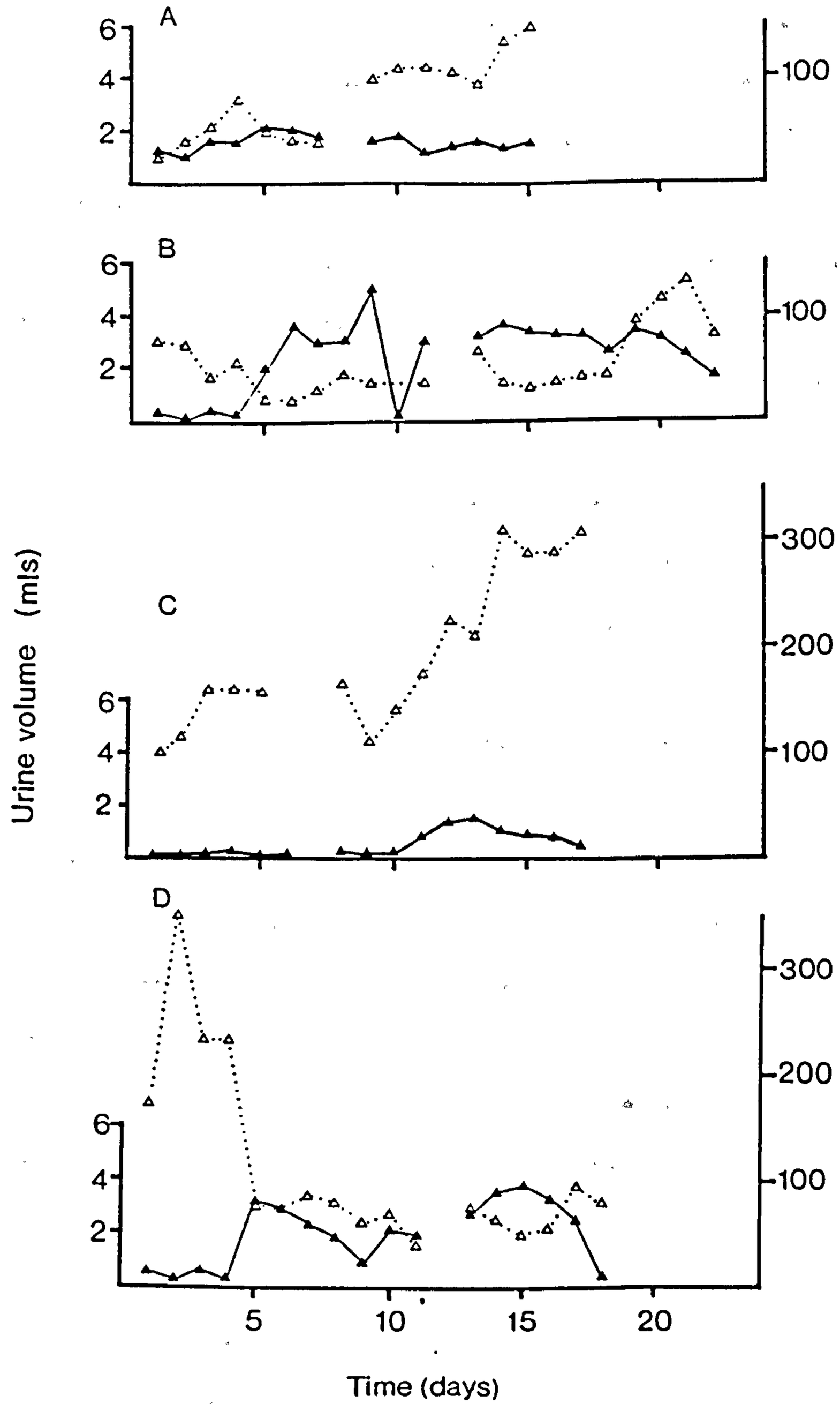

A
B
C
D
5
4
3
2
1
300
200
100
Urine production (mls)
Urine Osmolality (mOsm\kg)
Pre infection control
0
5
10
15
20
25
30
Time post infecfection (days)

Figure 5. Urine volume and osmolality in infected and control animals.

This figure shows the volume and osmolality of urine collected from two infected and two control animals (A & B and C & D respectively). The first part of the graph in each case represents a preinfection control period. The gap in each graph represents the period of infection during which no urine was collected (see text).

———Urine volume

.....Urine osmolality


A
B
C
D
6
4
2
100
200
300
5
10
15
20
Urine volume (mls)
Osmolality (mOsm/kg)
Time (days)

were followed in two infected and two control animals. The first infected animal died seven days post infection with no sign of increased urine flow following infection. Urine osmolality increased during the period of infection reaching a value of 295 mOsm/kg at death.

The second infected animal died 27 days post infection and again showed no gross increase in urine flow. Urine osmolality remained more or less constant. The two control animals died 24 days after re-attachment to the apparatus. Urine production remained more or less constant in both animals. Urine osmolality appeared to increase four days before death in one animal.

Figure 5. shows the rate of urine production and urine osmolality in four animals, two infected and two control (A&B and C&D respectively.). The length of the preinfection control period varied from animal to animal.

Both infected animals died showing signs of Aph astaci infection, animal B, 8 days post infection and animal C, 11 days post infection. Neither of the two infected animals showed a marked increase in urine flow before death, indeed in B, urine production remained more or less stable after infection, whilst in C, urine production began to decline 4 days before death. Urine osmolality increased before death in both animals, although in animal C it decreased sharply before death.

One control animal (D) died 6 days after re-attachment to the apparatus whilst the other animal (A) was still alive 10 days after re-attachment. In D, urine

production decreased before death as it did in one of the infected animals. This animal also showed an increase in urine osmolality before death.

DISCUSSION.

Neither urine production, nor urine osmolality remained stable for long periods during these experiments and no obvious differences were seen between infected animals and controls. However a trend towards increased urine osmolality in the period before death was noted in both infected and control animals.

In previous studies involving the collection of urine from the crayfish Procambarus clarkii, a similar technique was used and urine was collected over a period of 1 to 2 days (Kamemoto and Ono 1968). The volume produced was between 1.8 to 3.2 mls or approximately 4.3% of body weight per day, but varied from day to day. The results obtained from control and test animals in these experiments agree broadly with this data but urine flow in the animals studied occasionally fell as low as 100 ul. for long periods and reached as high as 4.8 mls on one occassion. Urine osmolality was, in general, 2-4 times higher than values obtained for Austroptamobius pallipes urine in fresh water at a similar temperature (Mantel 1983).

Clearly the data presented here is insufficient to

draw firm conclusions about the effect of Aph astaci infection on urine production in these animals, and thus about the effect of the infection on osmoregulation in general. However, in the small number of animals studied, there appeared to be no clear effect of fungal infection on either the volume of urine produced on a daily basis, or on the osmolality of the urine, even around the time of death.

Histological studies (Chapter 3) showed that animals dying early on in infection experiments (and also those dying after chronic infection) often showed evidence of fungal invasion of the articulating membranes of the head region, including joints of the antennae. It is conceivable that infection spreading from these joints to the antennal glands and preventing their efficient function, could lead to impairment of osmoregulation, and consequent swelling and death. However, no evidence of antennal gland infection was seen in the histological examinations (Chapter 3).

If infection did lead to a breakdown in osmoregulation, one might expect a change in haemolymph osmolality in infected animals. Recently, measurements of the osmolality and ionic composition of the haemolymph of infected and uninfected Astacus astacus failed to show a significant change in haemolymph osmolality upon infection with Aph astaci (Jarvenpaa et al 1986). Thus, the results of the present study, and those of Jarvenpaa et al indicate that haemolymph dilution is unlikely to be prime

cause of death in plague infected crayfish.

Experiments were not pursued further because techniques for long-term urine collection seemed to be very much less reliable than initial trials of the technique had indicated.

THE EFFECT OF CRUSTACEAN SURFACE EXTRACTS ON THE GERMINATION OF APHANOMYCES ASTACI ZOOSPORES IN VITRO.

INTRODUCTION.

The aim of this study was to determine whether compounds of an anti-fungal nature exist on the surface of both Gammarus pulex and crayfish, and to determine the effects of such compounds on the secondary zoospores of Aphanomyces astaci.

The existence of antimicrobial compounds on the surface of the insect cuticle is well documented (Koidsumi 1957, Wada 1957, Evalokova and Chekourina 1962, Smith and Grula 1982).

For example, removal of the surface lipid layer from either Bombyx mori (the silk worm) or Chilo simplex (the rice stem borer), increased the organisms' susceptibility to attack by the pathogenic fungi Aspergillus flavus and Isaria farinosa. Further, extracts of the cuticle inhibited the growth of the fungi and it was suggested that the effect was due to octanoic and decanoic acids in the epicuticular lipid layer (Koidsumi 1957).

Pentanoic, octanoic and nonanoic acids are believed to be responsible for mycostatic activity in surface hexane extracts of the Corn Ear Worm, Heliothis zea tested against the fungus Beauvaria bassiana. Octanoic acid itself was shown to prevent conidial germination in the

same fungus (Smith and Grula 1982).

In addition, the toxic effects of fatty acids on micro-organisms have been studied by a number of workers. For example, members of a homologous series of alkanoic acids between methanoic and undecanoic acid were capable of inhibiting the growth of Pithomyces chartarum, Fusarium oxysporum, F. culmorum and Mortierella alpina. An unsaturated C11 acid, hendecanoic acid was also toxic to these organisms. Nonanoic, decanoic, undecanoic and hendecanoic acids were capable of inhibiting spore germination in these species. The inhibition of spore germinaton by undecanoic acid was most marked at pH 3.4 to 5.5, above pH 6 there was no evidence of an inhibitory effect.(Thornton 1963).

Fatty acids between ethanoic and dodecanoic were demonstrated to be toxic to Boletus varigatus (Pedersen 1969), whilst fatty acids and alcohols between C5 and C12 were toxic to Hormoconis (previously Cladosporium) resinae. Fatty acids and alcohols are also known to be toxic to bacteria (Teh 1974).

An extensive search of the literature has failed to reveal any reports of antifungal fatty acids in crustacean cuticle, possibly because previous studies concentrated on interactions between insect pests and possible biological control agents. However, preliminary studies carried out by Unestam and co-workers indicate the presence of compounds on the surface of crayfish that influence the formation of the germ tube in Aph. astaci (Unestam et al

1977). Further, Svensson (1978) reported that exudates from crayfish eggs or intact cuticle affected zoospores even in very high dilutions, although no further details were given.

In the crayfish cuticle a number of compounds may act against invading fungi. For example, certain quinones, which are the intermediates in melanization reactions and are also found tanning or cross linking the lipoproteins of the inner epicuticle (Ross-Stevenson 1985), have been demonstrated to be toxic to Aph. astaci in vitro (Soderhall and Ajaxon 1984).

The enzyme responsible for quinone production in both cases is phenol oxidase (PO). PO activity is found in the soft cuticle of intermoult crayfish (Unestam and Ajaxon 1976, Unestam 1981) and has been demonstrated in the intermoult epicuticle of the crab Menippe rumphii (Babu et al 1985) but was not found in the epicuticle of the crayfish Orconectes obscurus. If free quinones are present in the epicuticle during intermoult, they may act as antifungal compounds.

Melanin, the end product of phenol oxidation in crayfish defence mechanisms, has also been demonstrated to have some anti fungal activity (Soderhall and Ajaxon 1982).

A protease inhibitor that is active against Aph. astaci protease, has also been isolated from the soft cuticle of the crayfish Ast. astacus. The protease is thought to originate in the haemocytes and is believed to

play a role in defence against fungal invasion. (Hall 1983, Hall and Soderhall 1983).

Thus there exist in the crayfish cuticle, a number of compounds that may be active against invading fungi.

There is strong evidence to suggest that the epicuticle of crayfish plays a major role in protecting the animal against invasion by Aph. astaci, and possibly in the differential susceptibility of European and American crayfish species to the fungus.

The resistance demonstrated by some species of crayfish, to infection by Aph. astaci, can be explained to some extent by stronger melanization reactions in the cuticle (Unestam 1975, Unestam and Ajaxon 1976). However, Unestam and Weiss (1970) demonstrated that the epicuticle of both susceptible and resistant species also offered a major barrier to infection by Aph. astaci.

When portions of excised cuticle were incubated in zoospore suspensions, no penetration of the cuticle from the epicuticular surface was observed, in either susceptible or resistant species, although penetration occurred easily from the inner surface.

If the epicuticle was either peeled away or pricked, P. leniusculus was rendered susceptible to infection by Aph. astaci (Unestam and Weiss 1970).

Futhermore, in electron microscope studies of the infection process (Nyhlen and Unestam 1975) it was noted that the number of penetrations of the epicuticle of P. leniusculus by germinating Aph. astaci zoospores, was

fewer than in Ast. astacus, again suggesting that some barrier to penetration existed at the level of the epicuticle in P. leniusculus.

The inability to demonstrate susceptibility of aquatic arthropods, other than some decapod crustaceans, to Aph. astaci suggests that such organisms are strongly resistant to crayfish plague (Unestam 1969c and 1972). It was reported above (Chapter 3), that G. pulex is not only resistant to the disease, but that animals exposed to strong suspensions of Aph. astaci zoospores showed no evidence of chronic infections, such as those found in resistant crayfish species (Vey et al 1981, Persson and Soderhall 1983). This suggests that G. pulex is refractory to infection by Aph. astaci zoospores rather than able to stave off any infections that may become established, again suggesting that the mechanism of resistance operates at the level of the cuticle surface.

The mechanism of resistance to Aph. astaci therefore appears to involve at least two factors: one involving melanization of the organism within the cuticle, the other involving the epicuticle.

The epicuticle of arthropods is the tough outer layer of the cuticle. In the crayfish O. virilis it is approximately 7um thick on the branchiostergite, whilst the remainder of the cuticle is some 230 um. thick (Travis 1965). It is a multi layered structure, traversed by pore canals and by dermal gland ducts (Neville 1975).

In the past, nomenclature of the layers has proved

confusing, thus that of Neville (1975) will be used here.

The basic pattern has four layers visible under the light microscope with appropriate staining, although modifications exist, and more layers may be visible by electron microscopy (Green and Neff 1972 and illustrations in Nyhlen and Unestam 1975).

The inner layer, known as the inner epicuticle, is believed to consist of tanned proteins or lipoproteins, whilst above this is the trilaminar outer epicuticle. The wax layer is found on top of the outer epicuticle and may be secreted by the pore canals. In some aquatic arthropods the wax layer is probably absent. In arthropods that do have a wax layer it is thought to play a major role in the animals water relations.

Above the wax layer is the cement layer. This layer consists of tanned proteins and polyphenols and is believed to act as protection for the underlying layers, although it is not always present.

Both resistant and susceptible species are more susceptible to infection by Aph. astaci during the post moult period than during intermoult (Unestam and Ajaxon 1976) and this may give a clue to the nature of the epicuticular factor responsible for the protection against Aph. astaci.

Travis (1965) pointed out that during the period immediately post moult, the epicuticle is incomplete, its lower layers, the inner and outer epicuticle, being secreted by the epidermis during pre-moult stage D2. It is

completed by the addition of materials after moult. These materials probably constitute the wax and cement layers where present (Neville 1975). Thus it may be the materials added after moulting, the outer layers of the epicuticle, that render intermoult animals more resistant to attack by Aph. astaci.

The aim of this study was to demonstrate whether anti-fungal compounds exist on the surface of both G. pulex and crayfish, what effects such surface compounds may have on the germination of the zoospores of Aph. astaci and whether such compounds contribute to the differential susceptibility of crayfish and G. pulex to infection by Aph. astaci. The report is divided into 5 sections dealing with: the effects of solvent treatment and abrasion of the epicuticle on infection of crayfish; the development of an assay to detect compounds effective against Aph. astaci zoospores in crustacean solvent extracts; using this assay to detect activity in G. pulex extract; the detection of activity in crayfish extracts; and the analysis of these extracts.

SECTION 1.

The effect of solvent treatment and abrasion of the epicuticle on Aphanomyces astaci infection in a resistant and susceptible species.

The aim of this experiment was to determine whether abrasion or solvent treatment of the surface of crayfish arthrodial membranes enhanced the ability of Aph. astaci to infect the treated membranes; and therefore to determine whether solvent soluble or purely mechanical factors could be detected on the surface of crayfish, that protected them against Aph. astaci infection.

METHODS.

Ten Ast. leptodactylus and two P. leniusculus were used in this experiment. The proximal joint of two legs were used for each of four treatments on each animal and were assigned randomly.

The proximal arthrodial membranes of all eight walking legs were cleaned with a damp cotton wool bud. Two were then wiped with a cotton wool bud soaked in hexane, two with a cotton wool bud soaked in a 1 : 1 chloroform / methanol (C/M) mixture and two were lightly rubbed with sand paper. The two remaining leg membranes of each animal were rubbed with a cotton wool bud only and served as controls.

After treatment, animals of both species were placed in a 15 litre plastic trough containing one litre of a zoospore suspension in distilled water. The suspension contained approximately 1,000 swimming zoospores per ml.

After 14 hrs the two species were separated and placed in separate troughs containing 10 litres of aerated tap water that had been allowed to stand over night. The animals were observed daily and after seven days an assessment of the number of infected leg bases was made.

Leg bases were considered severely infected if the infection was visible to the naked eye as a creamy discolouration of the usually translucent arthrodial membrane.

RESULTS.

Figures 1. and 2. show the appearance of Ast. leptodactylus and P. leniusculus respectively. After seven days, infected leg bases was clearly visible since they were whiter than the non infected ones, and had become opaque. Under a binocular microscope (X 100) hyphae could clearly be seen ramifying through the arthrodial membrane.

Table 1 shows the number of infected leg bases for each treatment.

Figure 1. Ventral view of Astacus leptodactylus challenged with Aphanomyces astaci after solvent treatment or abrasion of the proximal leg joints.

This figure shows the effect of solvent treatment or abrasion of the proximal leg articulation on Aph. astaci infection in Ast. leptodactylus. The infected joints appear to have lost their sheen and are a milky white colour. Note the melanization of the articulations treated with C/M

Figure 2. Ventral view of Pacifastacus leniusculus challenged with Aphanomyces astaci after solvent treatment or abrasion of the proximal leg joints.

This figure shows the effect of solvent treatment and abrasion of the leg joints on Aph. astaci infection in P. leniusculus. None of the leg joints are infected severely but one pair, those treated with sand paper, are becoming melanized.

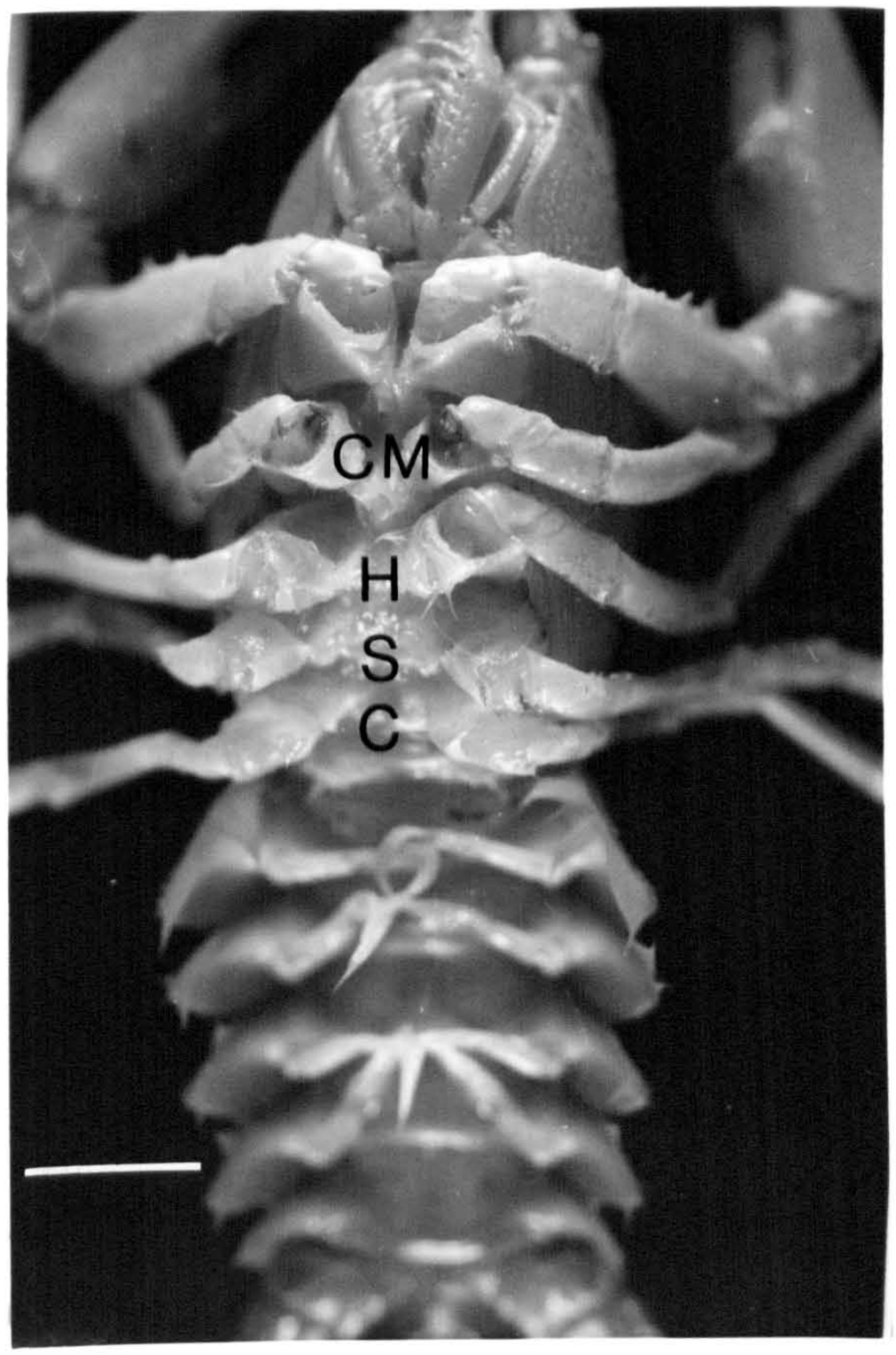
CM = 1 :1 chloroform : methanol

H = Hexane

S = Sandpaper

C = Control

Scale bar = 10 mm


CM
H
S
C

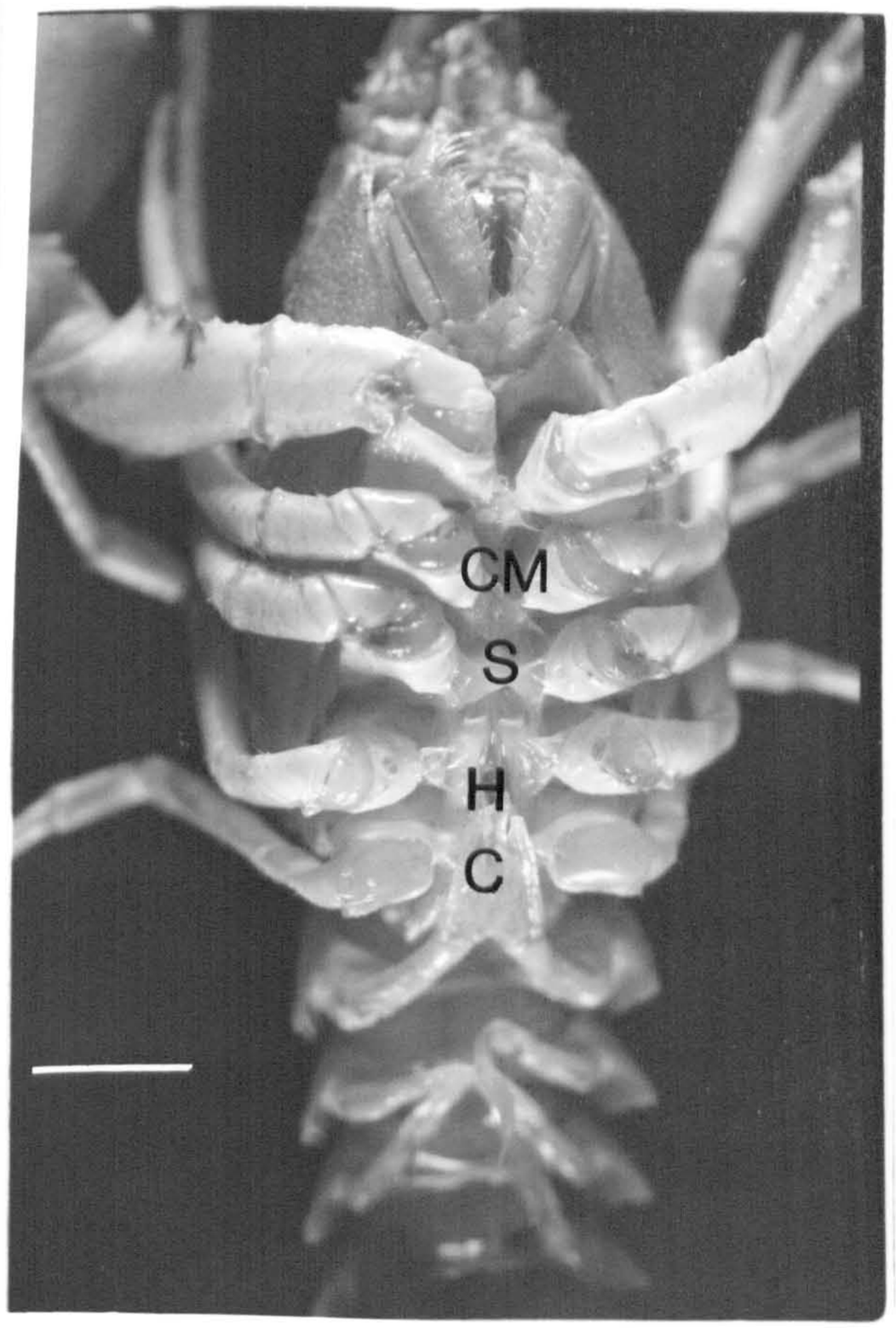

CM
S
H
C

Table 1. The effect of solvent removal of surface compounds on the infection of crayfish leg bases with Aphanomyces astaci.

	No. of leg bases with strong infections.
Ast. leptodactylus (10 animals)	
Control	9/20
Chloroform / methanol	20/20
Hexane	12/20
Sandpaper	0/20
P. leniusculus (2 animals)	
Control	0/4
Chloroform/ methanol	0/4
Hexane	0/4
Sandpaper	0/4

Since the leg bases abraded with sand paper became strongly melanized, it was difficult to see any fungal hyphae in this area. However, using a binocular microscope, single melanized hyphae could be seen close to the surface, and ramifying away from the melanized area, in leg bases treated with sand paper in both P. leniusculus and in two out of the ten Ast. leptodactylus. In neither species could a severe infection be detected.

After 7 days all the C/M treated leg bases of Ast. leptodactylus were strongly infected, whilst only nine of the control membranes were affected. Twelve out of twenty

hexane treated arthrodial membranes were heavily infected. The difference between control and solvent treated legs was tested statistically using the sign test (Siegel 1956). The number of C/M treated leg bases infected was significantly different to controls (P=0.1) whilst hexane treatment did not cause a significant increase in infection.

In P. leniusculus there was no infection visible to the naked eye at either the solvent treated or control sites.

After 10 days, all treated Ast. leptodactylus were dead. By the time of death most animals had begun to develop infections in those leg bases in which infections had not previously been visible.

Both treated P. leniusculus were still alive after 6 months and showed no signs of infection to the naked eye. The only sign of response was noticed two weeks after initial treatment; the treated leg joints had become a pale grey brown colour but this colour was gradually lost over the next few weeks.

DISCUSSION.

Treating Ast. leptodactylus leg arthrodial membranes with C/M, significantly increased the probability of their infection with Aph. astaci, whilst hexane caused no significant increase.

C/M treatment did not significantly affect the

ability of Aph. astaci to infect P. leniusculus.

Hexane wiping does not significantly enhance the ability of Aph. astaci to infect the proximal arthrodial membranes of either Ast. leptodactylus or P. leniusculus.

If a C/M soluble antifungal barrier exists at the level of the epicuticle in crayfish, its removal with C/M would reduce the resistance of the animal to fungal infection, and may account for the more severe infections in leg bases treated with C/M. Such a barrier may be either mechanical, in the form of an inpenetrable layer, or chemical in the form of anti fungal compounds.

Solvent soluble antifungal activity has been demonstrated in earlier studies of insects. Wiping of the surface of either C. simplex or Bombyx mori with tetrachloromethane greatly enhanced the susceptibility of these insects to fungal infection and this effect was ascribed to the removal of anti fungal medium chainlength fatty acids from the cuticle (Koidsumi 1957).

There may be a further explanation for the increase in infection seen in this case. Arthropod cuticles contain a number of lipids which serve a water proofing function (Hackman 1971, Neville 1975, Hadley 1982). In crustaceans however, the cuticular lipids have, been little studied (Hackman 1971, Stevenson 1985).

Since lipids are soluble in C/M, wiping the cuticle surface with this solvent may remove waterproofing lipids and reduce the water resistant properties of the cuticle. It may also increase the leakage of compounds from the

crayfish, to which Aph. astaci is chemotactically attracted (Cerenius and Soderhall 1982).

Thus, it is possible that more zoospores are attracted to treated legs than to untreated legs causing the infection in these legs to be stronger. Treatment of the epicuticle with sandpaper might be expected to have similar effects, however, no infection could be detected with the naked eye in abraded leg bases of either crayfish species, although invasion could be detected at the microscopic level.

In contrast, Koidsumi (1957) was able to enhance the susceptibility of the rice stem borer, C. simplex, to the pathogenic fungus Isaria farinosa by abrading the surface of the animals with alumina. The increase in susceptibility was believed to be due to the removal of the surface lipid layer of the animal, which contained fungicidal free fatty acids.

Unestam and Weiss (1970) showed that peeling or pricking the epicuticle of P. leniusculus led to an increase in the susceptibility of the animals to infection by Aph. astaci and that similar treatment of Ast. astacus caused these animals to succumb to otherwise sub-lethal doses of zoospores. However, superficial wounding of crayfish by the removal of pieces of epicuticle, causes aggregation of hemocytes at the wound site and eventual melanization (Nyhlen and Unestam 1980).

Strong melanization did indeed occur at the site of the abrasions and it may have been this coupled with the

toxicity of melanin intermediates to Aph. astaci (Soderhall and Ajaxon 1982) that prevented infection in these areas becoming severe.

SECTION 2.

The development of an assay to detect the presence of compounds affecting the germination of Aphanomyces astaci zoospores.

2.1. Development of a germination medium.

The first stage of infection of the host with Aph. astaci is the encystment of the zoospore on the epicuticular surface of the animal followed by germination of the spore. Thus, a technique was developed to determine whether solvent extracts of the epicuticle of crayfish or of whole G. pulex, contained a compound that inhibited the germination of Aph. astaci zoospores.

Svensson and Unestam (1975) demonstrated that Aph. astaci zoospores could be induced to germinate by exposure to lake water solutions of calcium, potassium, sodium or rubidium chlorides and mannitol. They suggested that the effect was, at least in part, due to the osmotic effects of the solutions on the zoospores, and demonstrated that the stimulatory effect was maximal for sodium and calcium chlorides and mannitol at around 150 mOsm \ kg water.

Accordingly, initial experiments on the effect of

G. pulex extracts on the germination of Aph. astaci zoospores, were carried out using sodium chloride solutions made up in distilled water to an initial osmolality of 300 mOsm / kg water. When equal quantities of zoospore suspension and this sodium chloride (GPR) solution were incubated together, germination frequencies of between 25% and 40% were obtained.

In later experiments, using the same isolate of Aph. astaci, but a different batch of sodium chloride, (GPR) the zoospores failed to germinate. No germination was obtained in solutions of Analar sodium chloride with osmolalities between 50 and 500 mOsm / kg.

Addition of a few mg. of calcium chloride to the solution restored the ability of the zoospores to germinate.

2.1.1. The effect of calcium chloride concentration on the germination of Aphanomyces astaci zoospores.

This experiment was carried out to determine the optimum concentration of calcium chloride for germination of Aph. astaci zoospores in media maintained at 300 mOsm / kg (150 mOsm / kg after addition of zoospore suspension). Since solutions of sodium chloride failed to stimulate germination of zoospores, the osmolality of test media was maintained at this level with sodium chloride.

METHODS.

Aph. astaci (Isolate 28-1083(4)) was grown on river water agar (Appendix 4) at 18°C and zoospore suspensions were prepared by incubating 10 to 15 small pieces of agar (approximately 5mm square) from the edge of the colony in 50 ml of sterile distilled water, in a sterile plastic Petri dish. The agar pieces were incubated overnight (approximately 14 hrs) at 18°C and the zoospores used the following day.

Haemocytometer counts showed that these suspensions contained between 2,000 and 20 000 swimming zoospores per ml.

Solutions of calcium and sodium chloride were prepared, both with an osmolality of 300 mOsm / kg. By mixing the two in varying proportions, media containing between zero and 116 mM calcium chloride and an osmolality of 300 mOsm / kg were obtained.

Five, fifty microlitre aliquots of each incubation medium were placed in a sterile 96 well tissue culture plate and 50 ul of zoospore suspension was added to each, giving a final osmolality of 150 mOsm / kg overall. The cultures were incubated at 18°C for 4 hrs.

The germination percentage was assessed by counting 100 zoospores in each well, using a Union Mic 1360 inverted microscope. Zoospores were considered germinated when the germ tube was at least the length of the zoospore diameter (Svensson and Unestam 1975).

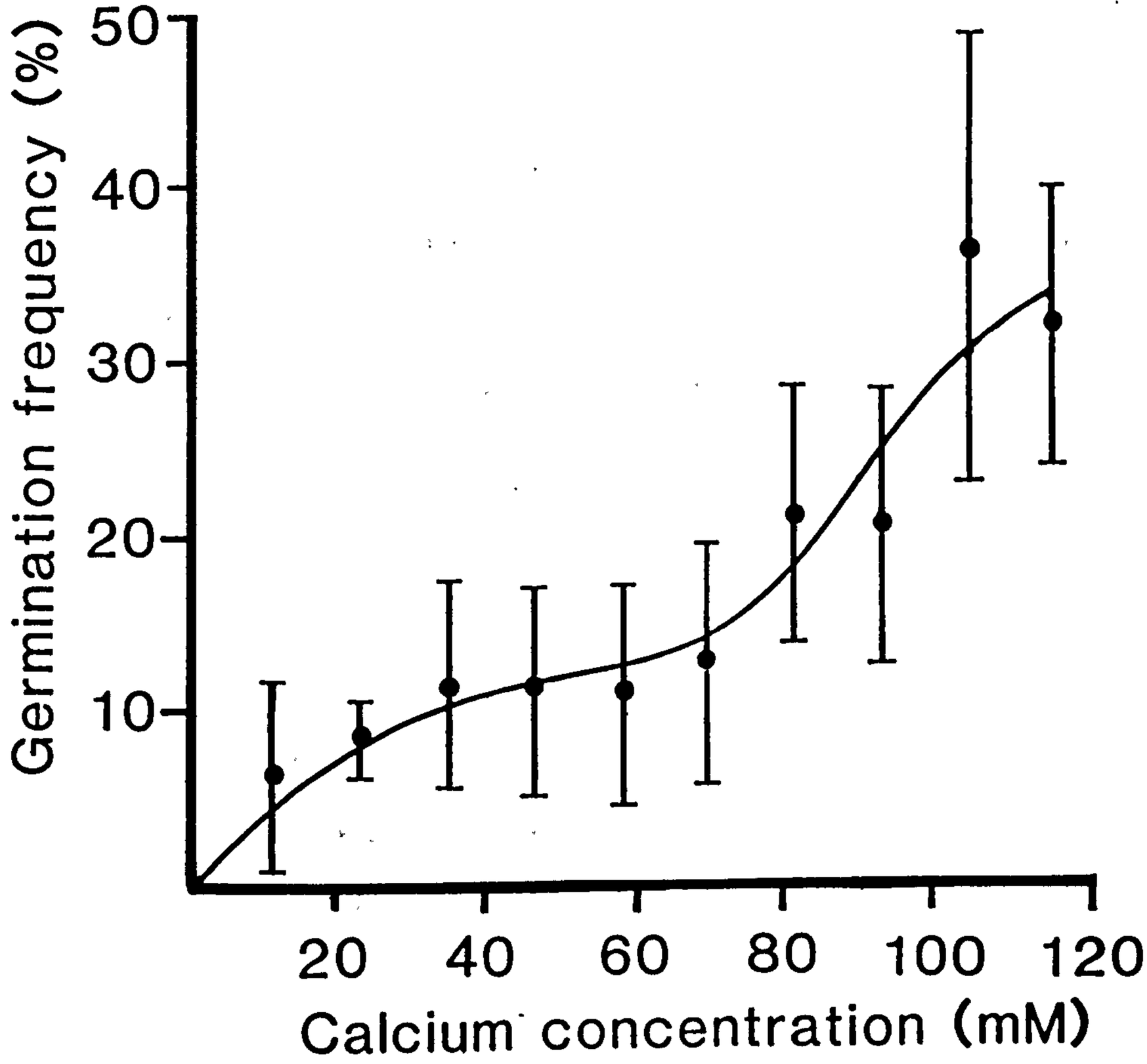
RESULTS.

Figure 3. shows the effect of calcium chloride concentration on the germination of Aph. astaci, when the osmolality of the germination medium was maintained at approximatly 300 mOsm / kg with sodium chloride.

Germination in sodium chloride solution alone was less than one percent, however in germination media containing a final calcium chloride concentration of 5.8 mM germination was 6%. In germination media containing a final calcium chloride concentration of between 17.4 and 29 mM, germination frequency was stable at around 12%, thereafter it increased with increasing calcium chloride concentration reaching 35% at 116 mM. Concentrations above this were not tested since the osmolality of the solutions of calcium chloride greater than 300 mOsm / kg were inhibitory to germination (see below)

Figure 3. The effect of calciun chloride concentration on the germination of Aphanomyces astaci zoospores.

This figure shows the effect of calcium chloride concentration on the germination of Aph. astaci zoospores in media maintained at 300 mOsm / kg with sodium chloride. Each point is the mean of five replicates and the bars represent standard deviations.


Germination frequency (%)
50
40
30
20
10
20
40
60
80
100
120
Calcium concentration (mM)

2.1.2.The effect of osmolality on the germination of Aphanomyces astaci zoospores in a germination medium containing calcium chloride.

METHODS.

The optimum osmolality for germination in calcium chloride solution was ascertained by incubating zoospores in calcium chloride solutions, with osmolatities between 50 and 525 mOsm / kg. Germination percentages were assessed after 4hrs incubation at 18°C.

RESULTS.

Figure 4. shows the effect of osmolality of the germination medium on the germination of Aph. astaci zoospores in two experiments, one of which used fresh zoospores, the other used a zoospore suspension that had been stored overnight at 4 C The optimum osmolality for germination of fresh zoospores was between 250 and 300 mOsm / kg, whilst the optimum osmolality for germination of one day old zoospores was somewhat less.

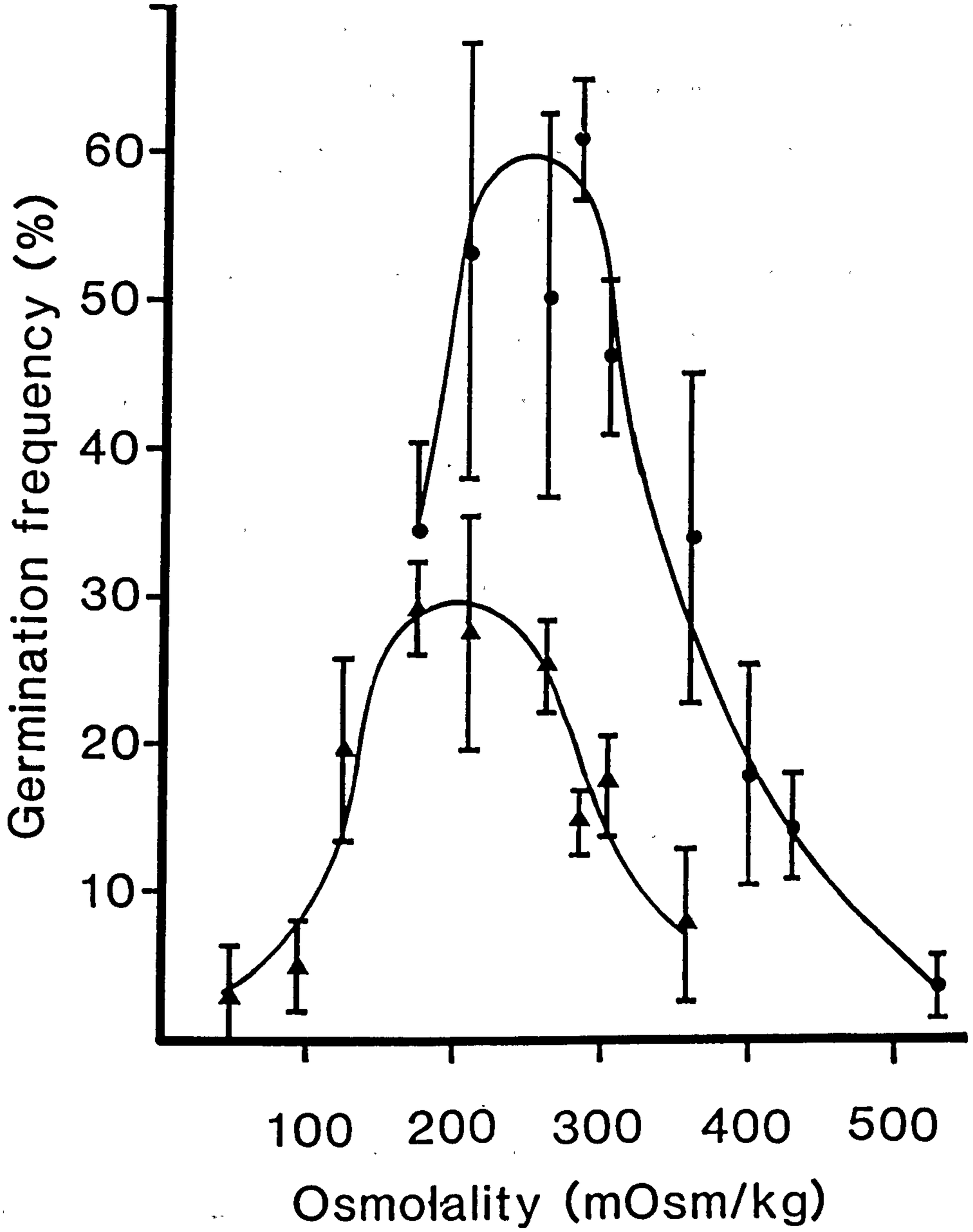
Whilst germination frequencies varied from experiment to experiment, within an experiment, variation in germination frequency between replicates was usually small.

Figure 4. The effect of the osmolality of the calcium chloride solution on the germination of Aphanomyces astaci zoospores.

This figure shows the effect of osmolality on the germination of both fresh Aph. astaci zoospores and those stored overnight at 4 °C.

● Fresh zoospores

▲ Zoospores stored at 4 °C


Germination frequency (%)
60
50
40
30
20
10
100
200
300
400
500
Osmolality (mOsm/kg)

2.2. The use of acetone as a dispersing agent in surface extract assays.

In the experiments described below, extracts of crayfish epicuticle or G. pulex were dispersed in 20 ul. of acetone and then added to 500 ul. of germination medium. Aliquots of this solution were mixed with an equal volume of zoospore suspension and incubated before germination was assessed. The addition of acetone allowed insoluble compounds present in the extracts to be dispersed and allowed soluble compounds to be dissolved. Without acetone addition, it was difficult to demonstrate any inhibition in later experiments. However, acetone addition initially increased the osmolality of the germination media by 350 mOsm / kg.

2.2.1. Evaporation of acetone from culture vessels.

Since incubations were carried out either as drop cultures in Petri dishes or in tissue culture wells as described above, it was possible for the acetone to evaporate, thus changing the osmolality of the germination medium. Therefore the rate of decrease in osmolality was compared in each type of incubation vessel.

METHODS.

A number of 100 ul. aliquots of half strength incubation medium, containing 4% acetone were incubated at 18°C and the osmolalities measured every hour.

RESULTS.

In Petri dishes, evaporation of acetone leads to a drop in osmolality to control levels during the first hour, whilst in tissue culture wells, where there is a smaller air space above the culture, osmolality takes five hours to drop to control levels (Figure 5).

2.2.2. The effect of acetone on the germination rate of Aphanomyces astaci zoospores in vitro.

METHODS.

In order to determine whether the addition of acetone to the germination medium affected the germination of Aph. astaci zoospores, hourly measurements of germination percentage were made. Zoospore suspensions were incubated in calcium chloride incubation medium containing 4% acetone, in both Petri dish drop cultures and tissue culture wells. Cultures were set up as previously described. Control cultures contained no

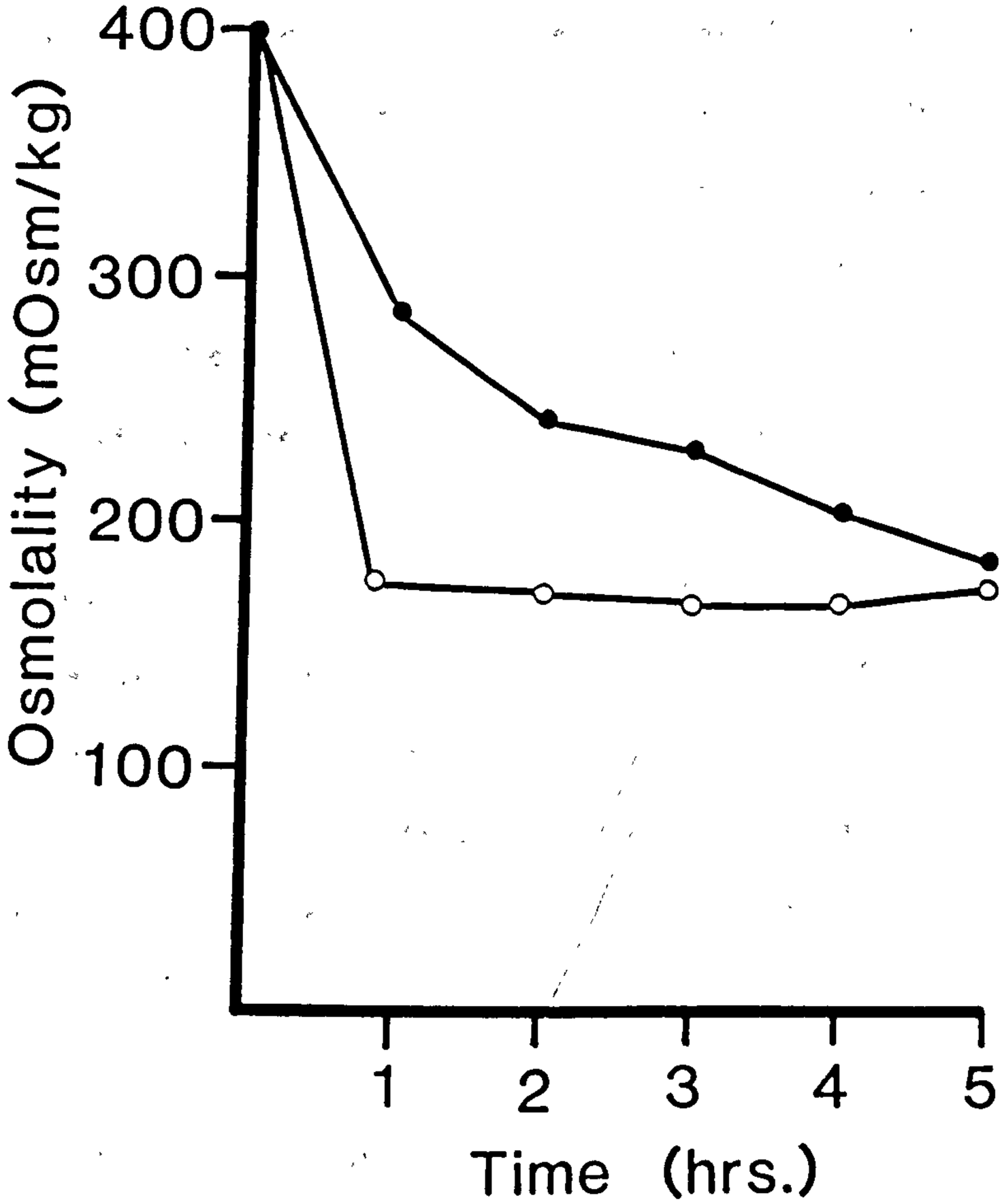
Figure 5. Effect of acetone on the osmolality of the germination medium.

This figure shows the change in osmolality with time in a 2% v/v acetone solution made up in 150 mOsm / kg calcium chloride.

The experiment was carried out in both drop cultures in Petri dishes, and in tissue culture wells.

○ Petri dishes

● Tissue culture wells.


400
300
200
100
Osmolality (mOsm/kg)
1
2
3
4
5
Time (hrs.)

acetone.

RESULTS.

Figure 6. shows the rate of germination in Petri dishes and tissue culture wells, with and without added acetone. In both cases, germination in cultures to which acetone had been added lags behind those to which acetone was not added. However, in Petri dishes the lag is most noticeable after one hour, becoming reduced to 1.5% after 5 hrs.

In contrast, in tissue culture wells, germination was much reduced after 3 hrs in cultures with added acetone, and was still noticably less than controls after 5 hrs. After 24 hrs germination percentages were similar in control and acetone treated cultures both in Petri dishes and in tissue culture wells, although final germination frequencies were higher in petri dishes than in tissue culture wells.

2.3.The effect of solvent residues on the germination of Aphanomyces astaci zoospores in vitro.

In initial experiments, general purpose (GPR) grade solvents were used as extraction media, however, later experiments showed that residues from GPR solvents, especially chloroform, contained impurities that were

Figure 6. The effect of acetone on the rate of germination Aphanomyces astaci zoospores in vitro.

This figure shows the effect of acetone on the rate of germination of Aph. astaci zoospores in both Petri dish drop cultures and in tissue culture wells. The final concentration of acetone in the germination medium was 2% v/v. Controls contained no acetone.

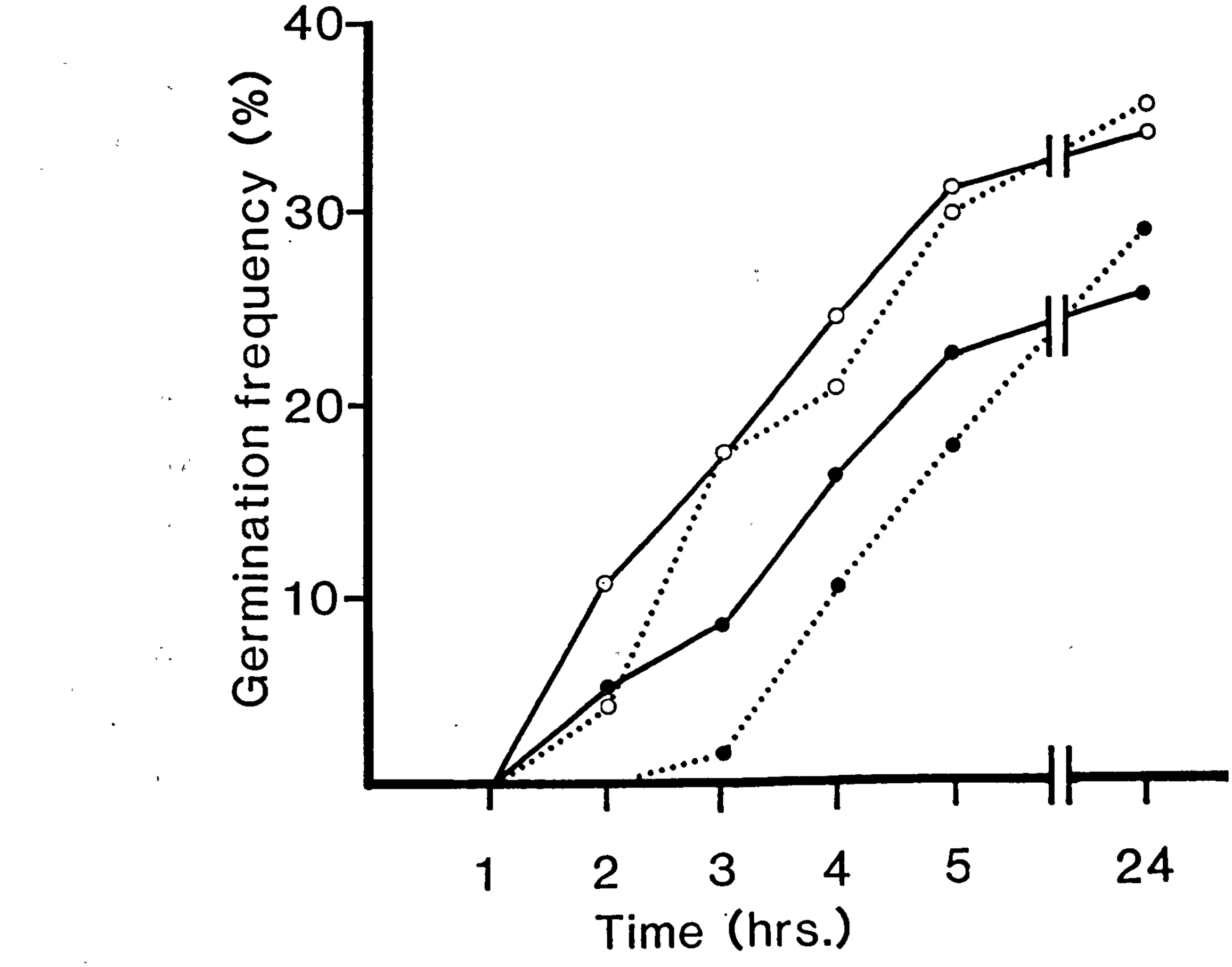
Each point is the mean of five replicates. Standard deviations are not added for the sake of clarity, but are presented in appendix 10.

○ Petri dishes

● Tissue culture wells

·····2% acetone

——Control


40
30
20
10
Germination frequency (%)
1
2
3
4
5
24
Time (hrs.)

inhibitory to zoospore germination. Thus experiments were carried out to determine the best grades of solvents to use in extraction experiments.

METHODS.

Five millilitre aliquots of Analar hexane, Aristar chloroform, Aristar methanol and a 1 : 1 mixture of Aristar chloroform and Aristar methanol were evaporated to dryness with a stream of nitrogen, the residues were dissolved in 20 ul of acetone and dispersed in 500 ul of 300 mOsm calcium chloride solution.

This solution was then used as the germination medium and tested against Aph. astaci zoospores as above.

RESULTS.

Figure 7. shows the effect of residues from 5mls of evaporated solvents on the germination of Aph. astaci zoospores. The figures represent the pooled results of six experiments.

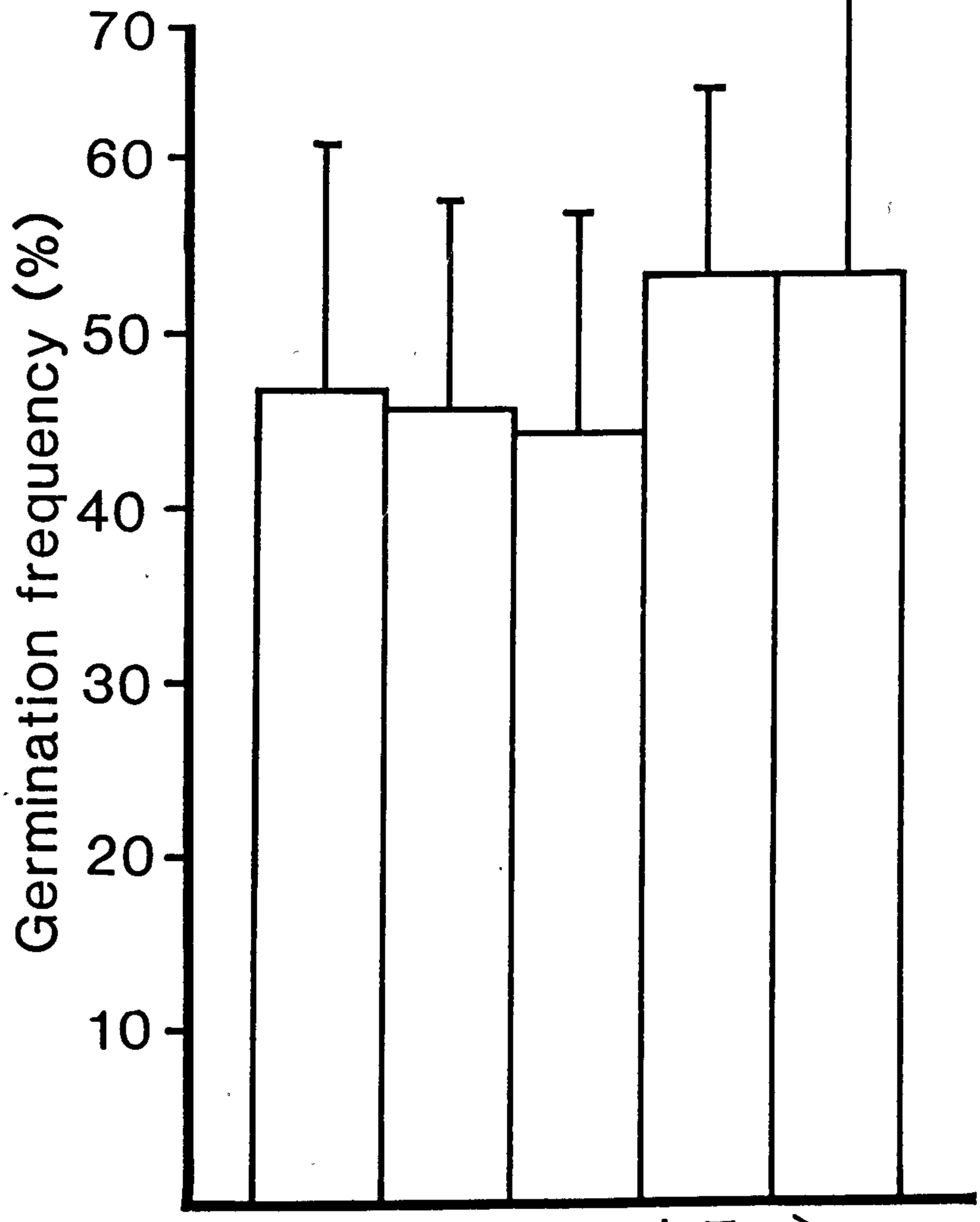
There was no significant difference between media containing residues of either Analar hexane or Aristar chloroform and control media (P=0.05).

Residues from Aristar methanol increased the germination frequency of zoospores (P=0.05), as did residues of the chloroform / methanol mixture.

In a single experiment, residues of Analar

Figure 7. The effect of solvent residues on the germination of Aphanomyces astaci in vitro.

This figure shows the effect of residues from 5 mls. of solvents of different grades, on the germination of Aph. astaci. The figure represents the pooled results of six experiments. Bars represent standard deviations.


70
60
50
40
30
20
10
Germination frequency (%)
Control
Analar hexane
Aristar chloroform
Aristar methanol
Chloroform/ methanol

chloroform and Analar methanol were tested. In this experiment, both increased the germination frequency of the zoospores (P=0.05).

CONCLUSIONS.

In the light of the above results calcium chloride solutions with osmolalities of between 250 and 300 mOsm / kg were used in spore germination assays and all experiments were carried out using controls consisting of calcium chloride solution containing 4% GPR acetone (v/v).

In experiments where large numbers of treatments were carried out, incubations were carried out overnight in tissue culture wells. This had the added advantage of taking up less space, keeping the cultures together and making the results easier to read. Fresh zoospores were always used.

Since later experiments showed that the residue from large volumes (150 ml.) of Analar hexane had antifungal activity, solvent extraction was carried out in Aristar chloroform, Aristar methanol and four times redistilled Analar hexane.

SECTION 3.

The effect of Gammarus pulex hexane extracts on the germination of Aphanomyces astaci in vitro.

METHODS.

Animals were collected from the river Mimram at Tewin, shortly before use and all particulate matter was removed from them. They were then rinsed in distilled water and blotted dry, before being weighed.

18g of gammarids (approximately 40 animals) were placed in a 250 ml. beaker in 50 ml. of four times redistilled Analar hexane. The beaker was allowed to stand for 90 minutes at room temperature. The hexane was then decanted and evaporated to dryness in a rotary evaporator at room temperature. The extract was used immediately.

Twenty microlitres of acetone was added to the dried extract to dissolve and disperse it, and to this was then added 500 ul. of 285 mOsm / kg calcium chloride solution. This was used as the germination medium.

Fifty microlitres of germination medium were then added to fifty microlitres of zoospore suspension prepared as above. The culture was incubated overnight and the germination frequency assessed.

RESULTS.

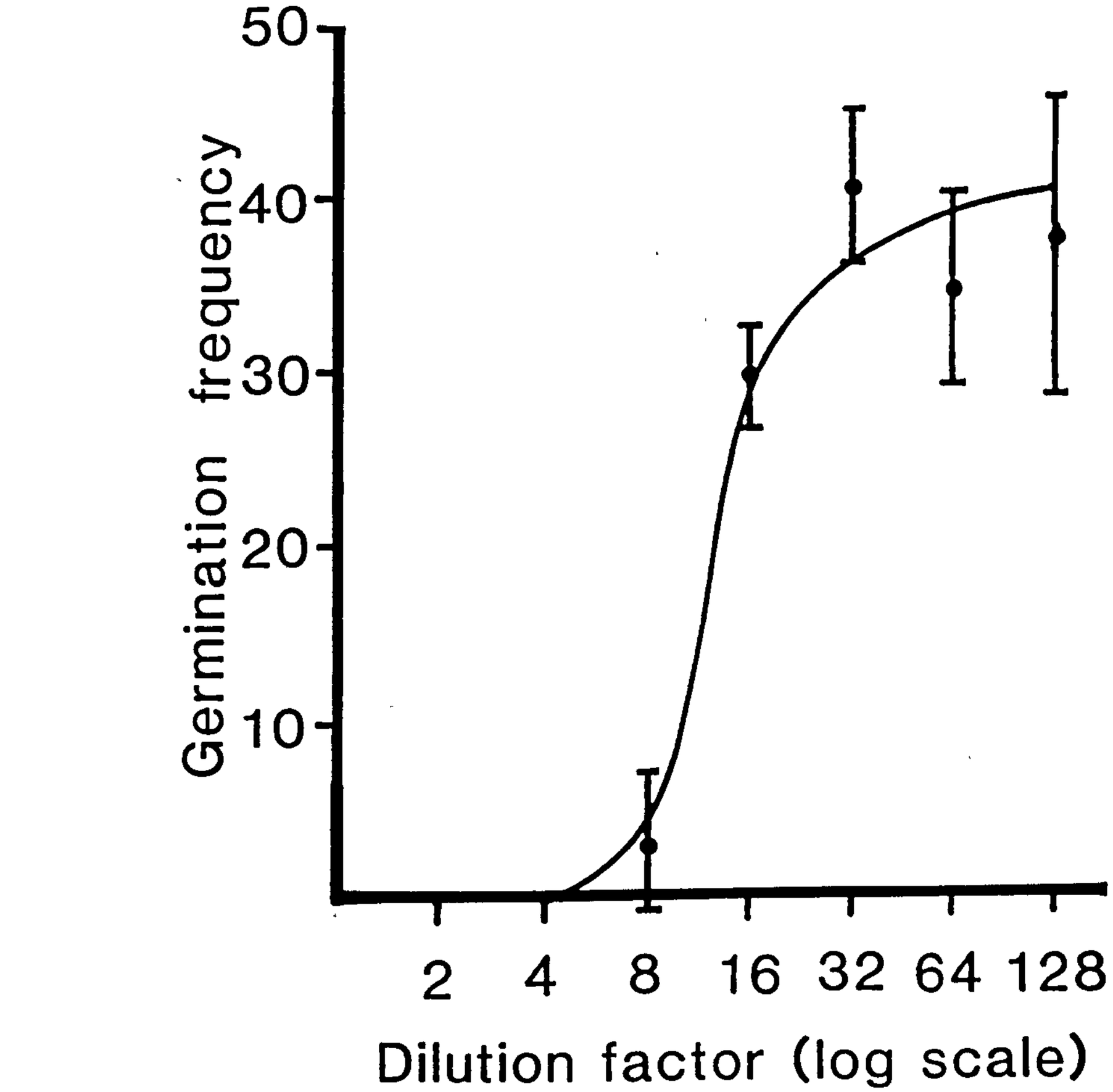
G. pulex hexane extract was a dark yellow oily residue. It dissolved in the acetone readily but much of it was insoluble in the calcium chloride solution and came to rest on top of the solution after centrifugation in a Beckman Microfuge. This top layer was dark yellow and the germination medium was removed from below it, taking care not to transfer any of the yellow layer with the germination medium.

Figure 8. shows the percentage of Aph. astaci zoospores germinating after incubation overnight in the presence of full strength and diluted G. pulex hexane extracts. In the presence of full strength hexane extract the spores failed to germinate whilst in control conditions the germination frequency was 36.8%. Zoospore germination was inhibited completely in dilutions of up to four times but returned to approximately control levels in media containing extract diluted 32 times.

Thus G. pulex hexane extract contains compounds that are inhibitory to the germination of Aph. astaci zoospores. It is likely that these compounds are water soluble since the removal of the insoluble compounds at centrifugation did not remove the inhibition.

Figure 8. The effect of Gammarus pulex hexane extract on the germination of Aphanomyces astaci zoospores in vitro.

This figure shows the effect of serial two fold dilutions of G. pulex hexane extract on the germination of A. astaci zoospores. Each point was the result of five replicates and bars represent standard deviations. Control germination (without added extract) was 36.8 ± 7%.


50
40
30
20
10
Germination frequency
2
4
8
16
32
64
128
Dilution factor (log scale)

SECTION 4.

The effect of crayfish epicuticle extracts on Aphanomyces astaci zoospores.

4.1. The effect of epicuticular hexane and chloroform / methanol extracts of Astacus leptodactylus and Pascifastacus leniusculus on the germination of Aphanomyces astaci in vitro.

These preliminary experiments were carried out to determine whether inhibitory activity towards the germination of Aph. astaci could be detected in hexane or C/M extracts of the epicuticle of a resistant and a suceptible species of crayfish.

In later experiments the inhibitory activity of the two species was compared.

METHODS.

The ventral abdominal epicuticle was removed from a number of crayfish of each species (weight 25-30g) as follows.

The ventral abdominal epicuticle of each animal was cleaned using a damp cotton wool bud then dried with a tissue paper. A gentle score was then made down the mid-line of each intersegmental membrane using a scalpel blade. Fine forceps were then used to lift the edge of the

epicuticle and peel it away from the underlying procuticle. The epicuticle pieces were extracted in 2 X 2.5 mls. of hexane, or 1 : 1 C/M, using a glass Potter homogenizer.

The hexane was evaporated to dryness using a stream of nitrogen and the dry extract dissolved in 20ul of acetone. This solution was then dispersed in 500 ul. of 300 mOsm calcium chloride solution and spun at 3600 rpm in an MSE Minor centrifuge to remove undissolved matter.

The C/M extracts were vacuum filtered using a sintered glass filter before being evaporated under a stream of nitrogen, incorporated into calcium chloride and centrifuged as above.

Fifty microlitre aliquots of the supernatant were then placed on the bottom of a plastic petri dish and 50 ul of zoospore suspension, prepared as above, was added.

Controls consisted of 4% acetone in calcium chloride to which the zoospores were added.

Cultures were incubated overnight at 18 C and the germination percentages were then assessed as above.

Since Unestam and Weiss (1970) suggest that epicuticle removed in this way is probably contaminated with underlying procuticle, every effort was made to keep the epicuticular peelings as thin as possible.

To determine how much of the underlying cuticle was removed with the epicuticle by this method, ventral abdominal cuticles with half the epicuticle removed from each segment were fixed overnight in Davidsons fixative

(Appendix 6) and subsequently dehydrated in an alcohol series and embedded in paraffin wax. Sagittal sections, 6 um. thick, were stained with haematoxylin and eosin and observed under a microscope.

RESULTS.

The epicuticle of P. leniusculus was a mustard yellow colour when removed from the animal, and was easier to peel away from the underlying cuticle than that of Ast. leptodactylus, which was a fawn colour. Epiphytic growth was also easier to remove from the epicuticle of P. leniusculus.

In initial experiments, hexane extracts were made in a Potter homogeniser, however, the epicuticles of neither species broke up under these conditions, rather they remained as individual pieces. Thus in later experiments the epicuticle was allowed to stand in 5 mls. of hexane for 90 mins. before the solvent was decanted off and evaporated.

When epicuticles from either species were treated with C/M, they readily broke up into small particles and the suspension appeared to swell. However, the solvent extract was readily recovered by vacuum filtration.

Hexane extracts of P. leniusculus were occasionally a pale yellow in colour, whilst those of Ast. leptodactylus were clear. C/M extracts of both species were clear.

C/M extracts were not fully soluble in calcium chloride solution. The insoluble matter, was readily removed by centrifugation and settled on top of the aqueous layer, which was removed from below it using a pipette.

Table 2. The effect of epicuticular solvent extracts on the germination of Aphanomyces astaci in vitro, expressed as a percentage of control values.

	Hexane		C/M	
	wt. of sample	% control	wt. of sample	% control
P. leniusculus	24.8	40.2	35.3	0
A. leptodactylus	37.0	8.6	36.3	0

weights in mg.

Table 2 shows the percentage of zoospores germinating in cultures containing epicuticular extracts. Since the results are from separate experiments, germination is expressed in terms of percentage control values. The weight of epicuticle is also given to enable a degree of comparison to be made between experiments. In controls, between 17 and 41 percent of spores germinated.

In cultures containing C/M extracts of either Ast. leptodactylus or P. leniusculus, germination of zoospores was totally inhibited, whilst in hexane extracts, inhibition was not so strong. The weaker inhibition in the medium containing P. leniusculus epicuticle extract may in part have been due to the smaller amount of epicuticle

used.

Histological examination of the areas from which epicuticle had been peeled away showed that the thickness of cuticle peeled away varied between 5 um., in areas where only the epicuticle had been removed, and 30 um, in areas where quite substantial portions of the procuticle had been removed with the epicuticle.

There was no obvious difference between the two species of crayfish in this respect, and the amount of cuticle removed appeared to depend only on the depth of the initial score mark.

Observation (i)

In two experiments involving Ast. leptodactylus C/M extract, bacterial contamination in the control medium was traced to the original zoospore inoculum. However, in cultures containing C/M extract there was no bacterial growth, suggesting that some degree of activity against bacteria was was also present in the C/M extract.

Observation (ii)

Animals stripped of sub-abdominal epicuticle were placed in a recovery tank separate from stock animals. The recovery rate was high and most animals went on to moult. After moult the melanin pigment that formed within two days post operation, was largely although not completely,

lost. A number of animals did however succumb to opportunistic, Saprolegnia and Aphanomyces (not Aph. astaci) infections sub-abdominally, which subsequently killed the animal (Figure 9.). No animals in the stock tanks, which were treated identically to the recovery tanks and had the same water supply, succumbed to such infections, suggesting, that such gross removal of the epicuticle predisposes the animals to fungal infection.

4.2. Comparison of the inhibitory activity of hexane and chloroform / methanol extracts of Astacus leptodactylus and P. leniusculus towards germination of Aphanomyces astaci zoospores in vitro.

METHODS.

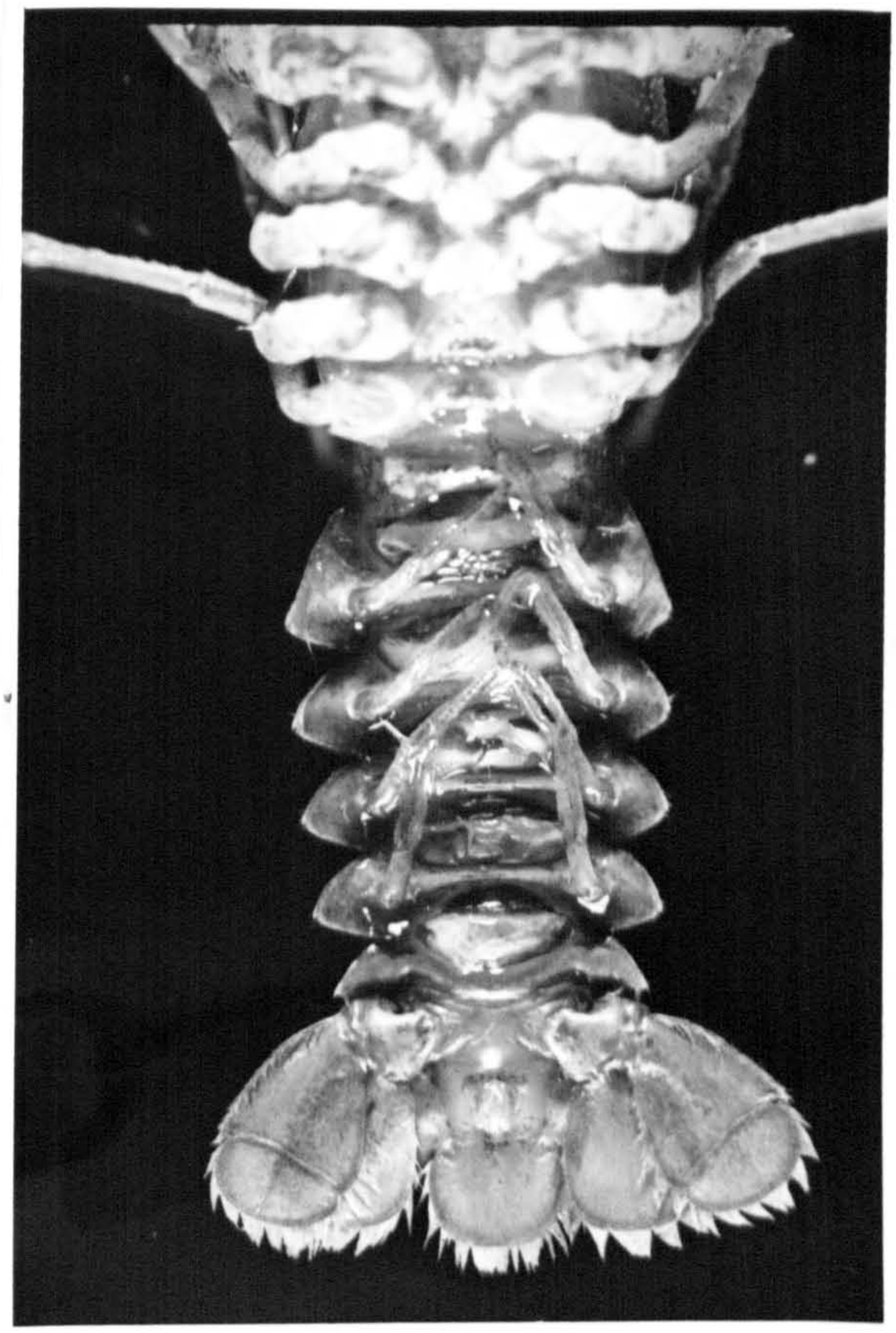
The experiments described in 4.1. were carried out to determine whether inhibitory activity existed in extracts of the outer layers of crayfish cuticle. Experiments described in this section were carried out to ascertain whether extracts of crayfish that are resistant to Aph. astaci were more active than those of susceptible species.

In an attempt to standardize the starting material between species, epicuticles were obtained from 10 Ast. leptodactylus and 10 P. lenuiusculus, of similar size, as above. By using epicuticle from intermoult animals of the same size, and by using the same weight of epicuticle from

Figure 9. Ventral view of Astacus leptodactylus with a fungal infection of the abdomen.

The epicuticle of this animal was peeled off and the animal allowed to recover. It subsequently developed a fungal infection of the abdomen. In this case the fungi isolated were Saprolegnia and Aphanomyces spp. (not Aph. astaci). The dark areas of cuticle are those that have been peeled and subsequently became melanized.

Actual size.



each species, it was hoped to use a similar area of epicuticle from each species.

The pooled epicuticles were weighed and equal weights of epicuticle from each species used in the experiment. The pooled epicuticles from 10 animals weighed approximately 30 mg, although those from Ast. leptodactylus often weighed slightly more than those from P. leniusculus, and appropriate adjustment was made.

Both hexane extracts and C/M extracts were carried out on the same epicuticle. Hexane extractions were carried out first, by immersing the epicuticles in 5mls of hexane for 90 minutes. The hexane was then decanted and the residual hexane removed by evaporating it in a stream of nitrogen. Subsequently chloroform methanol extractions were carried out as above (3.2.).

In individual experiments, the germination percentage in media containing full strength extract was often zero, thus, in order to illustrate the comparative inhibitory properties of the extracts it was necessary to construct a dilution series. Incubations were carried out in serial two fold dilutions of the full strength extract, made with 4% (v/v) acetone in calcium chloride solution.

The incubations were carried out as above, in 96 well tissue culture plates. Five replicates of each treatment were prepared.

In one experiment, after incubation overnight, the germination medium was removed from zoospores treated with Ast. leptodactylus chloroform methanol extract, they were

washed twice in 100 ul. of fresh, half strength germination medium and then re-incubated for a further 24 hrs in 100 ul. of fresh, half strength germination medium (to account for water added with the zoospores in the original incubation).

In half the original replicates, incubation was continued in the original inhibitory medium, and these acted as controls.

RESULTS.

Figures 10. and 11. show the mean germination frequencies of Aph. astaci zoospores in germination media containing hexane or C/M extracts respectivly.

Table 3 shows the mean germination frequencies of zoospores from four experiments. The results have been

Table 3. The mean germination frequencies of Aphanomyces astaci zoospores in media containing full strength epicuticular extracts.

	mean germination frequency + SD	
	hexane	C/M
Ast. leptodactylus	64.1 ± 34.9	23.8 ± 25.8
P. leniusculus	64.7 ± 37.5	46.5 ± 27.8

expressed in terms of percentage of control values, and pooled. Control germination frequencies varied between 37.8% and 57.9%. The osmolality of media containing

Figure 10. The effect of hexane extracts of crayfish epicuticle the germination of Aphanomyces astaci zoospores in vitro.

This figure shows the effect of serial two fold dilutions of Ast. leptodactylus and P. leniusculus hexane extracts on the germination of Aph. astaci zoospores.

The figure represents the results of a single experiment using similar weights of epicuticle from each species, and each point is the mean of five replicates. The bars represent standard deviations and the control germination frequency was 47.1 ± 9.3%.

—●— Ast. leptodactylus

··■·· P. leniusculus

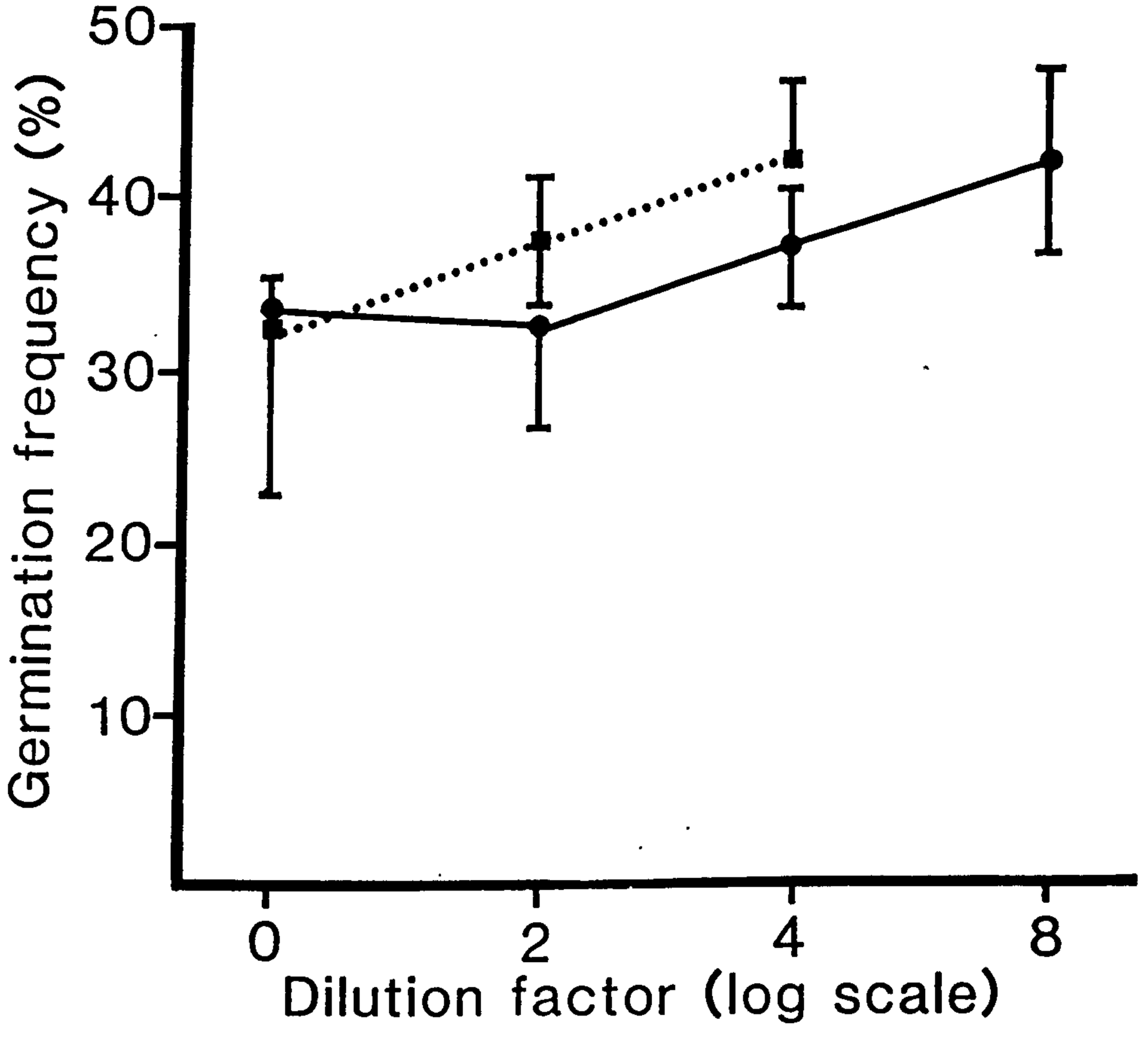

Germination frequency (%)
50
40
30
20
10
0
2
4
8
Dilution factor (log scale)

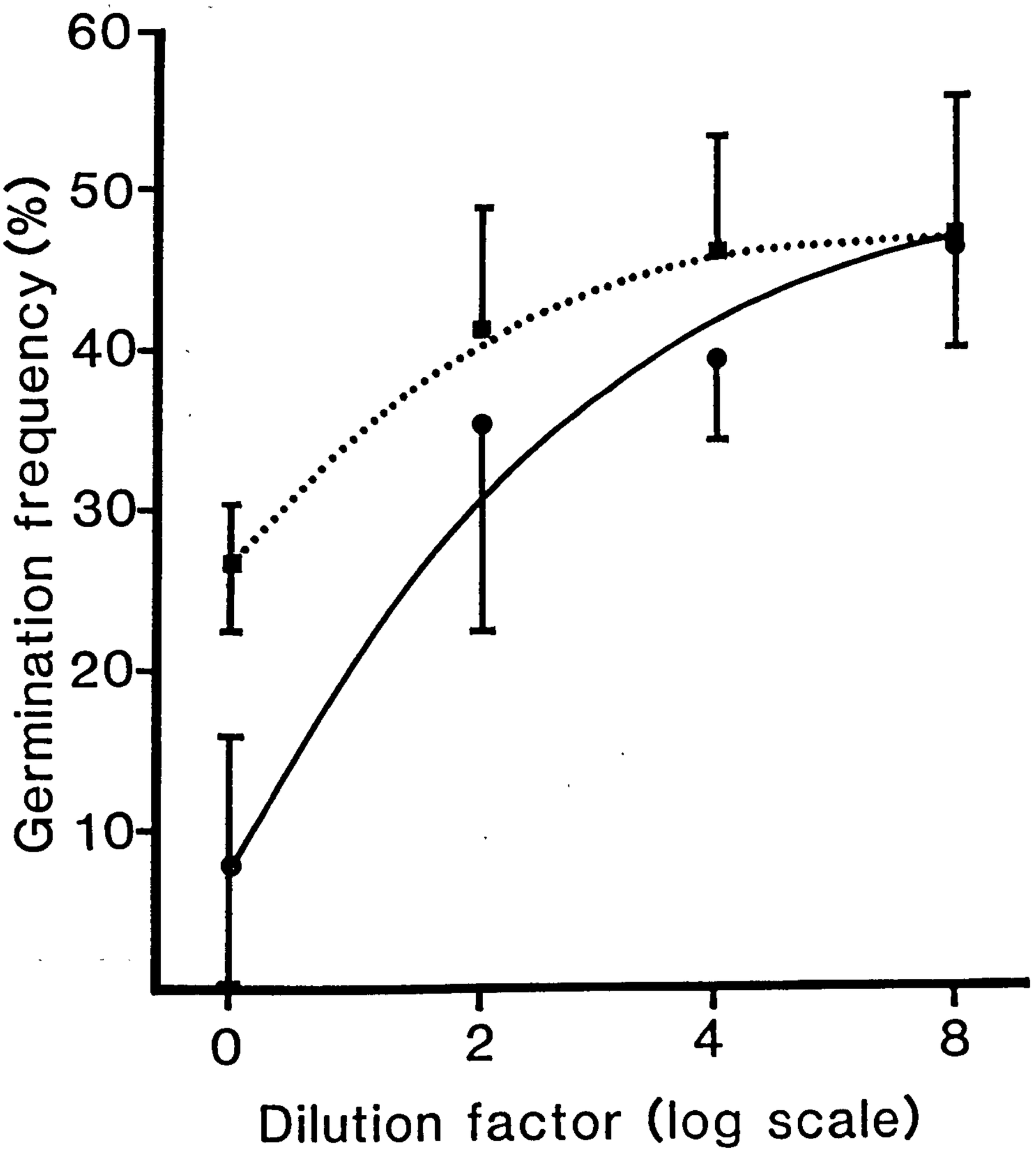
Figure 11. The effect of crayfish chloroform / methanol extract on the germination of Aphanomyces astaci in vitro.

This figure shows the effect of serial twofold dilutions of Ast. leptodactylus and P. leniusculus C/M extracts on the germination of Aph. astaci zoospores.

The figure represents results from the same experiment as those in figure 10. Each point is the mean of five repilcates. Bars represent standard deviations and the control germination frequency was 47.1 ± 9.3%.

—●— Ast. leptodactylus

··■·· P. leniusculus


60
50
40
30
20
10
Germination frequency (%)
0
2
4
8
Dilution factor (log scale)

extracts was not more than 15 mOsm / kg higher than controls.

i)Hexane extracts.

In individual experiments, full strength hexane extracts of both P. leniusculus and Ast. leptodactylus caused a significant reduction in the germination of Aph. astaci zoospores ($P<0.05$), but there was no difference between the extent of inhibition in media containing extracts of Ast. leptodactylus and those containing extracts of P. leniusculus ($P=0.05$).

Although there was no difference in the inhibitory effect of extracts between species, from one experiment to the next, the degree of variation in inhibition was considerable, and ranged from 12.6% to 94.5%.

ii)Chloroform / methanol extracts.

There was a significant reduction in the germination of zoospores, with both Ast. leptodactylus extracts and P. leniusculus extracts ($P<0.01$).

In individal experiments the reduction in germination frequency with Ast. leptodactylus extracts was greater than with extracts of P. leniusculus ($P<0.05$). This is also apparent in the pooled data (Table 3).

From table 3., it is apparent that the full strength Ast. leptodactylus C/M extract is approximatly

twice as effective at inhibiting zoospore germination in vitro, as full strength P. leniusculus extracts made in the same manner, from the same weight of epicuticle.

Statistical analysis of the data was carried out using analysis of variance and the means were compared using a least significant difference technique (Balaan 1972)

iii)Observation.

It was noticed that a proportion of spores in the medium containing full strength Ast. leptodactylus C/M extract (45.7%), were misshapen. On closer examination it was apparent that these spores had failed to encyst. No cyst wall was visible and they often retained their flagellae (Figure 12).

The proportion of non-encysted zoospores in media containing full strength extract (45.7%) was similar to the germination frequency in controls (57 ± 7.9%), suggesting that the nonencysted zoospores in media containing full strength extract, were those that would have gone on to germinate.

This observation suggests that the inhibitor or inhibitors present in the Ast. leptodactylus C/M extract kill the zoospores before encystment and in this way reduce the germination frequency. However, since these results are based on only one observation of the phenomenon, further investigation is necessary before a

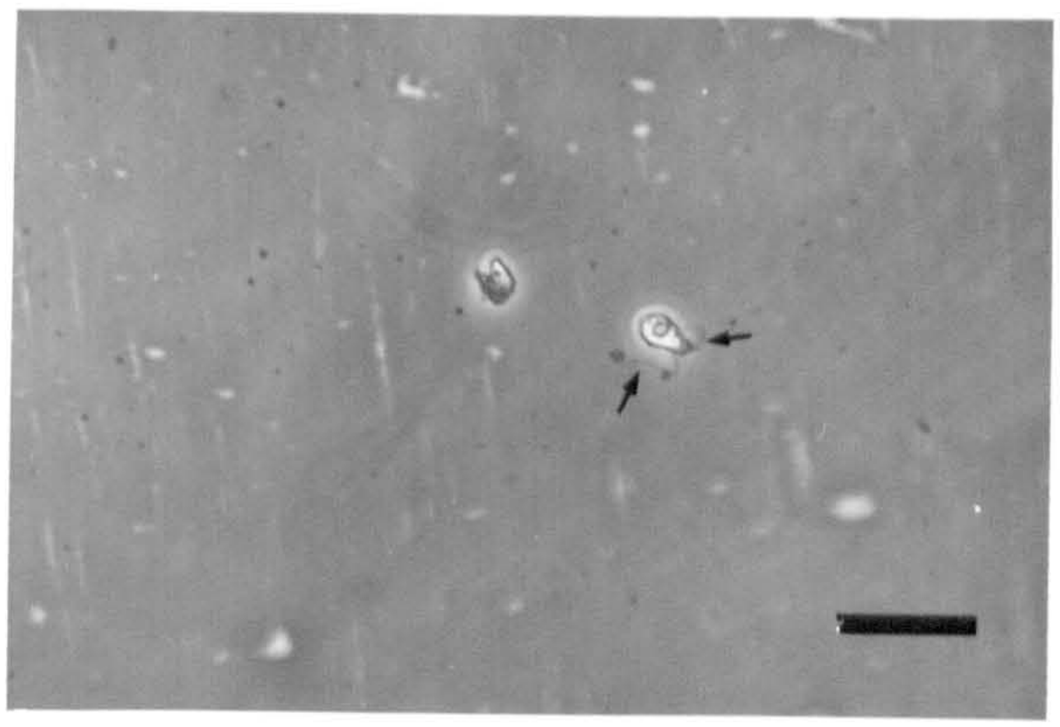
Figure 12. Non encysted spores resulting from incubation in media containing Astacus leptodactytlus chloroform / methnol extract.

Figures 12a. and b. show spores from cutures containing C/M extracts of Ast leptodactylus epicuticle and were taken 24 hrs. after incubation. The spores have failed to encyst in the calcium chloride medium containing C/M extract, and the flagellae can still be seen (arrows) attached to the zoospore.

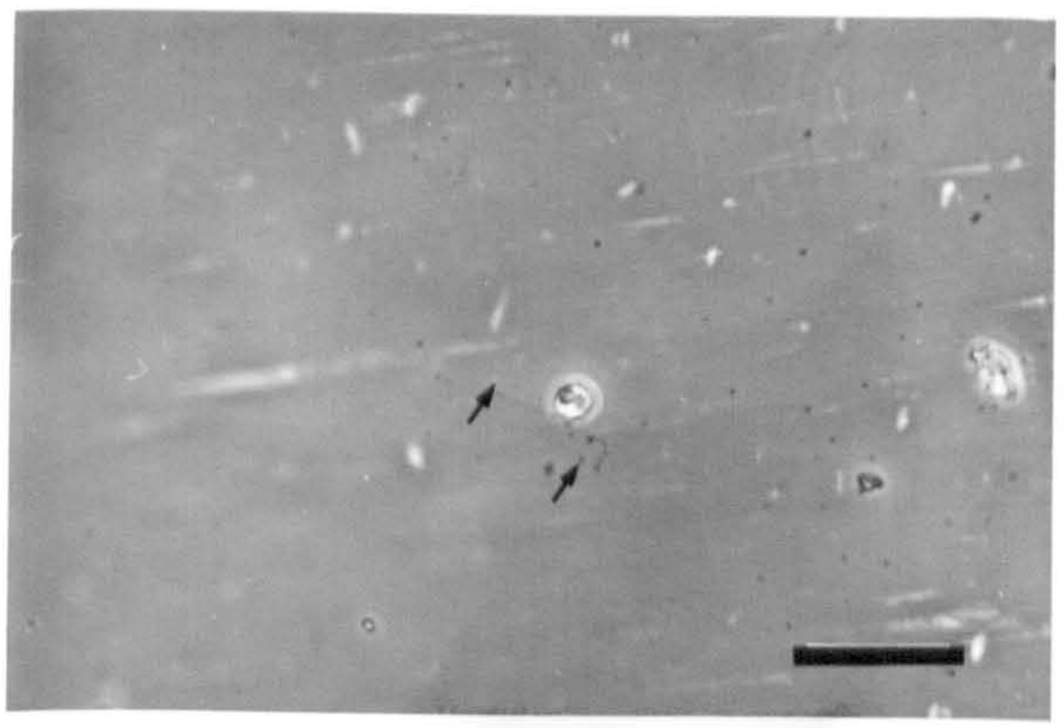
Figure 12c. shows an encysted secondary zoospore from a control culture.

Scale bar 50 um.

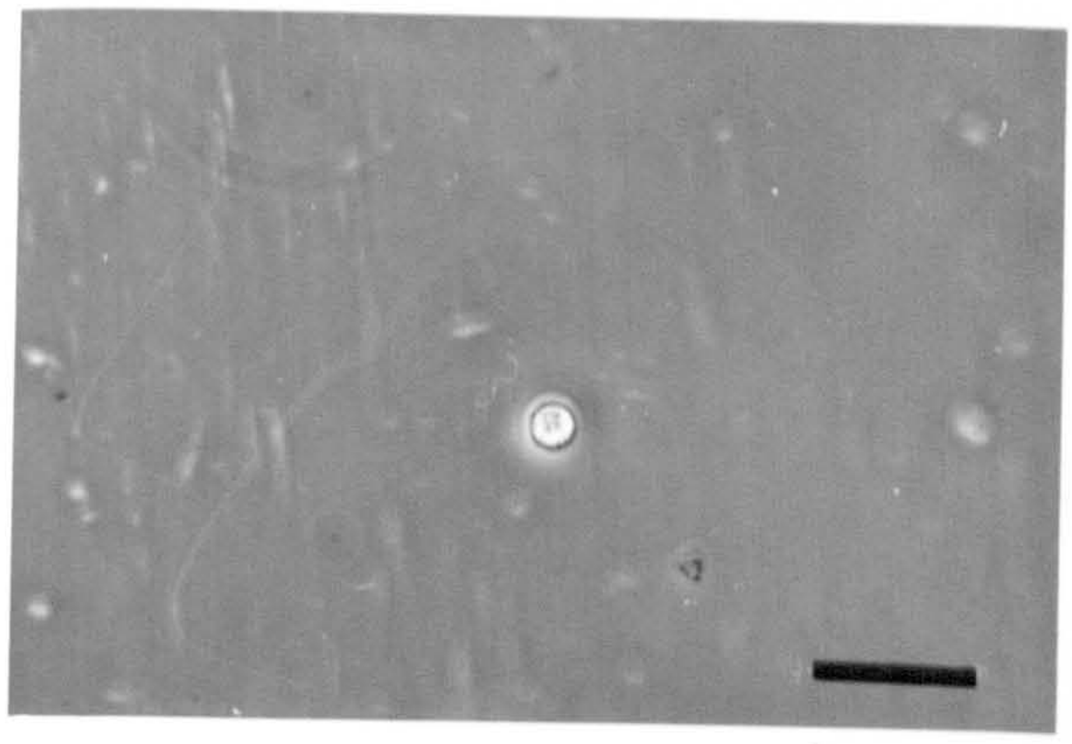
a



b



c



conclusion can be drawn.

iv) The effect of replacing the medium.

After 6hrs incubation in fresh medium, none of the zoospores originally inhibited by Ast. leptodactylus C/M extract had germinated, nor was there any germination in spores maintained in inhibitory medium. However, after 24 hrs in fresh medium, 19.6% of the encysted spores had germinated. The nonencysted spores did not germinate, nor did they encyst. After 24 hrs, control germination in inhibitory medium was still zero.

Table 4 summarizes the observations in sections iii) and iv) above.

Table 4. Obsevations on the effects of Astacus leptodactylus C/M extract on the germination of Aphanomyces astaci zoospores in vitro.

Control germination	57 ± 7.9%
Germination percentage in medium containing full strength extract.	zero
Percentage of totai zoospores unencysted	45.7
Percentage of encysted zoospores germinating in fresh medium.	19.6

4.3. The effect of calcium chloride extracts of the residual epicuticle on the germination and growth of Aphanomyces astaci zoospores in vitro.

METHODS.

After the hexane and C/M extractions had been carried out, the residual epicuticle was washed in 3 mls. of Aristar chloroform, in order to remove residual methanol, and allowed to dry.

The dried residue was then washed in two, 1.5 ml. aliquots of 300 mOsm calcium chloride solution which was filtered through a sintered glass filter.

Fifty microlitres of zoospore suspension was then added to 50 ul. aliquots of the resulting filtrate. The cultures were incubated for 4 hrs and germination percentages were assessed as above.

In these experiments it was noted that, if the incubation period was extended to 24 hrs, the sporelings incubated in calcium chloride extracts had grown to many times the length of those in control cultures, containing calcium chloride alone.

In order to compare the effects of Ast. leptodactylus extracts and those of P. leniusculus, calcium chloride extracts were made as above and a dilution series was prepared. Fifty microlitres of zoospore suspension was then added to 50 ul. aliquots of the full strength or diluted extract. The cultures were

then incubated overnight.

Drawings were made of ten sporelings from each of the cultures using a camera lucida and the lengths of each sporeling, including any branches, was measured.

RESULTS.

Table 5 shows the germination frequencies of zoospores incubated in control media, and in media containing full strength calcium chloride extracts of both Ast. leptodactylus and P. leniuscilus.

Table 5. The effect of calcium chloride extracts of residual crayfish epicuticle on the germination of Aphanomyces astaci zoospores in Vitro.

	Germination percentage.
Control.	26.1 ± 6.8
Ast. leptodactylus.	36.7 ± 6.1
P. leniusculus.	37.1 ± 8.3

Whilst the control germination frequency was 26.7%, germination frequency in Ast. leptodactylus and P. leniusculus reached 36.6 and 37.1 respectively.

Analysis of variance suggested that the increase in germination frequency in media containing epicuticle extracts was significant ($P<0.05$), although the difference between species is not.

Thus full strength extract can promote an increase

in germination frequency of 39%.

After incubation, the osmolality of both control and extract containing media was between 140 and 150 mOsm / kg, and that of full strength calcium chloride extract was only 3 mOsm / kg above the calcium chloride solution before incubation.

Figure 13. shows the effect of serial dilutions of calcium chloride extracts on the length of Aph. astaci germlings after 24 hrs. incubation.

Sporelings in full strength extract are far longer than the controls (Figure 15b). Maximal sporeling lengths however, occur in half strength dilutions of both Ast. leptodactylus and P. leniusculus extracts, in subsequent dilutions, the length of the sporelings becomes progressively less. In repeat experiments maximum sporeling length occurred in either half or quarter strength extract.

There is no significant difference between the length of sporelings in extracts of Ast. leptodactylus and P. leniusculus in half strength medium (P=0.05). Comparison with controls shows that the stimulatory effect of the extract is still present in dilutions of up to 64 times.

Figure 15. shows germlings in control and calcium chloride extract.

Sporelings in calcium chloride extract diluted between two and 256 times, tended to show hyphal anomalies (Figure 15c and d.) whilst control sporelings did not.

Figure 13. The effect of calcium chloride extracts on the growth of Aphanomyces astaci germlings in vitro.

This figure shows the effect of serial twofold dilutions of calcium chloride extracts from epicuticular residues from Ast. leptodactylus and P. leniusculus on hyphal growth in Aph. astaci germlings.

Each point is the mean of ten separate measurements. Standard deviations are not given for clarity. Control germlings were 62 ± 26 um long.

—●— Ast. leptodactylus

···■··P. leniusculus

Figure 14. The effect of calcium chloride extracts on the occurrence of hyphal bulges in Aphanomyces astaci germlings.

Figure 14. shows the effect of of serial twofold dilutions of calcium chloride extracts from residues of Ast. leptodactylus and P. leniusculus epicuticles on the occurrence of hyphal bulges in Aph. astaci.

The results are from the same experiment as those of figure 13. and are derived by counting the number of germlings out of 100 that show hyphal bulging. in each dilution of extract. Control spores did not show signs of hyphal bulging.

—●— Ast. leptodactylus

···■··P. leniusculus

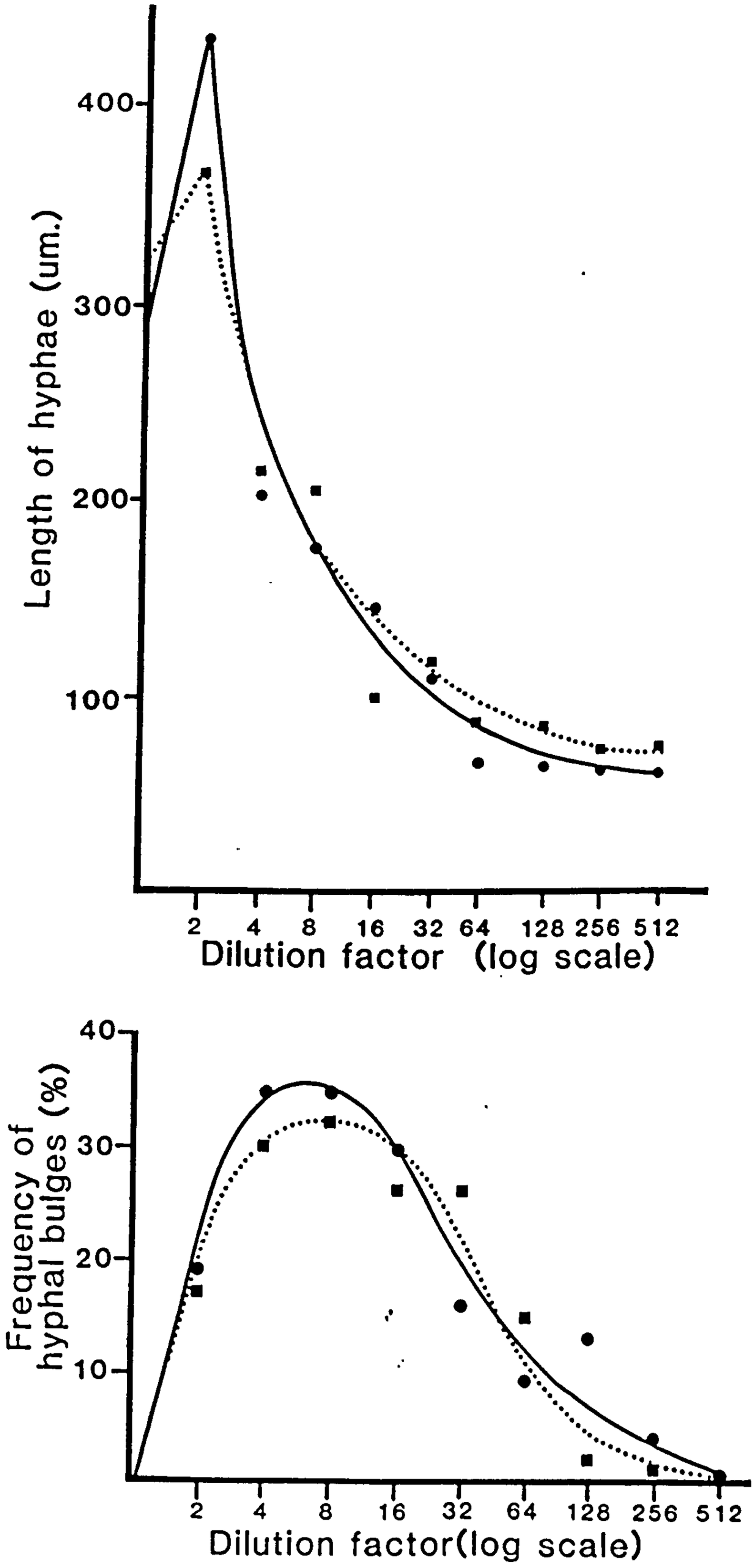

Length of hyphae (um.)
400
300
200
100
2 4 8 16 32 64 128 256 512
Dilution factor (log scale)
Frequency of hyphal bulges (%)
40
30
20
10
2 4 8 16 32 64 128 256 512
Dilution factor(log scale)

Figure 15. Comparison of germlings from cultures containing calcium chloride extracts with those from control cultures.

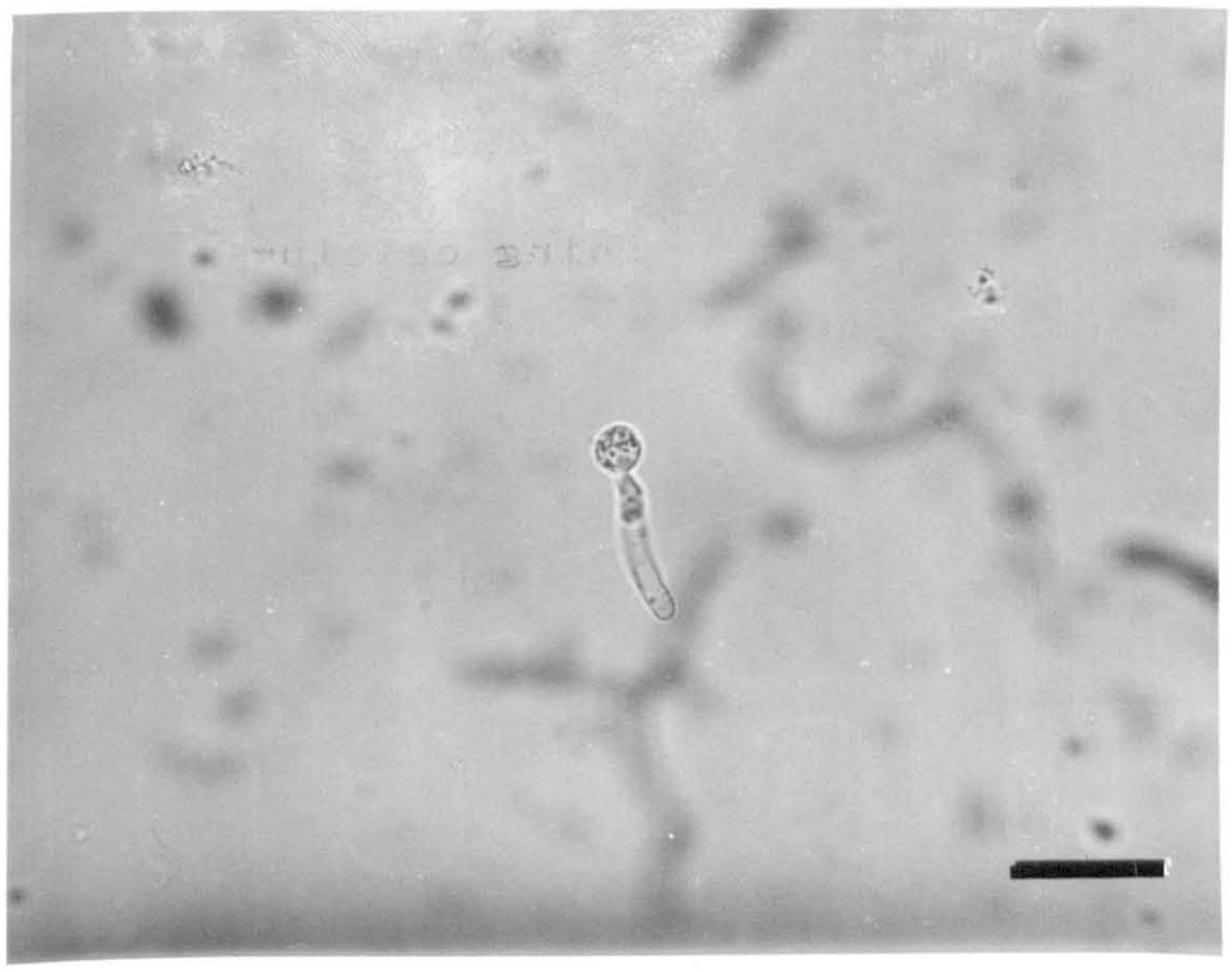
a. Control germling.

b. Germling from culture containing calcium chloride extract.

c and d. Germlings from calcium chloride extract showing hyphal bulges.

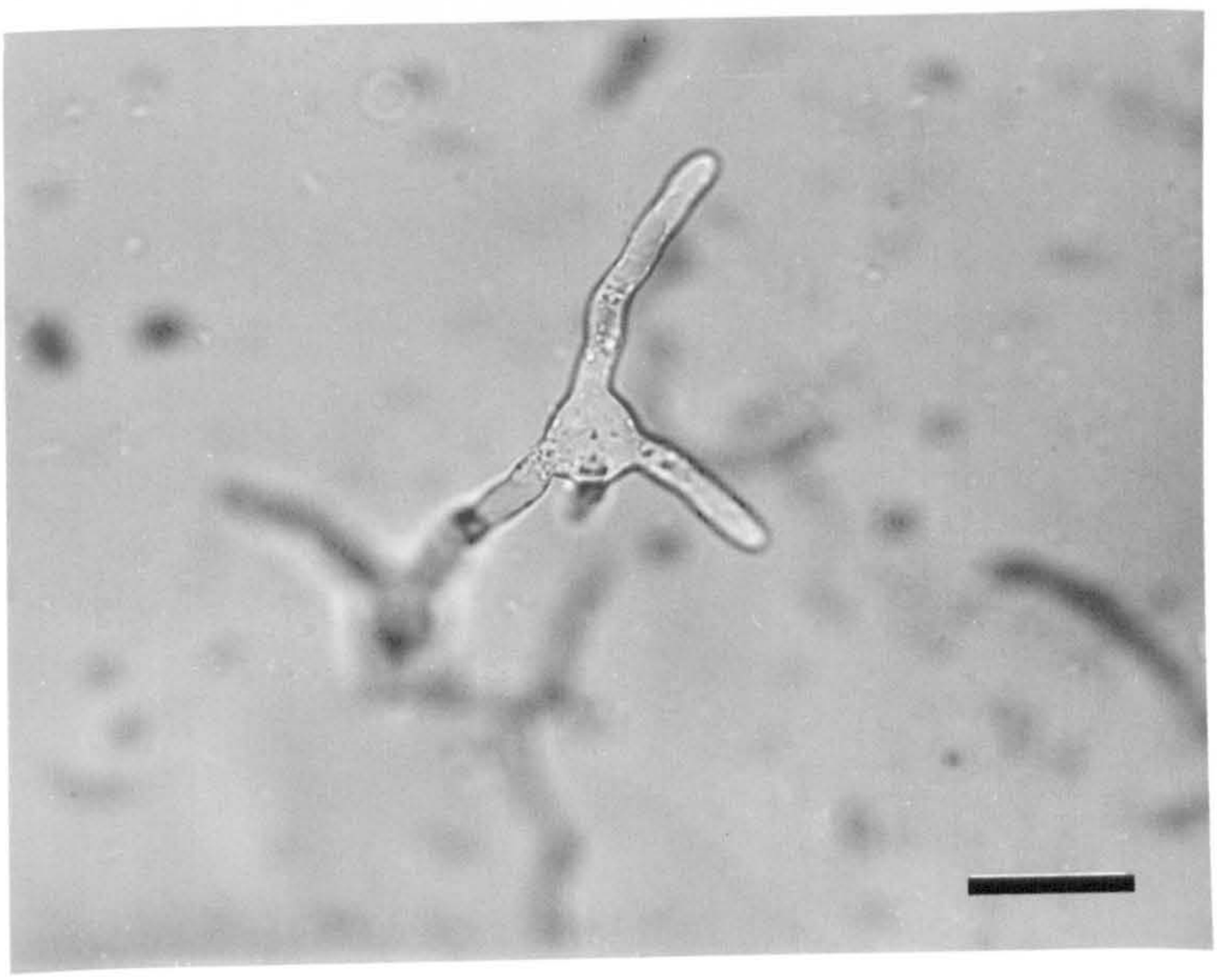
Scale bar = 25 um.

a



b

c



d

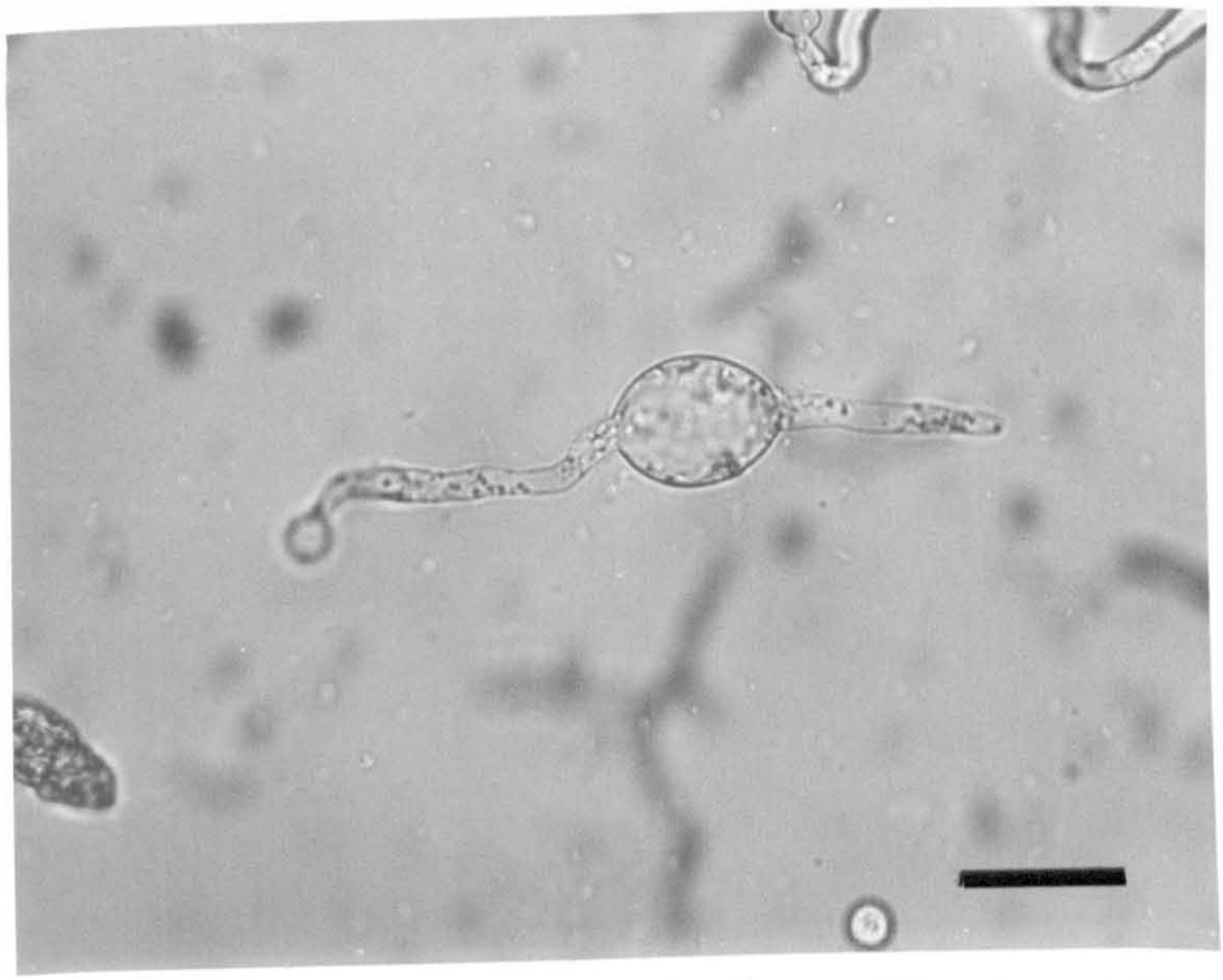


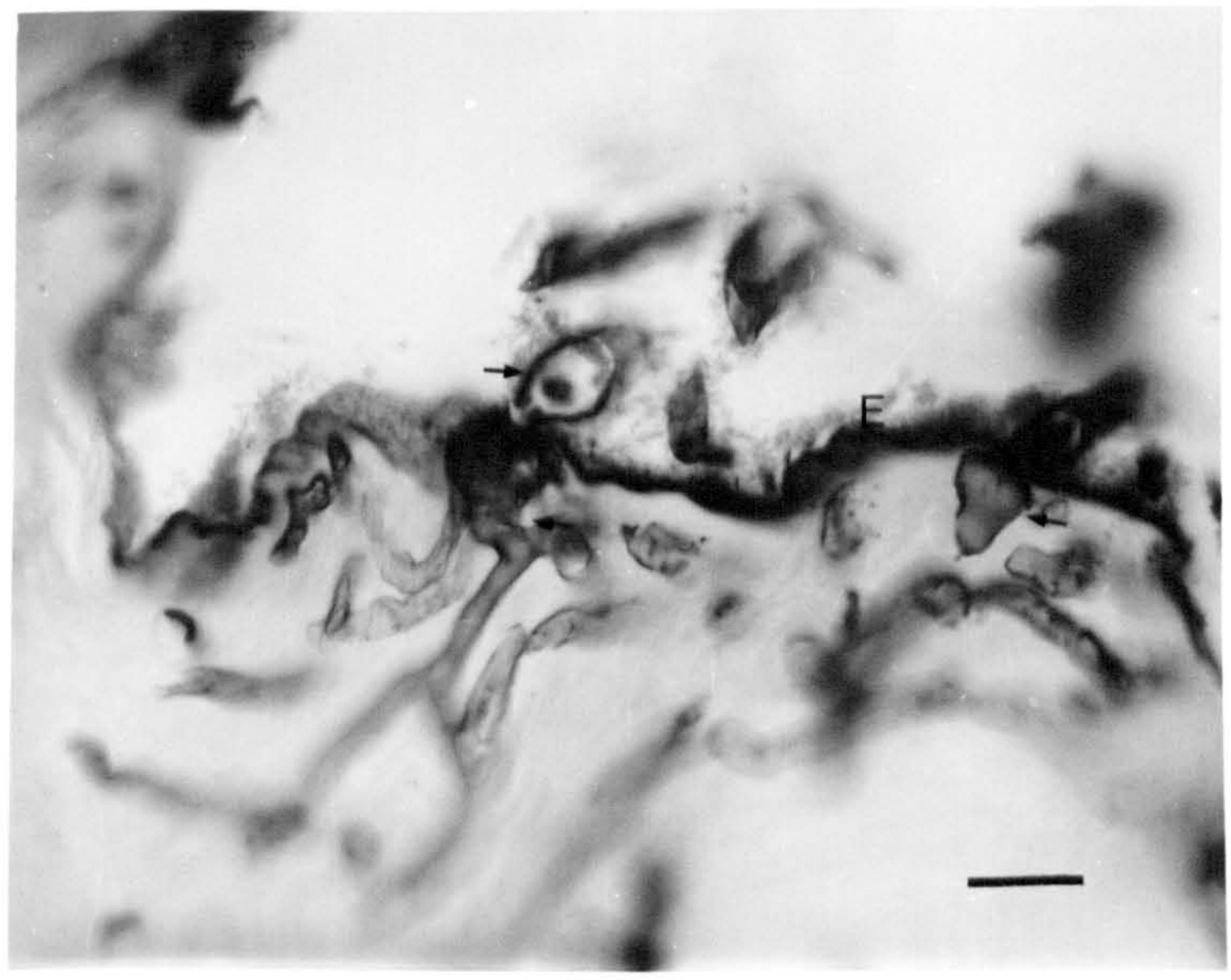
Figure 16. Hyphal bulges in Aphanomyces astaci in vivo.

This photomicrograph shows bulges in Aph. astaci hyphae (arrows) in a leg articulation of Ast. leptodactylus. The preparation was stained with Grocott's modification of Gomori's silver stain for fungi and counterstained with haematoxylin and eosin.

The hyphal bulges can be seen where the hypha comes into contact with the epicuticle both internally and externally.

EP = epicuticle

Scale bar = 50 um.


E

These anomalies were similar to the hyphal bulges seen where the hyphae impinge on the epicuticle *in vivo* (Figure 16.).

The number of such bulges per 100 sporelings was assessed in each dilution of extract. The results presented in figure 14. represent the occurrence of hyphal bulges in serial dilutions of extract and are derived from the same experiment as figure 13.

The incidence of bulges increases with dilution, however, in contrast to sporeling length, it reaches a maximum at between four times and eight times dilution, there-after decreasing with dilution. Again there was no significant difference between the effects of *Ast. leptodactylus* extract and those of *P. leniusculus*.

Section 5.

Analysis of extracts.

5.1. Ultraviolet/visible light (UV/Vis.) spectrophotometry.

Methods.

Preliminary examination of extracts was carried out by UV/Vis. spectrophotometry. *G. pulex* hexane extract was examined at full strength whilst *Ast. leptodactylus* and *P. leniusculus* hexane and C/M extracts were evaporated to

dryness, redissolved in 0.5 ml of the appropriate solvent and examined in cuvettes of 0.7 ml. volume.

Calcium chloride extracts were examined at full strength. A Cecil instruments C.E. 595 double beam spectrophotometer was used to measure the absorbance of the samples, in silica cuvettes, against blanks of the appropriate solvent.

RESULTS.

The UV/Vis. spectra of G. pulex hexane extract and Ast. leptodactylus and P. leniusculus C/M and calcium chloride extracts are illustrated in figures 17., 18 and 19. The instrument was not sensitive enough to detect any absorbance in either Ast. leptodactylus or P. leniusculus hexane extracts.

1. G. pulex hexane extract.

In G. pulex hexane extract, absorbtion maxima occur at 260, 271, 280, 290, 430, 450 and 474 nm.

The strong yellow / orange colour of the extract and the appearence of the characteristic triple peak in the visible range, suggests that the G. pulex hexane extract contains one or more carotenoid pigments. The pigments may have been initially bound to proteins as carotenoproteins or to lipoproteins to give carotenolipoproteins, however solvents have been shown to

Figure 17. UV/Vis spectrogram of Gammarus pulex hexane extract.

This figure shows a UV/Vis spectrogram of full strength G. pulex hexane extract. Peaks of absorption occur at 260, 271, 280, 290, 400, 450 and 474 nm.

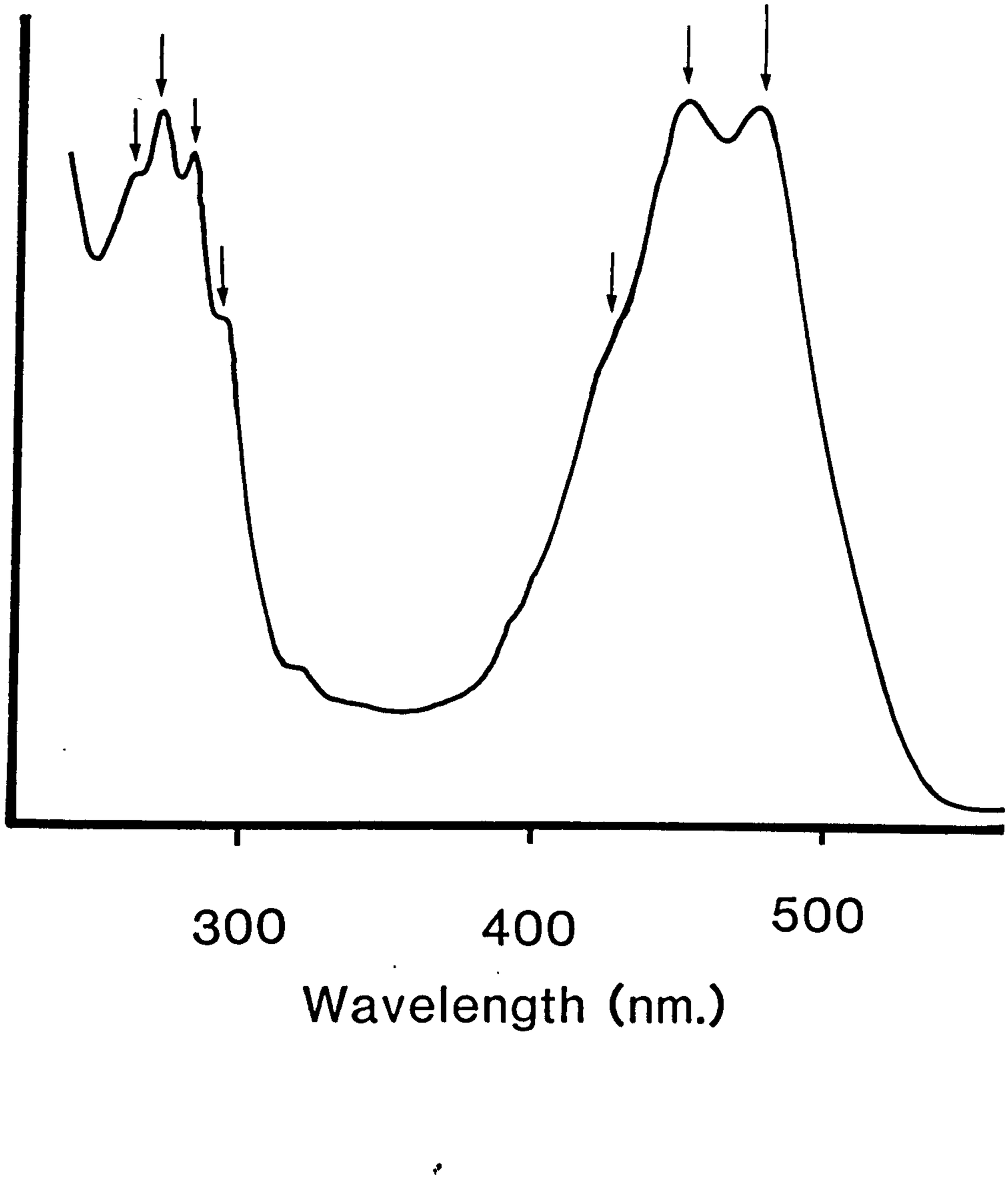

300
400
500
Wavelength (nm.)

Figure 18. UV/Vis spectrogram of crayfish chloroform / methanol extracts.

This figure shows the UV/Vis spectrograms obtained from a. Ast. leptodactylus and b. P. leniusculus C/M extract, dissolved in 500 ul. of C/M.

Extracts of both species show peaks at 240 and 270 nm. although there appears to be more of the compounds responsible in Ast. leptodactylus extract.

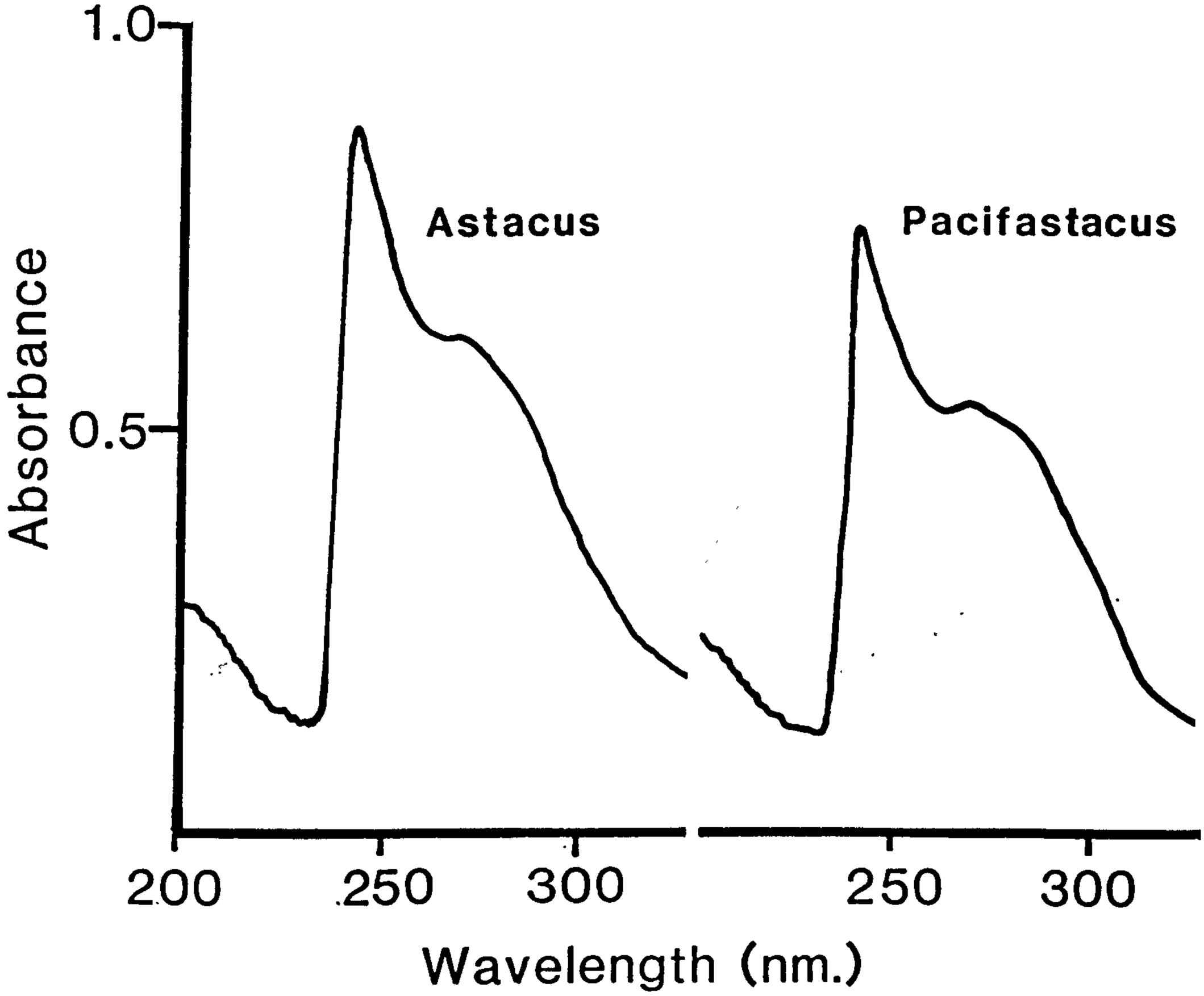
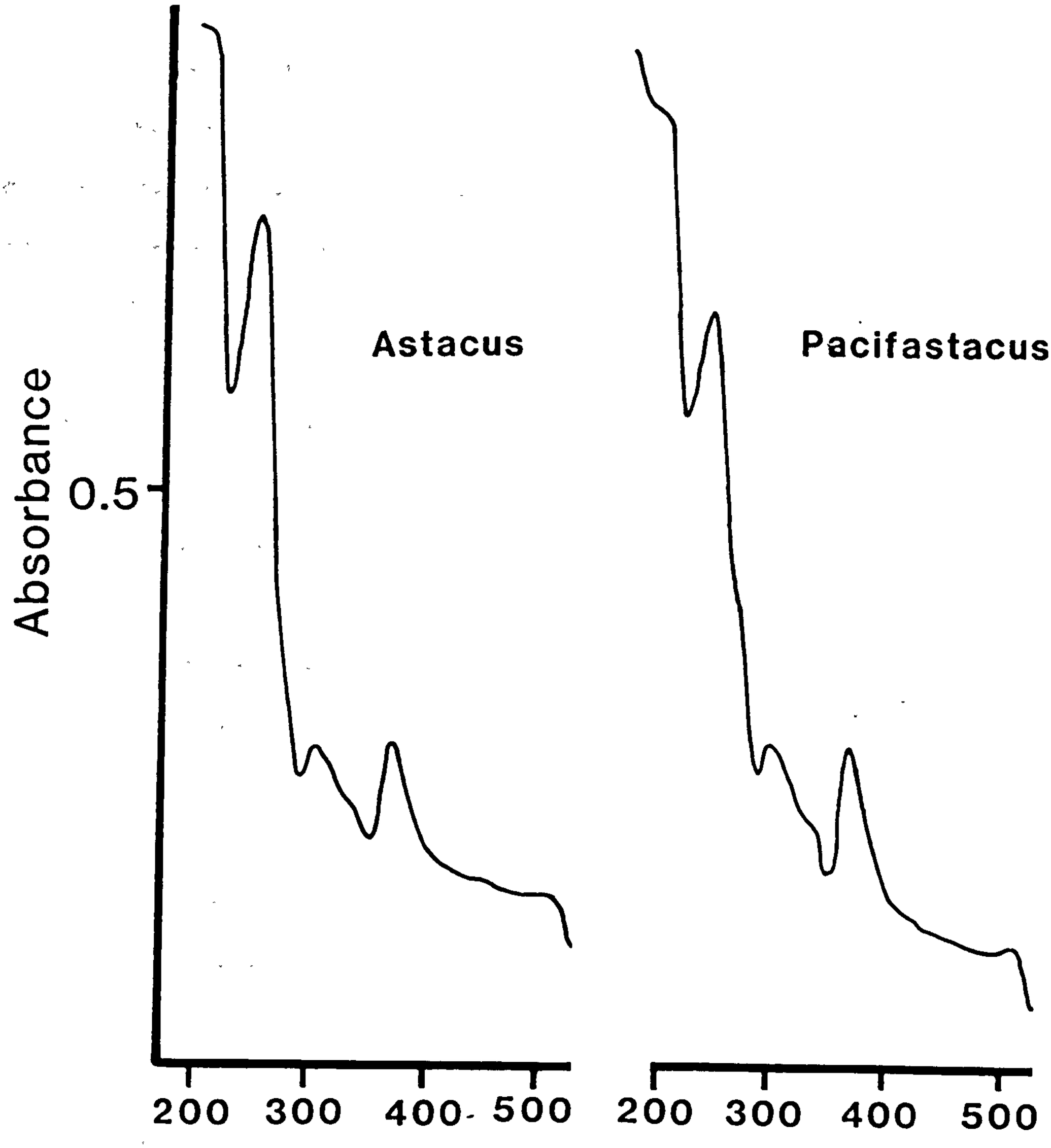

1.0
0.5
Absorbance
Astacus
Pacifastacus
200
250
300
250
300
Wavelength (nm.)

Figure 19. UV/Vis spectrogram of calcium chloride extracts of crayfish epicuticle residues.

This figure shows the UV/Vis spectrograms obtained from a. Ast. leptodactylus and b. P. leniusculus calcium chloride extracts.

Major peaks of absorption occur at 280, 332 and 402nm. in both species.


Absorbance
0.5
Astacus
Pacifastacus
200
300
400
500
200
300
400
500
Wavelength (nm.)

break down carotenoprotein complexes releasing the yellow / orange carotenoid (Ghidalia 1985).

In the crustacean cuticle, carotenoids are present in the pigmented layer. This layer is found immediately below the epicuticle, and thus the presence of these compounds in the hexane extract indicates that extraction from layers deeper than the epicuticle has occurred.

When hexane extract was added to calcium chloride solution, an orange insoluble layer was formed on the surface (2.5. above). This probably contained the free carotenoid, since carotenoproteins and carotenolipoproteins are generally soluble in water, whilst carotenes are not (Brittton 1983, Ghidalia 1985). Since the free carotenoids were removed by centrifugation, it is unlikely that it was these compounds that caused the observed inhibition.

The group of peaks at 260, 271 and 280 nm. are very similar to those produced by conjugated trienoic acids, such as alpha eleostearic acid (cis-9, trans-11, trans-13 octadecatrienoic acid), a common constituent of seed oils. (Morton 1975, Gurr and James 1980).

Similarly C18 trienoic acids are insoluble in water as well as in acetone (Weast 1973), suggesting that these compounds, if they exist in the extract, are unlikely to contribute to inhibition of zoospore germination under these conditions.

ii. C/M extracts.

Ast. leptodactylus and P. leniusculus C/M extracts had similar absorbtion spectra. Both extracts absorbed strongly at 240 nm. as well as exhibiting absorbance between 260 and 280 nm.. The compounds responsible for the absorbance at these wave lengths are unknown, however the spectra are similar to those for hexadecanoic (palmitic), octanoic (caprylic), octadecanoic (stearic) and cis-9-octadecanoic (oleic) acids (Figure 20.) in that these compounds all display absorbance maxima at 240 nm. Octanoic, octadecanoic and cis-9-octadecanoic acids also exhibit absorbance at wavelengths between 260and 280 nm.

The absorbance at 240 nm. and between 260 and 280 nm.. was always higher in Ast. leptodactylus than in P. leniusculus.

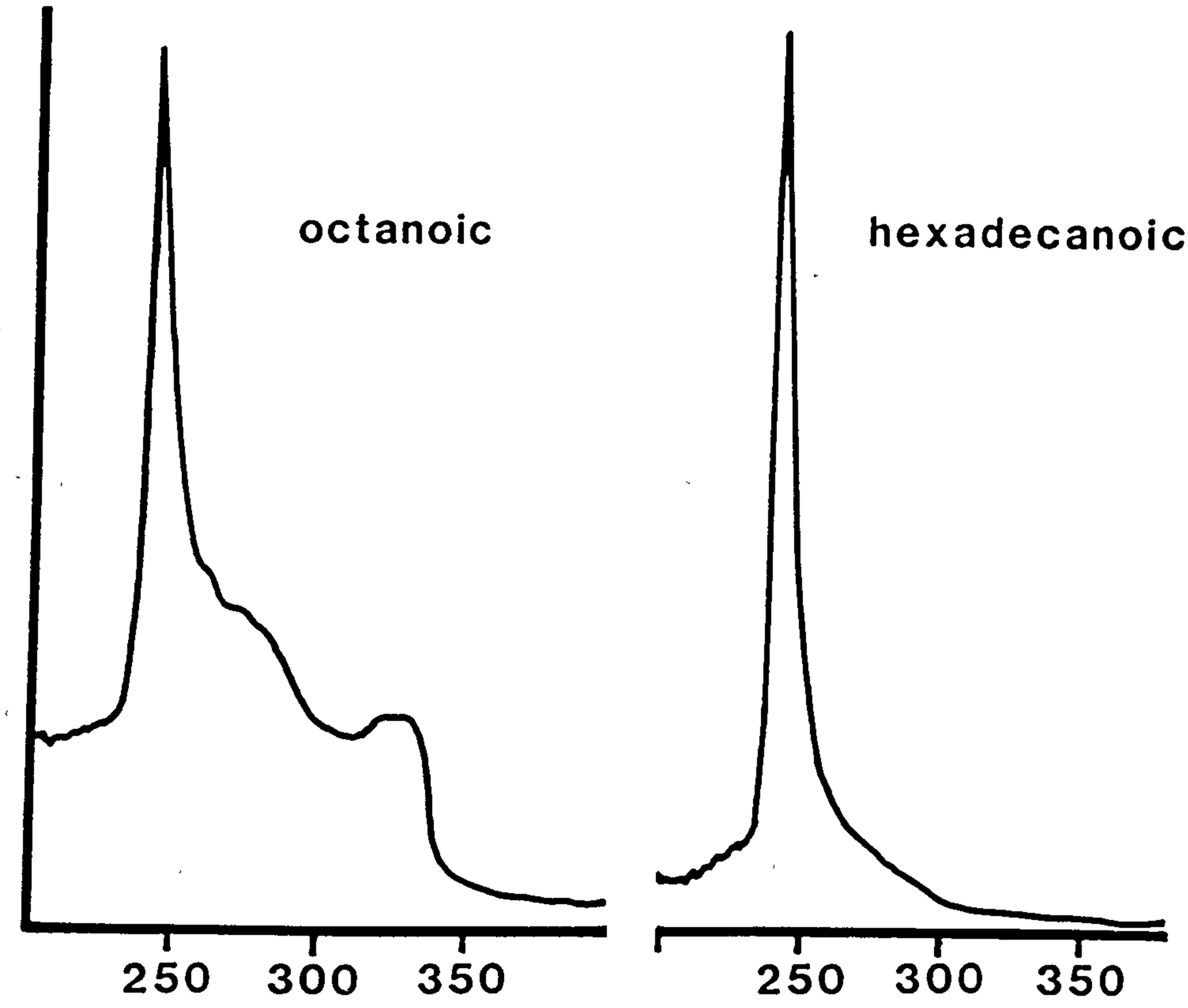
iii. Calcium chloride extracts.

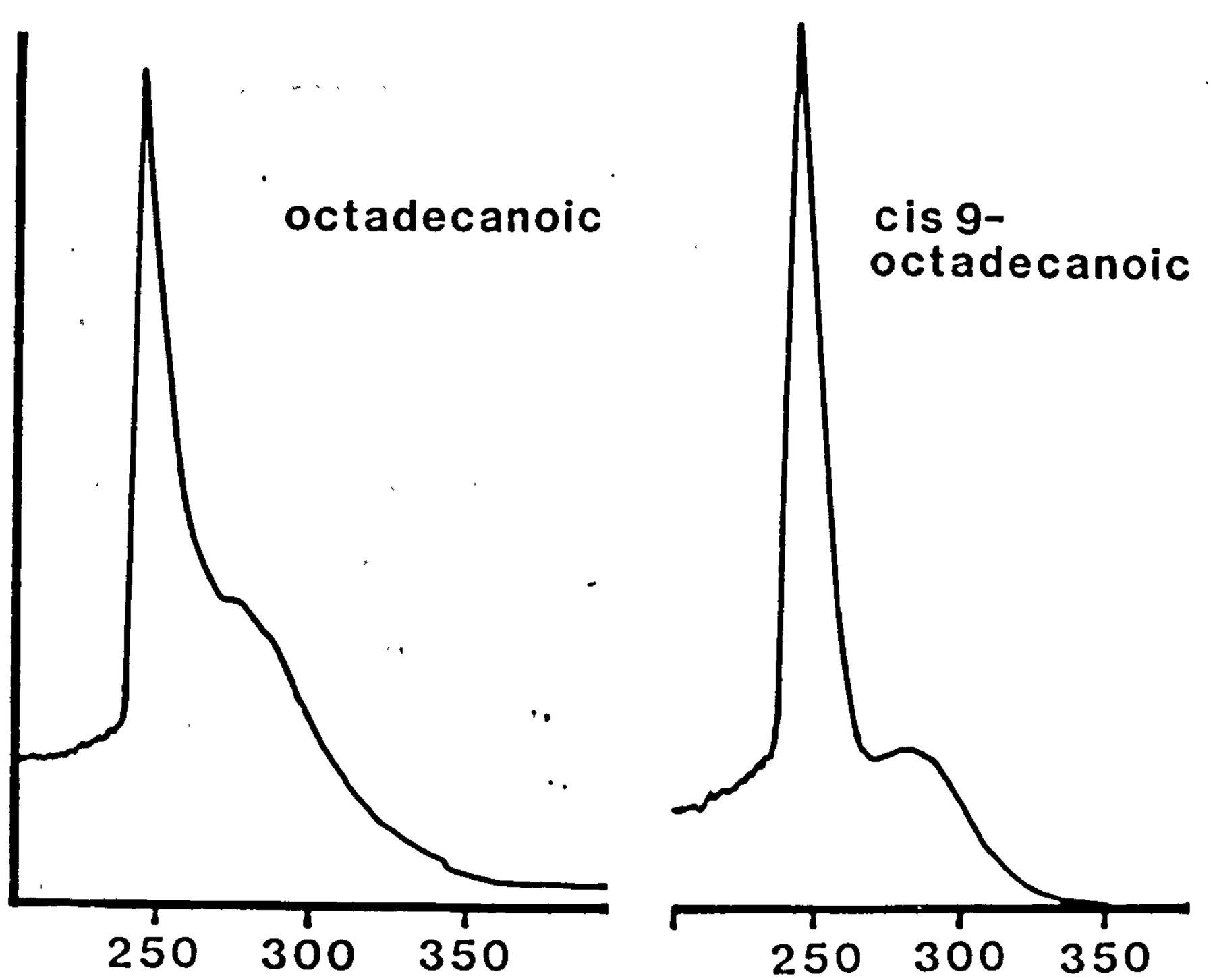
Calcium chloride extracts of both Ast. leptodactylus and P. leniusculus absorbed strongly at 280 nm., suggesting the presence of protein in the extracts. They also both absorbed at 332, 400 and 588 nm. but the compounds responsible for absorbances at these wavelengths were not identified.

The absorbance at 280 nm. was stronger in Ast. leptodactylus extracts than in those of P. leniusculus.

Figure 20. UV/Vis spectrograms of four fatty acids.

This figure illustrates the UV/Vis spectrograms obtained from a. octanoic acid b. hexadecanoic acid c. octadecanoic acid and d. cis 9-octadecanoic acid. All compounds were disolved in hexane and read against a hexane blank.


octanoic
hexadecanoic
250
300
350
250
300
350


octadecanoic
cis 9-
octadecanoic
250
300
350
250
300
350
Wavelength (nm.)

5.2. Analysis of calcium chloride extracts.

Calcium chloride extracts were analysed to determine whether potential nutrients such as proteins, amino acids, carbohydrates or lipids could be detected, in an attempt to explain the lush growth of Aph. astaci sporelings in these extracts.

METHODS.

Proteins were assayed using the BCA protein assay, total carbohydrates were assayed using the anthrone test and amino acids and residual lipids were screened using TLC.

In the anthrone test for total carbohydrate, two hundred microlitres of 5% trichloroacetic acid was added to 200 ul. of full strength extract in a microcentrifuge tube and allowed to stand for 20 minutes. The sample was then spun for 30 seconds in a Beckman microfuge and two 200 ul. aliquots were removed for analysis.

Eight hundred microlitres of anthrone reagent (2% anthrone in sulphuric acid) were added to each aliquot and the solution was placed in a boiling water bath for 10 minutes. The resultant solution was read in a Cecil 590 spectrophotometer at 620 nm. and compared to a calibration graph prepared using glucose solution.

For TLC, extracts were freeze dried redissolved in a minimum amount of distiled water and spotted onto

activated silica gel G plates.

In tests for amino acids, the plates were developed in Butanol : acetic acid : water (4:1:1). After drying, the plates were sprayed with ninhydrin and heated to 110°C for 10 minutes to visualize the spots. A variety of amino acids were used as standards.

To ascertain whether residual lipids were present in the extract, the plates were developed in Chloroform : methanol : water (80:25:3) Spots were visualized by spraying the dried plates with ammonium molybdate in perchloric acid (Appendix 7) and heating them to 110°C for 15 minutes. Hexadecanoic acid was used as a control.

RESULTS.

The BCA protein assay gives a protein concentration of between 1.8 and 2.8 mg/ml. protein for Ast. leptodactylus calcium chloride extracts and between 1.1 and 2.0 mg/ml. for those of P. leniusculus.

Ast. leptodactylus extracts therefore contain more protein than those of P. leniusculus. This is in agreement with the results obtained for UV spectra of the extracts above.

The anthrone method gives a total carbohydrate level for Ast. leptodactylus extract of 9.0 ug./ ml, and for P. leniusculus 8.6 ug./ ml.

No spots staining as amino acids or as lipids were present on chromatograms of calcium chloride extracts of

either species.

5.3. Thin layer chromatography of Gammarus pulex and crayfish solvent extracts.

METHODS.

Further analysis of solvent extracts was carried out by thin layer chromatography in order to determine whether free fatty acids or melanin pathway intermediates could be detected.

Hexane, C/M and calcium chloride extracts were made as described above. Solvent extracts were evaporated to dryness with a stream of nitrogen, redissolved in approximately 50 ul. of the appropriate solvent, in the case of crayfish extract and 150 ul. in the case of G. pulex extract and then spotted onto a 0.25 mm silica gel plate (Polygram Sil G, Macherey-Nagel) that had previously been activated at 80°C.

The whole of the crayfish extract was added to the plate but, in the case of G. pulex, only 50 ul. was added to avoid overloading the plate.

Development of solvent extracts was carried out in hexane : diethyl ether (70:30) and spots were visualized using either a spray of 1% dichlorofluorescene in ethanol (DCF) or Schweppes reagent (Appendix 8). Octanoic, hexadecanoic, octadecanoic and cis-9-octadecanoic acids were run in parallel with the

extracts, in order to locate potential fatty acid spots. 1-4 napthoquinone, P-benzoquinone, 2 methyl 1, 4 napthoquinone and 2, 7 dihydroxynapthaline were used to locate intermediates of melanin synthesis.

RESULTS.

Table 6 details the Rf values of the fatty acid standards and melanin intermediates used, as well as identifying characteristics of the compounds.

Table 6. Mean Rf values of fatty acid standards and melanin synthesis intermediates.

Standard	Rf	Characteristics
Octanoic acid	0.56	UV fluorescent (DCF)/ Brown (Schwepps)
Cis 9-octadecanoic acid	0.55	UV fluorescent (DCF)/ Brown (Schwepps)
Hexadecanoic acid	0.53	UV fluorescent (DCF)/ Brown (Schwepps)
Octadecanoic acid	0.57	UV fluorescent (DCF)/ Brown (schwepps)
1,4 Napthoquinone 2 Methyl	0.54	UV absorbant (DCF)
1,4 napthoquinone	0.60	UV absorbant (DCF)
p-Benzoquinone	0.45	UV absorbant (DCF)
2,7-Dihydroxy-napthalene	0.06	Black staining (DCF)

Table 7 lists the sensitivity of DCF visualization

to the quinone standards used. This was estimated by spotting dilutions of the standards onto a TLC plate and spraying with DCF.

Table 7. Sensitivity of Dichlorofuorescene staining technique for melanin pathway intermediates.

Compound	Sensitivity
1,4 napthoquinone	0.6 ug.
2 methyl 1,4 napthoquinone	1.0 ug.
p benzoquinone	1.0 ug.
1,7 dihydroxy napthalene	0.1 ug.

Figure 21. shows the arrangement of spots on a chromatogram of G. pulex hexane extract visualized with DCF followed by Schweppes reagent. A maximum of 10 spots were seen using this developement technique, their mean Rf values and identifying characteristics are listed in Table 8.

No spots staining in the same manner as any of the melanin intermediates were seen, suggesting that such compounds, if present in the extract were present in amounts smaller than that detectable by this method (see table 7).

One spot, with a mean Rf value of 0.56 and staining in the same manner as fatty acids with both DCF and Schwepps reagent, was present. Its mean Rf value coincided

Figure 21. Thin layer chromatogram of Gammarus pulex hexane extract.

This figure illustrates a chromatogram of G. pulex hexane extract on silica gel G, developed in 70 : 30 hexane : diethyl ether and visualized both by dichloroflourescene and Schweppes reagent. For the Rf values of the individual componants see table 8.

Fatty acid standards

F1 = octadecanoic acid

F2 = octanoic acid

F3 = cis 9-octadecanoic acid

F4 = hexadecanoic acid

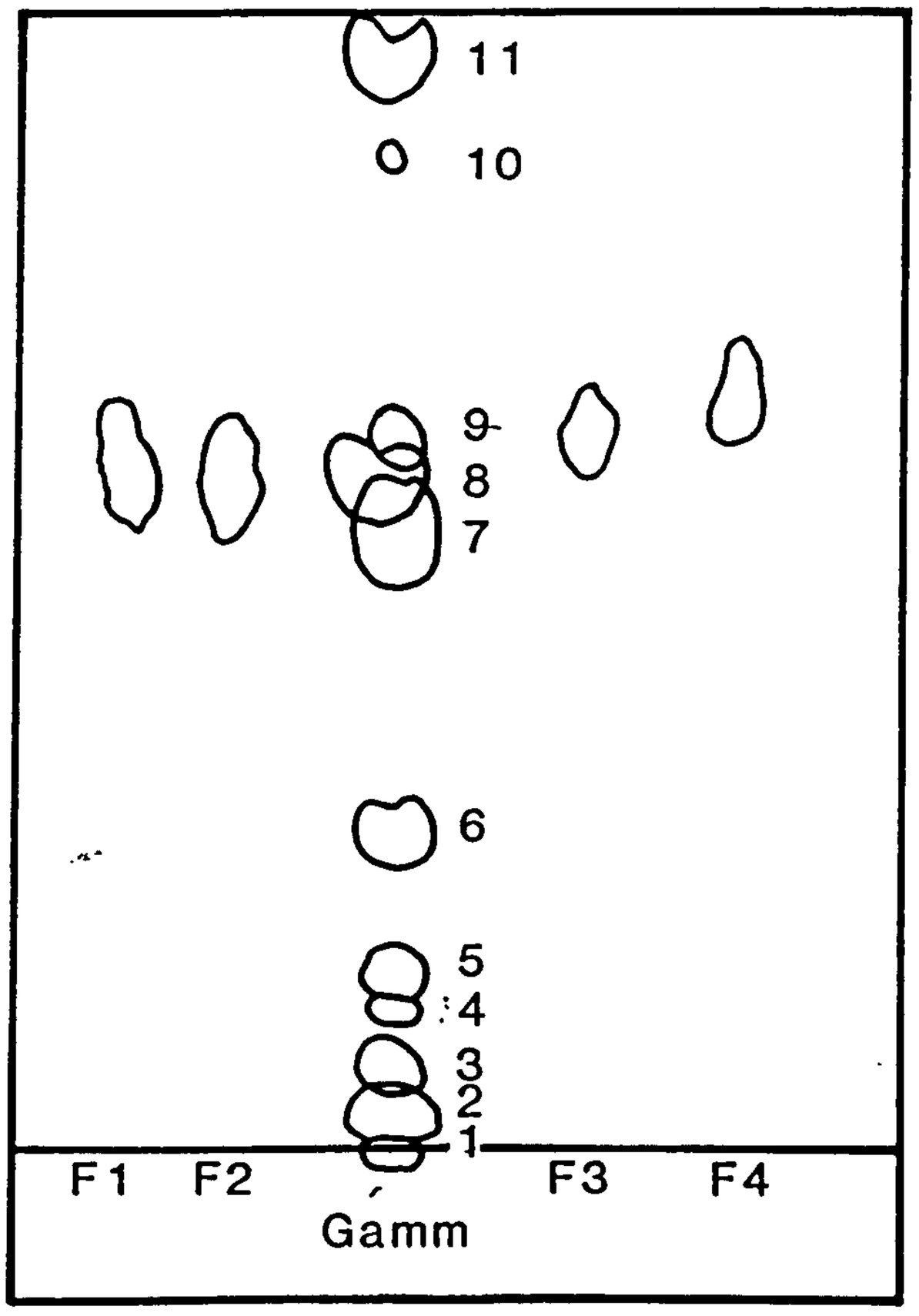

11
10
9
8
7
6
5
4
3
2
1
F1
F2
F3
F4
Gamm

Table 8. Mean Rf values of constituents of G. pulex hexane extract.

Spot number	Rf value.	Characteristics
1	0	Orange pigment
2	0.03	Orange pigment
3	0.08	Orange pigment
4	0.12	Orange pigment
5	0.16	UV fluorescent (DCF)
6	0.27	Pale yellow
7	0.54	UV fluorescent (DCF)
8	0.56	UV fluorescent (DCF) Brown (Schweppes)
9	0.71	UV fluorescent
10	0.87	UV fluorescent (DCF)
11	0.97	Pale yellow

with that of octanoic acid.

Figure 22. shows the the arrangement of spots on a chromatogram of hexane extracts from the two crayfish species, their Rf values and identification characteristics are listed in Table 9.

As for the G. pulex extracts above, there were no spots staining in a manner similar to that of the quinone samples in either crayfish species. A number of compounds that were UV fluorescent when visualized with DCF were present, although none of these had Rf values similar to those of the fatty acid standards used.

Figure 22. Thin layer chromatogram of crayfish hexane extracts.

This figure illustrates a chromatogram of crayfish epicuticular hexane extracts on silica gel G, developed in 70 : 30 hexane : diethyl ether and visualized with dichloroflourescene. For the Rf values of the individual componants see table 9.

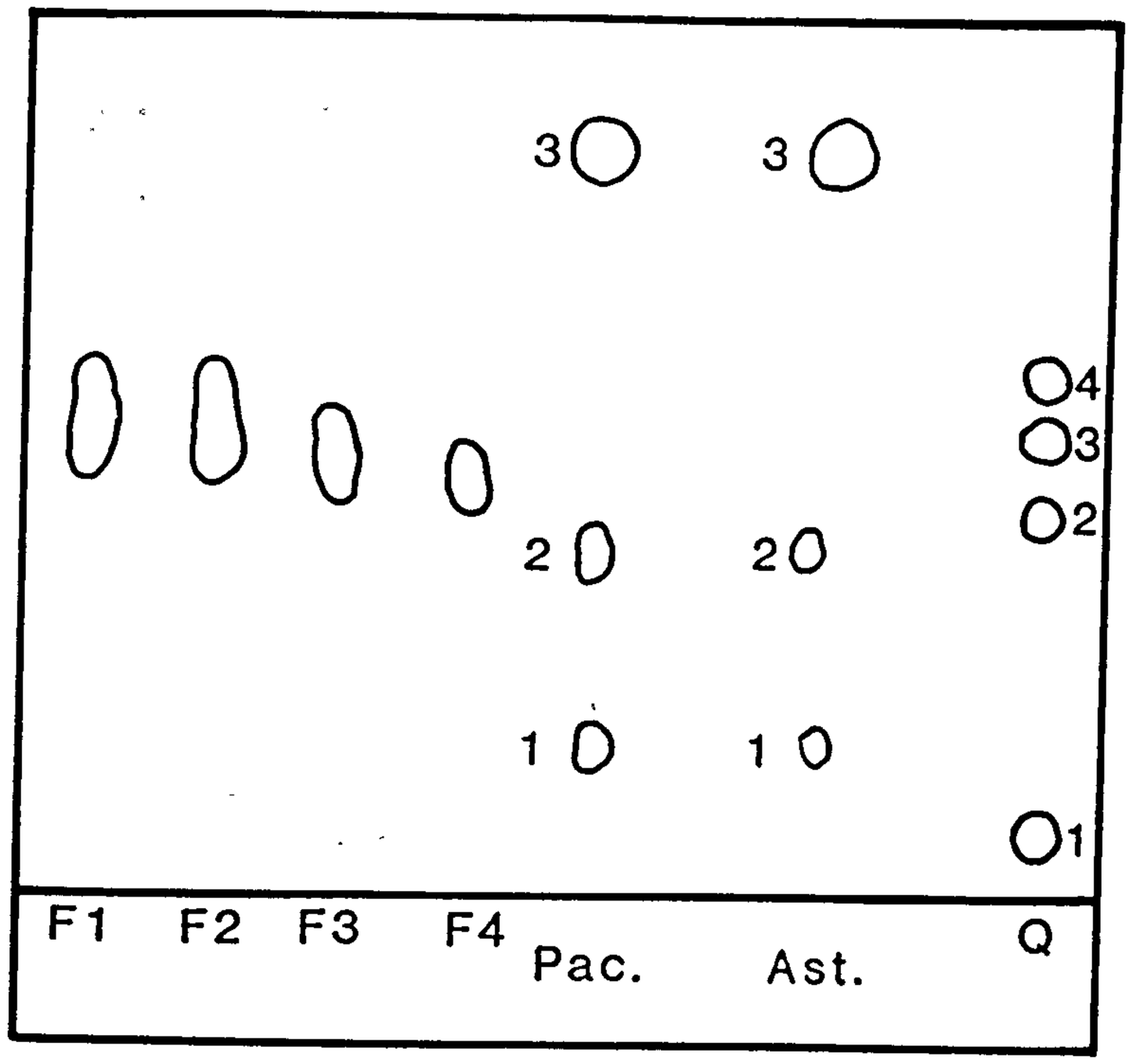
Quinone standards	Fatty acid standards
Q1 = 2,7 dihydroxy napthalene	F1 = octadecanoic acid
	F2 = octanoic acid
Q2 = p-benzoquinone	F3 = cis 9-octadecanoic acid
Q3 = 1,4 naptho-quinone	F4 = hexadecanoic acid
Q4 = 2 methyl 1,4 napthoquinone	

A. = Ast. leptodactylus P. = P. leniusculus

Figure 23. Thin layer chromatogram of crayfish chloroform / methanol extracts.

This figure illustrates a chromatogram of crayfish epicuticular C/M extracts on silica gel G, developed in 70 : 30 hexane : diethyl ether and visualized with dichloroflourescene. For the Rf values of the individual componants see table 10.

Quinone and Fatty acid standards as above.


3
3
4
3
2
2
1
1
F1
F2
F3
F4
Pac.
Ast.
Q

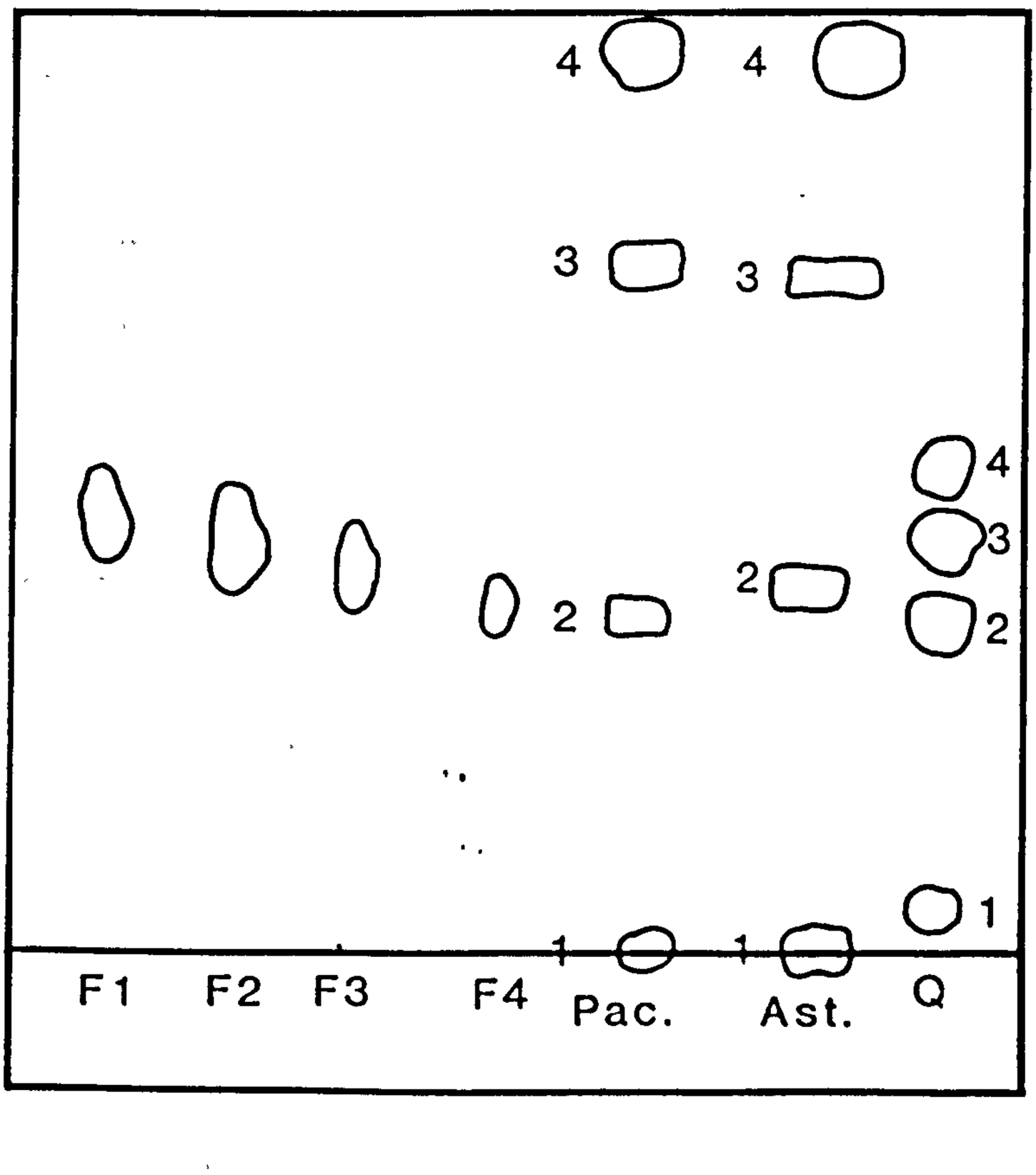

4
4
3
2
2
4
3
2
1
1
1
F1
F2
F3
F4
Pac.
Ast.
Q

Table 9. Rf values and characteristics of components of crayfish hexane extracts.

spot number.	Rf	Characteristics
A.1	0.16	UV fluorescent (DCF)
P.1	0.16	UV fluorescent (DCF)
A.2	0.39	UV fluorescent (DCF)
P.2	0.39	UV fluorescent (DCF)
A.3	0.83	UV fluorescent (DCF)
P.3	0.84	UV fluorescent (DCF)

A.= Ast. leptodactylus.
P.= P. leniusculus.

Figure 23. shows the arrangement of spots on a chromatogram of C/M extracts. The Rf values and identification characteristics are presented in table 10. Three spots are visible after spraying with DCF, in both crayfish species.

One spot in each extract has a similar Rf value to hexadecanoic acid, although the Rf value of the Ast. leptodactylus spot (0.39) differs from that in P. leniusculus (0.36). Both compounds, like hexadecanoic acid, are UV flurescent when sprayed with DCF. There are no spots visible, which stain in a manner similar to the melanin pathway intermediates used.

In one experiment, C/M extracts of the two species were separated by two way chromatography on silica gel G. The plate was developed first in 70 : 30 hexane : diethyl

Table 10 Rf values and characteristics of crayfish C/M extracts.

Spot number.	Rf	Characteristic
A.1	0.0	UV fluorescent
P.1	0.0	Pale orange Non UV fluorescent
A.2	0.39	UV fluorescent
P.2	0.36	UV fluorescent
A.3	0.74	UV fluorescent
P.3	0.75	UV fluorescent
A.4	0.97	UV fluorescent
P.4	0.97	UV fluorescent

A.= Ast. leptodactylus

P.= P. leniusculus

ether, and in the second direction, in 70 : 30 : 1 petrolium ether : diethyl ether : acetic acid. In the second solvent system the four standard fatty acids were run in parallel.

In both animals the putative fatty acid spot had an Rf value of 0.45 in the second solvent, which was similar, under these conditions, to the 4 standard fatty acids.

These results suggest that the hexane extracts of G. pulex and the C/M extracts of epicuticles of both crayfish species do indeed contain fatty acids, but that they contain no detectable free quinones such as those involved in the synthesis of melanin. Hexane extracts of neither crayfish species contained compounds that had

similar Rf values to the free fatty acids used as standards.

DISCUSSION.

Activity against Aph. astaci zoospores was found in hexane extracts of G. pulex. These extracts caused a marked reduction in germination frequency of the zoospores.

Absorption spectra of G. pulex hexane extract suggested the possible presence of both carotenoid pigments and trienoic fatty acids. Further, TLC of these extracts also suggested the presence of fatty acids, although their identity was not determined. No melanin pathway intermediates were detected by TLC.

Since the insoluble, free carotenoids were removed from incubation media by centrifugation, and any trienoic acids present would not be soluble in either water or acetone, it is unlikely that these compounds play any role in the inhibition of spore germination mediated by G. pulex hexane extract.

The site of the antifungal activity in vivo is unclear, since the presence of carotenoid pigments in G. pulex extracts indicates that extraction was not limited to the epicuticle, but had affected deeper layers.

Hexane extracts of both Ast. leptodactylus and P. leniusculus generally caused a less marked reduction in germination frequency than those of G. pulex. Moreover, it

was not possible to demonstrate known antifungal compounds such as quinones or fatty acids in these extracts.

This limited activity against Aph. astaci zoospores may explain why wiping the arthrodial membranes of the legs with hexane did not cause a significant reduction in protection against Aph. astaci in either Ast. leptodactylus or P. leniusculus.

Since no significant difference could be detected between the effects of extracts of the two species, it is unlikely that the compounds present in these extracts explain the difference in susceptibility to Aph. astaci between the two species of crayfish.

Epicuticular C/M extracts of both crayfish species were more effective at reducing the germination frequency of zoospores than hexane extracts. Osmolality measurements on incubation media after 24 hrs. suggested that the reduced germination was not due to an increase in osmolality in any of the media tested.

Limited observation of the effects of Ast. leptodactylus C/M extract on Aph. astaci zoospores, suggested that the extract caused the death of motile secondary zoospores before encystment, thus leading to a reduction in the germination percentage.

Replacement of the inhibitory medium with fresh calcium chloride solution resulted in the germination of some of the encysted spores.

The germination of a small number of spores in fresh medium can be explained if germination inhibition is

reversible in a small percentage of spores.

Cerenius and Sodderhall (1984a) showed that zoospores encysted by agitation, were competent to germinate for 15 minutes after encystment.

Thus a small proportion of encysted spores transferred with the swimming spores in this assay are likely to have been competent to germinate. The presence of a spore coat may have afforded these spores protection, whilst the nonencysted spores were killed. Removal of the antifungal agent with fresh medium may then have allowed germination of the spores.

A similar effect was seen when extracts of 4th instar larval silkworms (Bombyx mori) were tested against Aspergillus flavis spores. Whilst germination was strongly and irreversibly inhibited in high concentrations of extract, in low concentrations (1 in 2,000) inhibition of germination could be reversed by replacing the inhibitory medium with fresh germination (Koidsumi 1957).

Ast. leptodactylus C/M extract was twice as effective as P. leniusculus C/M extract at preventing the germination of zoospores, despite the fact that the latter is more resistant to crayfish plague (Unestam 1969 and 1972).

In contrast, ether extracts of the larvae or pupae of silkworm strains resistant to Asp. flavis, Asp. clavatus, Asp. niger or Mucor sp. had more antifungal activity than extracts of susceptible races (Koidsumi and Wada 1955).

Furthermore, in a later study (Wada 1957) it was demonstrated that extracts of field insects resistant to I. farinosa but susceptible to Spicaria pracina, contained volatile antifungal lipids more active against I. farinosa than against S. pracina and vice verca.

Wiping the surface of Ast. leptodactylus leg arthrodial membranes with C/M rendered these areas more susceptible to invasion by Aph. astaci, whilst in P. leniusculus there was no evidence of increased susceptibility after such treatment.

Thus the C/M soluble antifungal activity in Ast. leptodactylus epicuticle appears to confer a limited degree of resistance to Aph. astaci in vivo. However, whilst the epicuticle of P. leniusculus does contain C/M soluble antifungal activity, it is clear that resistance to Aph. astaci in this species is not dependent on such activity.

The compounds responsible for the antifungal activity of G. pulex hexane extracts or Ast. leptodactylus and P. leniusculus C/M extracts were not identified, however, evidence from TLC suggests that the extracts contain fatty acids.

Most lipids, including fatty acids, are easily soluble in mixtures of chloroform and methanol and many are soluble in hexane. Further some fatty acids and alcohols are sparingly soluble in water also (Weast 1973).

Spectra of C/M extracts of both Ast. leptodactylus and P. leniusculus and those of G. pulex hexane extracts,

show similarities to those of a number of fatty acids and extracts of Ast. leptodactylus, which are more effective at reducing the germination frequency of Aph. astaci zoospores than those of P. leniusculus, appear to contain more such compounds.

Moreover, TLC of C/M extracts from the epicuticles of both species of crayfish, as well as those from G. pulex hexane extract, indicated the presence of fatty acids, although they were not identified.

A variety of short chain fatty acids, alcohols and aldehydes have been shown to influence spore formation in Aph. astaci (Unestam 1966b).

Acids and alcohols were the most effective agents, and generally the longer chain length compounds were more toxic. Thus nonanoic acid and nonanol reduced spore formation considerably, whilst decanoic and dodecanoic acids, decanol and undecanol abolished it completely. Of the aldehydes tested, octanal, nonanal, decanal and undecanal reduced spore formation, whilst dodecanal prevented it completely.

Saturated, unsaturated and in some cases branched free fatty acids have been found in the epicuticles of insects (Lockey 1984). For example, mono and di-enoic acids have been found in Anabrus simplex (Baker et al 1960), as well as C-18 tri-enoic acids. Fatty alcohols are also present in the cuticles of most insects (Neville 1974).

Insect cuticular fatty acids vary from short chain

lengths such as pentanoic acid found in Heliothis zea (Smith and Grula 1982) to acids with around twenty carbon atoms. The precise composition of the fatty acid componant of cuticular lipids varies from species to species, and often from individual to individual (Toolson 1984). However, the predominant components are usually cis-9 octadecanoic (oleic) and cis, cis 9, 12 hexadecanoic (Linoleic) acids (Lockey 1984).

Extracts of the cuticle of C. simplex inhibit the growth of I. farinosa and Asp. flavis, this effect is probably due to the presence of octanoic and decanoic acids in the epicuticular lipids (Koidsumi 1957).

Pentanoic, octanoic and nonanoic acids present in surface hexane extracts of the Corn Ear Worm, H. zea, are mycostatic when tested against B. basiana (Smith and Grula 1982).

Further, it has been shown that fatty acids up to and including hendecanoic (C11) are toxic to P. chartarum (Thornton 1963) and those between acetic and dodecanoic (Lauric, C12) are toxic to Boletus varigatus (Pedersen 1969), whilst fatty acids and alcohols between C5 and C12 are toxic to H. resinae (Teh 1974).

The inhibition of fungal spore germination by fatty acids has also been demonstrated. Spore germination in P. chartarum is inhibited by fatty acids between nonanoic and hendecanoic (Thornton 1963) and octanoic acid prevents conidial germination in Beauvaria basiana.

The location of the antifungal activity within the

crayfish cuticle remains speculative. In insects the antifungal fatty acids appear to occur in the outer layers of the epicuticle since light abrasion with alumina and wiping with tetrachloromethane increases susceptibility to fungal diseasé (Koidsumi 1957). However, although the presence of fatty acids has been demonstrated histologically in the outer lamina of the cuticulin layer (probably corresponding to the outer epicuticle) of larval Calpodes ethlius (Locke 1966), lipids in general have proven difficult to locate histologically due to their solubility in the solvents used in preparation (Hadley 1982).

Certainly antifungal activity would be of most use to the animal on the outer surface of the epicuticle and the ability to increase susceptibility to Aph. astaci in Ast. leptodactylus by wiping the surface with C/M, suggests that this may be the location of the C/M soluble activity.

However, whilst long chain fatty acids and alcohols are insoluble in water, as the chain length becomes shorter the solubility increases. Thus the solubility of tetradecanoic (Myristic, C14) acid is 0.8 uM / litre and the solubility of octanoic acid is 2.2 mM / litre, whilst cis 9-octadecanoic (oleic) and cis 9, 12 octadecanoic (linoleic) acids are insoluble in water (Weast 1973).

The solubility of aliphatic alcohols is slightly greater; that of tetradecanol (C14) is 1.46 um / l, whilst that of octanol is 4.5 mM /l (Bell 1973). The

solubilities of fatty acids and alcohols thus have important implications for their possible presence on the epicuticular surface of aquatic arthropods.

Free fatty acids in the big stone fly, Pteronarcys californica, and its aquatic niaid form have been compared (Armold et al 1969). It was found that although the nymph had larger amounts of C14 acids (both unsaturated: 14:0, and monoenoic: 14:1) in its surface lipids, the adult insect had a larger proportion of acids less than C14 (2.7% of total free fatty acids) than the niaid (1.8% total free fatty acids).

Thus, although extracts of the terrestrial adult insect contained more short chain free fatty acids than the aquatic form, such compounds did exist in the aquatic form despite their solubility in water.

Incubation of Aph. astaci zoospores in calcium chloride extracts of either species of crayfish resulted in an increased germination frequency.

Observations that crayfish tissues enhance germination of Aph. astaci zoospores are not new. In early experiments (Rennerfelt 1936) it was reported that, whilst germination in water was low, in crayfish blood it reached 100%, although in 20% horse serum germination was not as high. Later experiments were unable to reproduce these results (Unestam 1966b and 1969b). When Aph. astaci zoospores were incubated in crayfish serum germination frequency was low (8-12%), however when zoospores were incubated in crayfish serum on the inner surface of

stripped and cleaned subadominal cuticle of either Ast. astacus or P. leniusculus, germination was often as high as 50% (Unestam 1969b). This phenomenon may have been caused by a cuticular germination enhancer or may have been the result of the stimulating effect of cuticular calcium.

In this study germination of Aph. astaci zoospores was shown to be related to calcium cloride concentration. Crayfish haemolymph contains in the order of 10 mM calcium (Jarenpaa et al 1986), which, on its own, would give a germination frequency of approximately 15% (Figure 1.). The increase in germination frequency when incubation was carried out on the inner surface of crayfish cuticle may have resulted from a local increase in the calcium concentration due to calcium being leached out of the cuticle.

In these experiments, although the presence of calcium from epicuticular deposits may have influenced the germination frequency, it is unlikely to have caused a large increase in germination, since the difference in osmolality between test and control media (3 mOsm / kg) was eqivalent to an increase in calcium chloride of only 1 mM. Neither was such a small increase in osmolality enough to cause a large increase in germination frequency by itself.

Aph. astaci sporelings grown in calcium chloride extracts of crayfish epicuticle were far larger than those in control media, and growth was most marked in half or

quarter strength extract. This suggests that an initial inhibitory effect of the medium was superimposed on the stimulatory effect.

It has already been suggested that the peeled epicuticles of crayfish contain a compound or compounds that are lethal to zoospores, in the concentrations involved in these experiments, and it was suggested that such compounds were soluble in water as well as in C/M. The appearance of an initial inhibitory effect on growth in full strength calcium chloride extract may thus be due to inhibitory compounds remaining in the epicuticle after hexane and C/M extraction.

A growth promoting effect of crayfish cuticle was demonstrated by Rennerfelt (1936) who noted that when Aph. astaci zoospores were germinated in water containing pieces of crayfish cuticle, the germinated spores grew larger than those germinating in water alone.

Increased growth of sporelings in calcium chloride extract may be due either to a stimulatory effect of the extract on the growth of the sporelings, or to nutrients in the epicuticular extracts.

Analysis of the calcium chloride extracts showed that they contained both protein and carbohydrate components but these were not identified. No amino acids or residual lipids were detected by TLC.

The epicuticle of arthropods contains both structural proteins such as lipoproteins, glycoproteins, and enzymes (Travis 1965, Neville, 1975 Babu et al 1984),

and these may have been the source of the proteins in the extracts.

Aph. astaci is capable of utilizing a number of mono and disaccharides as the sole carbon source, notably glucose, sucrose, maltose and lactose, although the ability to utilize certain substrates depended on the nitrogen source (Unestam 1965). In addition, chitinase and limited pectinase activity have been detected in culture filtrates (Unestam 1966a). The ability to utilize other polysaccharides has not been reported.

Carbohydrates have been localized in the epicuticle of crustaceans by several workers using histochemical techniques. Travis (1965) detected PAS positive material in the epicuticle of the crayfish O. limosus up until post moult (stage B). After this stage she suggests that the material is masked by other components of the developing epicuticle. No acid mucopolysaccharides were found.

Using alcian blue, Babu et al (1985) located acid mucopolysaccharides in the epicuticle of intermoult M. rumphii. Glycogen and traces of glycoproteins were also detected. The glycogen was derived from the reserve cells of the conective tissue and is thought to be the precursor of both epicuticular chitin and glycoproteins (Hackman 1971, Babu et al. 1985).

Extracellular, broad-spectrum protease activity is found in older mycelia of Aph. astaci (Soderhall and Unestam 1975). The protease has activity against chromogenic peptides containing aromatic amino acids and

is therefore believed to be important in degrading the cross links in arthropod cuticles (Hall 1983). Aph. astaci zoospores encysted with calcium chloride solution, are also capable of secreting a peptidase (Persson 1986).

The presence of proteins and carbohydrates in the epicuticular calcium chloride extracts and the ability of Aph. astaci to utilize such compounds as nutrients may therefore explain the lush growth of sporelings in these extracts.

In addition to both germination stimulating and growth promoting factors, it was demonstrated that both Ast. leptodctylus and P. leniusculus calcium chloride extracts contained factors that caused hyphal bulging in A. astaci sporelings.

Hyphal swellings have been noted in other fungi, for example Markham (1979) isolated a number of mutants of Asp. nidulans that showed either ballooning or gross hyphal swelling, and suggested that this phenomenon was due to extensive unpolarized wall synthesis rather than to a stretching of the walls due to internal pressure.

In Neurospora crassa hyphal swellings can be induced by replacing sucrose in the growth medium with sorbose. This also causes an increase in hyphal branching (Burnett 1978).

Hyphal swellings have also been reported in Aph. astaci infections of crayfish (Unestam and Weiss 1970 and chapter 3 above), and occur at the point were hyphae come into contact with the inner face of the epicuticle (Figure

16.). Such hyphal swelling is abolished in excised boiled cuticle. Disturbed growth and abnormal branching at the tip of melanized Aph. astaci hyphae has also been reported (Unestam and Nyhlen 1974).

The occurrence of hyphal swellings in Aph. astaci germlings germinating in extracts of peeled epicuticle suggests that the phenomenon is not a physical effect of the hypha impingeing on the inner epicuticle, but that it is an effect of compounds within the epicuticle.

Since there was no difference in the germination stimulating or growth or hyphal bulge promoting properties of epicuticular calcium chloride extracts of the two species, it is unlikely that these effects explain the difference in their susceptibilities to crayfish plague.

Thus compounds have been detected in hexane extracts of whole G. pulex and in C/M extracts of Ast. leptodactylus and P. leniusculus epicuticle, that reduce the germination frequency of Aph. astaci zoospores in vitro.

It is suggested that, in Ast. leptodactylus, these compounds act by killing the secondary zoospore before encystment and may also inhibit germination of recently encysted zoospores.

Evidence from TLC and UV / Vis. spectroscopy suggests that the antifungal extracts contain fatty acids and these maybe the compounds responsible for the antifungal activity.

Further, it has been demonstrated for Ast.

leptodactylus, that infection of arthrodial membranes can be enhanced by wiping them with a mixture of chloroform and methanol, suggesting that the C/M soluble antifungal compounds are active in vivo, and that they exist on or close to, the surface of the animal.

Since Ast. leptodactylus extract is approximately twice as effective as that of P. leniusculus, at preventing germination of Aph. astaci zoospores, a further mechanism of resistance must exist in P. leniusculus.

In addition, calcium chloride extracts of crayfish epicuticle have been demonstrated to enhance zoospore germination and sporeling growth as well as cause hyphal swellings, this later may explain the presence of hyphal swellings where hyphae come into contact with the inner epicuticle in vivo.

The increase in hyphal growth caused by calcium chloride extracts may be explained by the presence of proteins and carbohydrates in the extracts.

APPENDIX 1.

ANIMAL HUSBANDRY.

Ast leptodactlus were aquired commercially up till 1985 when the supply ceased due to the occurrence of crayfish plague in Turkey. Animals of this species were subsequently aquired from an introduced population in central London.

Commercially aquired animals were of consistant size, usually between 15 and 30g, whilst those from the local population were of wider size range.

P. leniusculus were aquired from a variety of comercial sources.

The two species were kept in separate rooms in fibreglass tanks with bottom area of 5 square meters. Hides were provided in the form of small terracotta plant pots and plastic guttering sections. Aeration was provided continuously through a number of air stones.

Stock animals were fed ad libitum on carp pellets supplemented with small pieces of par-boiled carrot or potato and tanks were cleaned out as necessary.

A 12 hr light / dark cycle was maintained.

APPENDIX 2.

STERILIZATION PROCEDURES AND ANIMAL DISPOSAL.

Alderman and Polglase (1985) have shown that both "Wescodyne" (Ciba Geigy) at 100ppm available iodine, and hypochlorite at 100ppm available chlorine, were effective disinfectants for use against Aph. astaci. Accordingly all equipment used in field experiments, including boots and nets, were sprayed with "Wescodyne" between sampling sites. Crayfish traps were allowed to stand in "Wescodyne" overnight before being washed and dried.

In laboratory studies, polythene troughs were disinfected after use by soaking them overnight in a plastic dustbin full of sodium hypochlorite bleach. Airlines, airstones and plastic crayfish refuges were treated in a similar way

When experimental tanks were cleaned, potentially infected water was poured into a plastic dustbin containing neat hypochlorite solution and left for at least 24 hrs. before being disposed of.

Before disposal, infected animals from both field and laboratory experiments were disinfected for a period of not less than one week, in "Lysol" (BDH chemicals). They were then disposed of by maceration.

APPENDIX 3.

UNESTAM'S GLUCOSE PEPTONE AGAR.

(Unestam 1965).

1. Trace element solution.

Copper sulphate pentahydrate	0.250g
Boric acid	0.286g
Magnesium chloride tetrahydrate	0.181g
Zinc sulphate septahydrate	0.022g
Molybdic acid	0.006g

Make up to 1 litre with distilled water.

Autoclave in 100 ml quantities.

2. EDTA solution.

Disodium EDTA	7.445g

Make up to 100 mls. with distilled water.

3. Basal medium.

Magnesium chloride hexahydrate	1.65g
Anhydrous calcium chloride	1.11g
Potassium chloride	3.73g
Ferric chloride hexahydrate	0.20g
EDTA solution	15 ml.
Trace element solution	10 ml.

Made up to 1 litre with distilled water.

Autoclave in 100 ml. quantities.

4. Phosphate buffer.

A. Disodium phosphate dodecahydrate	47.7g
B. Sodium dihydrogen phosphate	20.8g

Make up both compounds to 1 litre with distilled water.

Titrate A. against 1 litre of B. to pH 7.

Autoclave in 100 ml quantities.

5. Glucose peptone agar.

Glucose	6g
Peptone	3g
Basal medium	100 ml
Agar	12 g
Distilled water	800 ml

Autoclaved at 15 lb / sq. in. for 15 mins.

Phosphate buffer	100 ml

Autoclaved separately and added after cooling to pouring temperature.

APPENDIX 4.

RIVER WATER AGAR (RWA).

(Alderman 1982).

Glucose	5g
Yeast extract	1g
Oxolinic acid	10mg
Agar	12g
Filtered river water	1 litre

In practice, it was found that river water could be replaced with water from crayfish stock tanks and that yeast extract could be replaced with mycological peptone (Oxoid).

Oxolinic acid was used only in isolation proceedures.

APPENDIX 5.

DAVIDSON'S FIXATIVE.

(Shaw and Battle 1957).

Formaldehyde (35%)	20 parts
Glycerine	10 parts
95% IMS	30 parts
Glacial acetic acid	10 parts
Distilled water	30 parts

APPENDIX 6.

GROCOTT'S HEXAMINE SILVER NITRATE STAIN FOR FUNGI IN TISSUES.

(Grocott 1955).

Stock hexamine silver nitrate solution.

3% Hexamine	1 litre
5% Silver nitrate	5 mls.

Before use dilute 50% with distilled water and add 2mls of 5% borax per 100 mls of working solution.

Proceedure.

i) Dewax and bring to 100% ethanol.

ii) 2% cellulose nitrate in 1:1 diethyl ether : ethanol, 5 mins. Allow the sections to begin to dry in air before the next step.

iii) 80% ethanol 5 mins.

iv) Rehydrate sections.

v) 5% chromic acid, 60 mins.

vi) Wash in tap water three times.

vii) 1% sodium metabisulphite, 1 min.

viii) Running tap water, 5 mins.

ix) Rinse in distilled water three times.

x) Stain in hexamine silver solution for 30 mins. in

the dark at 50°C or until sections are tobacco brown.

xi) Rinse three times in distilled water, 3 mins each.

xii) 0.1% gold chloride, 3 mins.

xii) Rinse in distilled water

xiii) 2% sodium thiosulphate, 2 mins.

xiv) Rinse in running tap water, 5 mins.

xv) Counterstain in Coles haemotoxalin and 1% eosin

Impregnation with 2% cellulose nitrate immediately before the 80 % alcohol stage of dehydration prevented the cuticle, which was often hardened by processing, from coming off the slides during the staining proceedure.

APPENDIX 7.

AMMONIUM MOLYBDATE-PERCHLORIC ACID.

(Touchstone and Dobbins 1978).

a. 3g ammonium molybdate in 25 mls. distilled water.

b. 1M hydrochloric acid.

c. 60% perchloric acid.

The total of solution a. is mixed with 30 mls. of solution b. and 15 mls. of solution c. The TLC plate was sprayed and heated to 105 °C for 20 mins. Lipids produce blue-black spots.

APPENDIX 8.

SCHWEPPES REAGENT.

(Touchstone and Dobbins 1978).

a. 2mls of 100% glucose

b. 2mls. of aniline disolved in 2mls. of ethanol

Mix solutions a. and b. and dilute to 100 mls with n-butanol.

The TLC plate is sprayed with the reagent and heated to 125°C for 5-10 mins. Organic acids produce brown spots on a white background.

APPENDIX 9.

The effect of acetone on the germination of Aphanomyces astaci zoospores in tissue culture wells and Petri dishes.

Time (hrs)	Culture wells		Petri dishes	
	control	+ acetone	control	+ acetone
0	0	0	0	0
1	0	0	0	0
2	5.4 ± 1.3	<1	10.8 ± 3.0	4.5 ± 2.1
3	8.8 ± 1.7	1.8 ± 3.1	17.8 ± 4.3	17.6 ± 6.4
4	16.2 ± 1.3	10.3 ± 6.6	23.9 ± 4.2	20.4 ± 5.9
5	22.2 ± 5.1	17.4 ± 6.6	30.9 ± 5.0	29.6 ± 6.9
24	25.2 ± 4.7	28.6 ± 6.3	33.8 ± 7.6	35.0 ± 5.8

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