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Department of Biology

THE *SIMULIUM OCHRACEUM* AND *S.METALLICUM* SPECIES COMPLEXES IN MEXICO : IDENTIFICATION, DISTRIBUTION AND RELATION TO ONCHOCERCIASIS.

by Alice Lucy Millest

A dissertation submitted for the degree of Doctor of Philosophy

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Contents.

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ABSTRACT

FACULTY OF SCIENCE

BIOLOGY

Doctor of Philosophy

THE *SIMULIUM OCHRACEUM* AND *S.METALLICUM* SPECIES COMPLEXES IN MEXICO : IDENTIFICATION, DISTRIBUTION AND RELATION TO ONCHOCERCIASIS

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Both *S.ochraceum* and *S.metallicum*, which are vectors of onchocerciasis, are now known to be species complexes (Hirai *et al*, in preparation; Conn, 1988).

Larval collections were made in a variety of streams in the three onchocerciasis foci in Mexico and identified by cytotaxonomic criteria. *S.ochraceum* cytotype A was found in the Soconusco focus, cytotype B in the Oaxaca focus and cytotype C in the Chamula focus. Cytotype C was recorded in Mexico for the first time. *S.metallicum* cytotypes A, B, H and I were found in all three of the foci. Cytotypes B, H and I were recorded in Mexico for the first time. In addition a new cytotype, named X, was discovered in the Oaxaca focus. Attempts to analyse chromosomes of *S.ochraceum* adults were unsuccessful.

The larval head patterns and body colouration of different cytotypes of *S.metallicum* were examined and variations were found between them. Cytotypes A, B, H and I could be distinguished in the majority of cases on these morphological differences. Cytotype X however resembled cytotype B.

Cuticular hydrocarbon analysis by gas liquid chromatography, a technique to distinguish adult members of species complexes, was attempted on *S.ochraceum* and *S.metallicum* specimens. Some separation was achieved amongst *S.ochraceum* cytotypes A, B and C.

The distributions and abundances of the larvae of different members of the *S.ochraceum* and *S.metallicum* species complexes and other *Simulium* species were investigated in relation to selected environmental variables, by multivariate analyses. Different cytotypes of each vector complex were found to be associated with characteristic stream conditions.

The distribution of the different cytotypes of *S.ochraceum* and *S.metallicum* in relation to onchocerciasis is discussed.

Chapter 1.

Introduction.

1.1 General introduction.

Onchocerciasis is a disease caused by a parasitic worm, Onchocerca volvulus (Leuckart)(Nematoda, Onchocercidae), and it is transmitted by blackflies of the genus Simulium (Diptera, Simuliidae). With the exceptions of parasites indistinguishable from O.volvulus found in a spider monkey (Caballero and Barrera, 1958) and in a gorilla (Berghe et al, 1964), man is the only known natural reservoir of this disease. To date the disease has been found in sub-Saharan Africa, the Yemen, Guatemala, Mexico, Venezuela, Colombia, Brazil and Ecuador. Worldwide some 17.5 million people are estimated to be infected (WHO, 1987). High incidences of the disease are found near river valleys where the blackfly vectors breed, hence the common name for onchocerciasis - river blindness.

In Africa the disease is spread by members of two species complexes, S.damnosum Theobald and S.neavei Roubaud and, rarely, by S.albivirgatum Wanson and Henrard (WHO, 1987). In the Americas the vectors are members of the S.ochraceum Walker, S.metallicum Bellardi, S.callidum (Dyar and Shannon), S.exiguum Roubaud and S.oyapockense Floch and Abonnenc species complexes with S.quianense Wise, and some other potential vectors (Shelley, 1988). The S.ochraceum and S.metallicum complexes are the main subject of this dissertation; the significance of species complexes will be discussed in section 1.5. Where species complexes are being discussed in general terms, they are simply referred to by their species names, e.g. S.ochraceum and not S.ochraceum sensu lato.

In West Africa, where there are the largest and most severe foci of onchocerciasis, the disease has had a major socioeconomic impact with the inhabitants having to desert some of the most fertile areas because of the risk of blindness. As a consequence of this the World Health Organization Onchocerciasis Control Programme (OCP) was set up in 1974 incorporating parts of seven countries in West Africa. The aim of the project is to dramatically reduce the populations of Simulium damnosum by weekly larviciding of the rivers where they breed. The Programme is intended to last for 20 years so that the reservoir of the parasite in the human population may die out, hence breaking the transmission cycle. Despite problems of reinvasion of flies from outside the control area and pesticide resistance the programme is continuing to be successful and now incorporates a much larger area than at the outset.

Although the disease does not cover such large areas in the Americas it is an important health problem in the regions where it occurs. It was first discovered in Guatemala in 1915 and in Mexico in 1923 and in these two countries the disease foci are well known and appear to be stable. However, the disease was only discovered in some of the South American foci relatively recently and there is evidence that it is spreading (Anon, 1986; Guderian et al, 1988).

1.2 The organisms.

1.2.1 The life cycle of Onchocerca volvulus.

The parasite enters the human host as a third stage infective larva (L3) by penetrating through the blackfly mouthparts into the skin when the vector takes a blood meal. The larva makes its way through the superficial skin layers and is then free to move and develop in the subcutaneous layer of the human host. The parasite can

either live freely or in characteristic nodules, fibrous capsules formed around the nematode by the human host. These nodules normally contain one or more pairs of worms, the mature males averaging 2-5 cm in length and 0.02 mm in diameter and the females measuring 50-70 cm in length and 0.04-0.06 mm in diameter. Nodules generally appear 1 to 3 years after initial infection with the parasite (WHO, 1985).

The females are sexually active for an average of 8-12 years and during this time they can give birth to 500,000-1,000,000 live embryos each year. These microfilariae measure approximately 330 μm and may have a lifespan of up to 3 years. They migrate into the dermis, eyes and other parts of the body and are picked up by the vector during blood feeding (WHO, 1985).

Once inside the vector, the microfilariae pass into the midgut of the fly and through the peritrophic membrane into the haemocoel. They then enter the large flight muscle, the syncytium, where they pass through two moults to become third stage larvae. This process takes a minimum of 8 days in S.ochraceum and S.metallicum (Collins et al, 1977; Collins, 1979b), but the rate of development is temperature-dependent (Takaoka et al, 1981). The larvae then re-enter the haemocoel and move to the head of the fly where they become infective.

1.2.2 The life cycles of S.ochraceum and S.metallicum.

S.ochraceum deposit their eggs on floating vegetation while hovering over streams. The eggs are laid a few at a time on different substrates. The larvae emerge within 3 to 10 days and migrate to fast-flowing areas where they attach to vegetation by silk threads. The larvae filter-feed and pass through four to five instars in 7 to 15 days. The mature larvae spin cocoons on leaves or twigs and pupate. The

adults emerge 4 to 6 days later and mating takes place soon after emergence. The females need to take a blood meal in order to mature their eggs. This may be taken from either humans or animals. The female passes through several gonotrophic cycles taking a blood meal for each one. The maximum longevity of S.ochraceum females has been found to be at least 62 days (Dalmat and Gibson, 1952; Dalmat, 1955).

S.metallicum has a similar life cycle to S.ochraceum. However, between 150 and 200 eggs are laid in a single mass on floating vegetation. The larvae emerge in 13 to 20 days and then take 6 to 20 days to become pupae. The adults then emerge within 4 to 10 days and the females can survive for at least 85 days (Dalmat and Gibson, 1952; Dalmat, 1955).

1.3 The disease and its treatment.

The adult nematodes live in the body tissues and become encapsulated in nodules of scar tissue which measure from 0.5 cm to 10 cm. They produce microfilariae which migrate to the skin and eyes where they cause lesions. Some of the symptoms of the disease in the skin are itching, altered pigmentation and atrophy. There may also be lymphatic involvement but the most serious consequences are ocular lesions which may lead to partial or total blindness. The severity of the disease depends upon the duration and intensity of infection and many of the symptoms may be caused by dead microfilariae (Buck, 1974).

The manifestation of the disease varies from country to country. In Africa palpable nodules mainly occur around the pelvis and in the lower part of the body. In contrast, in Mexico and Guatemala the nodules predominate in the head, with some in the chest and arms and a few around or below the pelvic area. The skin and lymphatic lesions tend to be less severe in Mexico than in Africa but a condition known

as "mal morado", a decolouration of the skin, is seen. The nodules situated in the head may be associated with a higher risk of blindness (Buck, 1974).

The disease can be diagnosed by the presence of palpable nodules but more reliably by the detection of microfilariae in the skin or eyes. The reason for this is that although many nodules lie near the surface, deep ones will be undetected. Microfilariae may be found by examining skin snips, where the amount can be quantified, or by the "Mazzotti reaction" (Mazzotti, 1948). This is initiated by administering a 50mg dose of diethylcarbamazine (DEC) to the patient and observing the subsequent reaction. The drug kills the microfilariae, and the patient produces a reaction against the dead worms. If microfilariae are present pruritus, erythema, papular eruption and oedema will be witnessed in the patient during the next 24 hours. The test is sensitive but does lead to a few false positives and negatives. It may, however, cause severe reactions in heavily-infected patients and for this reason is not always readily accepted by the population under investigation (Buck, 1974).

There are two methods of treating the disease. The first is by drug therapy to kill the adult worms or microfilariae and the second by nodulectomy - the surgical removal of nodules. The two drugs which have most commonly been used for therapy are DEC and suramin. The former killing the microfilariae and the latter the adults. However, both drugs are hazardous, particularly if given to patients with a high level of infection, and should be used in a controlled environment. This is often impossible when the drugs are administered to large numbers of people in the rural areas where the disease occurs. Suramin is also a toxic drug and can be fatal in a small number of cases. Due to the severe reactions initiated by these drugs they are often refused by the inhabitants.

A new drug, ivermectin, is currently undergoing clinical trials in Africa and Central America. This drug leads to the rapid disappearance of microfilariae from the skin but a slower removal of microfilariae from the eye. As ivermectin reduces the microfilarial load in the eye more slowly than DEC, it causes less severe reactions after the drug is given. DEC can actually cause eye lesions by the sudden death of large numbers of microfilariae there. Ivermectin does not kill the adult worms but it destroys the embryonic microfilariae, leading to a suppressive effect on the release of microfilariae, probably lasting several months. This effect also reduces the amount of transmission in the community as there are no microfilariae in the skin for the vectors to take up (WHO, 1987).

The alternative to drug therapy is nodulectomy. This is a simple surgical procedure to remove superficial nodules and can be carried out in rural areas. The disadvantage of this technique is that it does not reduce the microfilariae already present in the skin or the nodules buried deeper in the body wall.

1.4 Onchocerciasis in Mexico.

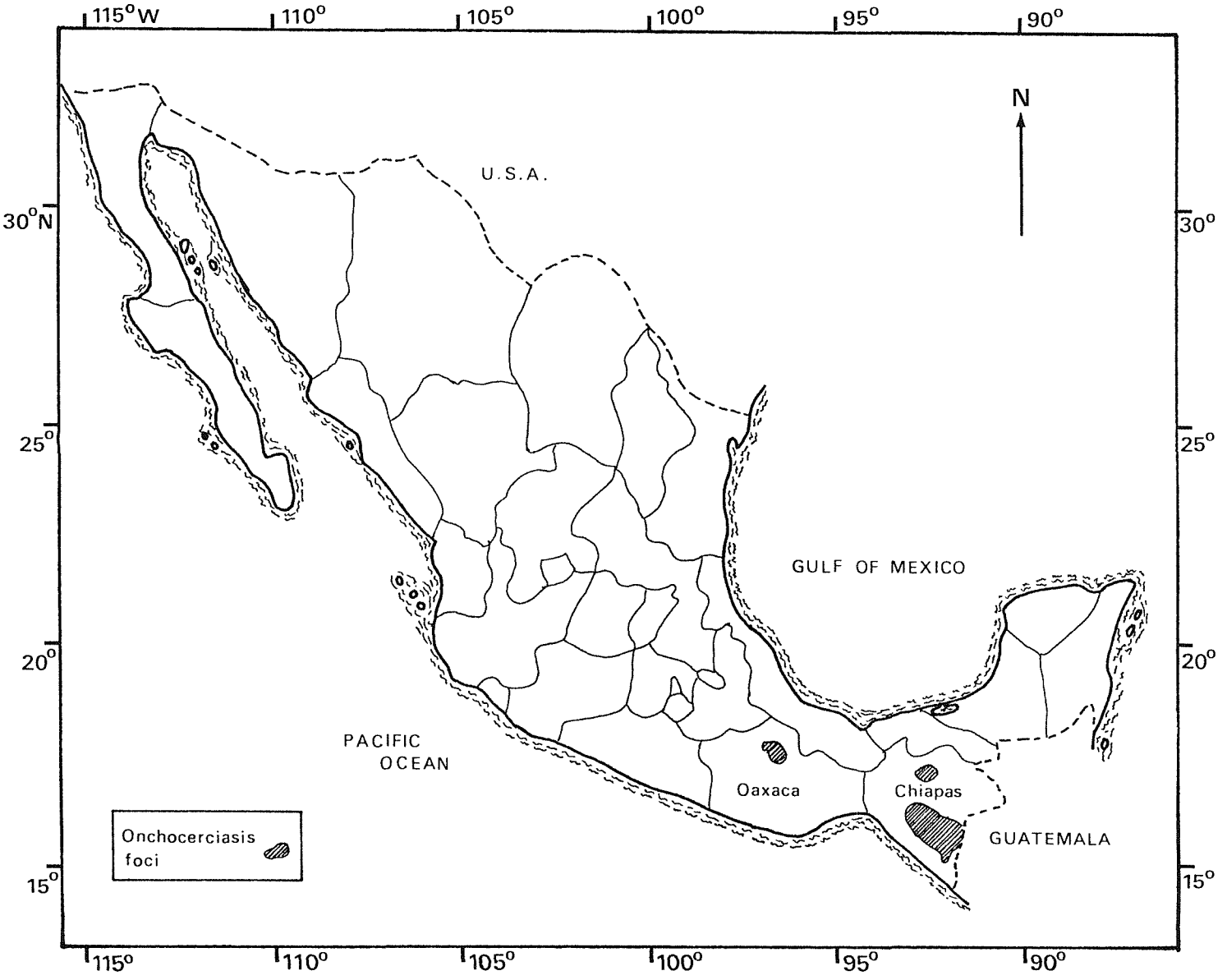
1.4.1 The foci.

The first case of onchocerciasis was identified from Mexico by Fullerborn (1923). There are three distinct foci of the disease (see Figure 1.1) :

(i) the south Chiapas or Soconusco focus
(ii) the north Chiapas or Chamula focus
(iii) the Oaxaca focus.

The following information on the foci is taken mainly from García Sánchez and Chávez Núñez (1962), Chávez Núñez (1963a), Davies (1968) and Martínez Reynoso (1979a) :

Figure 1.1. The three onchocerciasis foci in Mexico.



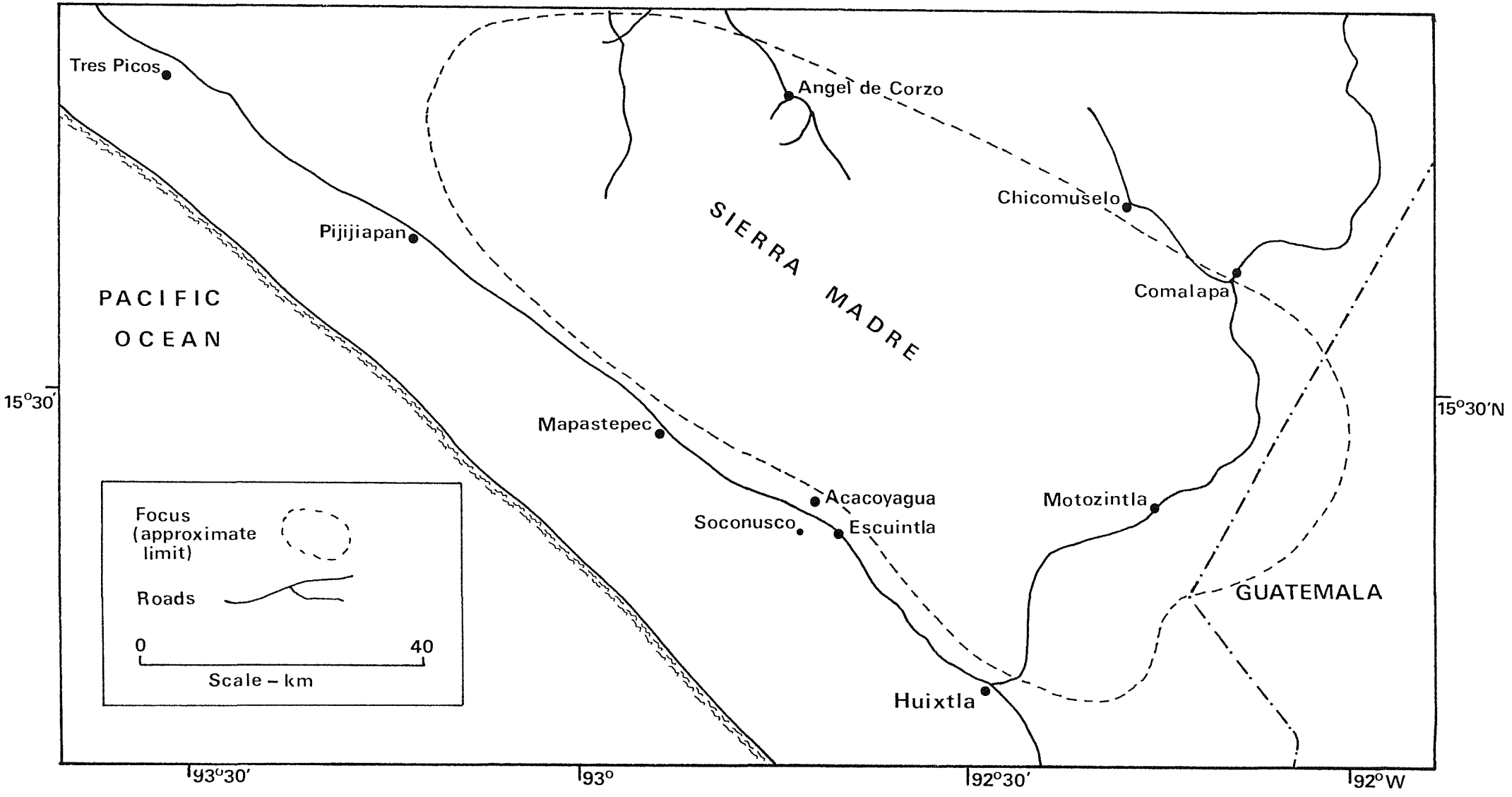
(i) The Soconusco focus (see Figure 1.2).

This focus is located on both sides of the Sierra Madre del Sur from the Guatemalan border to Pijijiapan. It is the largest of the three foci and is continuous with the Huehuetenango focus in Guatemala. The approximate area of the focus is 6,800 square km (García Sánchez and Chávez Núñez, 1962). The Pacific side of the mountain range consists of fifteen valleys separated by altitudes of 1600m to 2400m and each valley is divided by small ridges. The north side consists of ten valleys which are also divided into ridges. The slopes are steep and there is an abundance of small streams.

There is luxuriant natural vegetation and the climate is subtropical, warm and humid. The rainfall is abundant and is heaviest in the rainy season between June and September. Much of the original vegetation has now been removed as the area is ideal for growing coffee. Paulat (1979) has stated that the change from the high forest vegetation to cultivation of coffee has created a microclimate ideal for the Simulium vectors to breed. The human population consists of mestizos, people of mixed Spanish and Indian blood, whose primary occupation is growing coffee. On the lower slopes maize and beans are grown and on the higher slopes, up to 2000m, coffee is the main crop. As well as the resident population, 40,000 to 50,000 migrant workers arrive every year from other parts of Chiapas and from Guatemala to harvest coffee.

The principal zone where onchocerciasis transmission occurs is between 500m and 1200m, where the vectors are abundant. No transmission occurs above 1800m. The prevalence of the disease is however not uniform in this area. The highest levels of disease are at present found in the municipalities of Escuintla, Huixtla, Acacoyagua, La Concordia, Mapastepec and Pijijiapan. The disease has only

Figure 1.2. The Soconusco (south Chiapas) onchocerciasis focus.



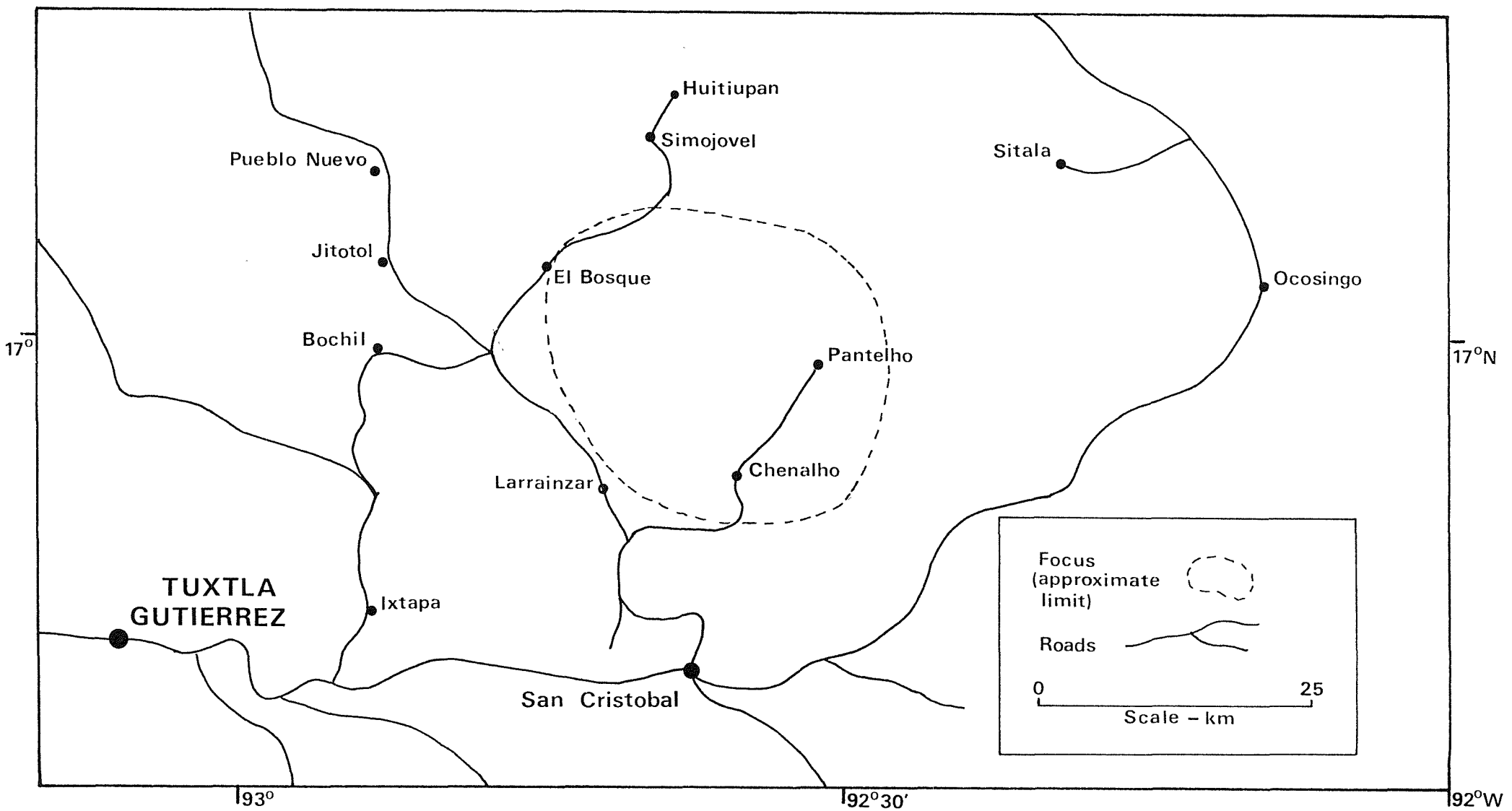
spread to the latter two areas in the last 40 years with the westward movement of people from hyperendemic zones. Formerly these areas were largely uninhabited and now it is possible that the focus could move further west. The disease focus is prevented from spreading to the north or south by areas where transmission is inadequate due to climatic barriers. To the east the focus combines with that in Guatemala. The area to the south-east of the focus is not yet affected but spread of the disease is a possibility as there is an abundance of vectors present.

(ii) The Chamula focus (see Figure 1.3).

This is the smallest focus, having an area of approximately 700km2, and is situated 60km north-east of Tuxtla Gutierrez. It is a mountainous region but the slopes are more gentle than in the other two foci. The area is surrounded by geographical barriers. To the south there is the mountain range and plateau of San Cristobál de Las Casas at 2400m. The plateau of Pueblo Nuevo is to the west at 2000m. The low valley of Rio Tacotalpa lies to the north of Simojovel. Although the region to the east of Pantelho is superficially similar to the focus, there are few streams and so spread of the disease is unlikely.

The Chamula focus is the most heavily cultivated and very little natural vegetation remains. The inhabitants, who are Tzetzil and Tzotzil Indians, grow maize and rear cattle and sheep. Coffee is also grown on a small scale and the climate is similar to the Soconusco focus. Every year there is a migration of workers, often accompanied by their families, to the south focus where they help to harvest the coffee. The Chamula focus is believed to have been established in the 1930s as a result of migrant workers contracting the disease in Soconusco and carrying it back to this area. In this region there is an abundance of vectors which could transmit the disease. Foci of

Figure 1.3. The Chamula (north Chiapas) onchocerciasis focus.



onchocerciasis have not been established in other areas of the state by migrant workers because the chief vector of the disease has a limited distribution.

The main prevalence of the disease in the north focus is currently in the municipalities of Pantelho and Chenalho, although the levels are much lower than in the other two foci.

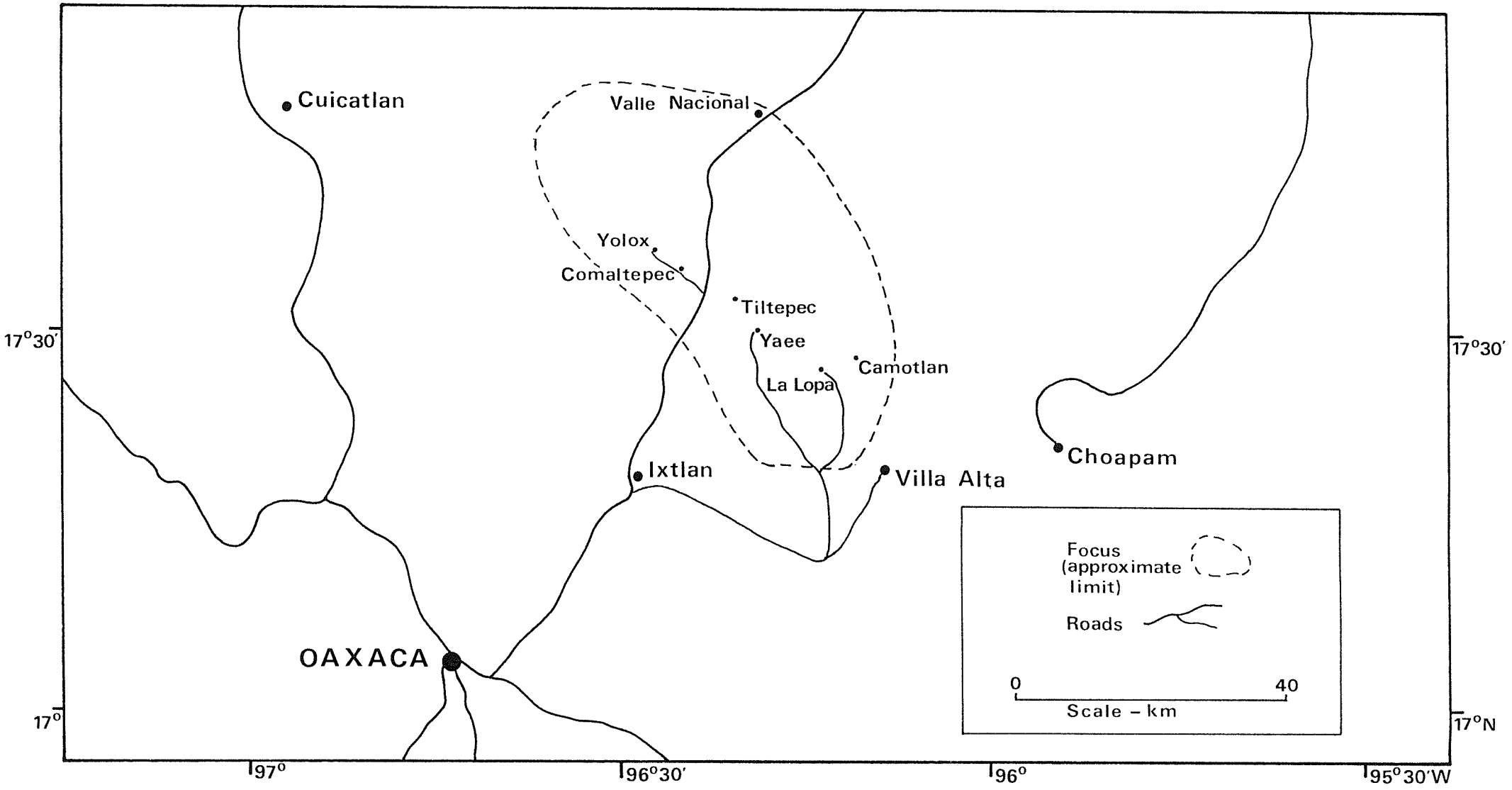
(iii) The Oaxaca focus (see Figure 1.4).

This focus lies 60km to the north-east of Oaxaca city and has an area of approximately 1400km2. The area consists of a U-shaped range of mountains with a ridge between 2,400m and 3000m. The focus is limited to the south by very high mountains and to the north by the low plains of Veracruz. It is possible that the disease could move east to Choapam but it is not known whether vectors occur in sufficient quantities there and the area is largely uninhabited. The disease has moved slightly to the west over the last few decades to the district of Cuicatlan but the ecological conditions in this area are not very favourable for the vectors.

The mountain slopes are very steep and there is dense deciduous forest on the upper slopes. The vegetation on the lower slopes approaches tropical rain forest and relatively small areas have been cleared for cultivation. The climate in the onchocerciasis zone is similar to the other two foci and the area is predominantly inhabited by Chinateco, Zapateco and Mazateco Indians. The crops grown are maize, beans and coffee.

The main prevalence of the disease is now in the municipalities of San Juan Yaee, San Pedro Yolox, Santiago La Lopa, Santiago Comaltepec and Santiago Camotlan. Paulat (1979) states that there has been movement away from areas where high transmission occurs, which is similar to the

Figure 1.4. The Oaxaca onchocerciasis focus.



situation found in Africa. This is the case in Tiltepec, which was one of the first areas where the disease was discovered, where the numbers of inhabitants have declined and coffee cultivation has been abandoned.

1.4.2. The history of the health campaigns to control onchocerciasis.

After the discovery of the disease in Mexico in the 1920s, "la Campaña contra la Oncocercosis" (the Campaign) was started in 1930 in Chiapas and in 1932 in Oaxaca. A preliminary survey was made of the distribution of the disease and nodulectomies were performed, along with various drug treatments including injection of mercury bichloride. The treatments were administered by special brigades who visited the communities.

In 1941, the brigades in the Soconusco focus visited 958 centres of population and examined 160,837 people for nodules and symptoms of disease. Of these 24,384 were infected and 14,040 had palpable nodules. A total of 26,742 nodules were removed that year (Martínez Báez, 1962). In 1946 and 1947 trials were made of suramin and DEC and from 1948 DEC was employed on a large scale. It was found that one year after treatment with DEC most of the patients were carrying nodules again. In 1951 there was an intensive campaign to administer DEC to everyone to test for the Mazzotti reaction and then continue the dosage to patients with a positive reaction. However, this only led to about 60% effective treatment, a major problem being the reluctance of the people to take the drug due to severe secondary reactions (Ortiz Mariotte, 1963). The brigades visit the most heavily infected communities every six months, although there have been interruptions in this treatment. In 1963 it was estimated that 93,000 people lived in the onchocerciasis zone in Soconusco and

approximately 30,000 of these had the disease, seventy-two being blind (Chávez Núñez, 1963a).

In the Oaxaca focus treatments with DEC and nodulectomy have also been performed every six months, with some interruptions, since 1948. In 1963 it was estimated that 5,800 of the 45,000 people in the disease zone had the illness, eighty-five of whom were blind mainly due to their reluctance to be treated (Chávez Núñez, 1963a). A similar Campaign has been carried out in the Chamula focus and in 1963 it was estimated that 4,000 out of a population of 22,500 were infected (Chávez Núñez, 1963a).

The treatments between 1932 and 1962 led to the following:
1. a reduction in the number and size of the nodules
2. the cutaneous lesions almost disappeared
3. blindness was halted.

In 1978, 67.4% of the population in the endemic areas of the three foci were examined and it was concluded that the number of people with the disease had diminished from 1962 until 1972 and then remained stationary (Martínez Reynoso, 1979a). In the Soconusco focus, 83,938 people (59.8%) were examined out of a total population of 140,380 in the endemic area and 15,628 (11.13%) of these were found to have onchocerciasis. In the Chamula focus, 11,277 people (66.6%) were examined out of a total population of 16,943 in the endemic area and only 199 (1.17%) of these were found to have the disease. In the Oaxaca focus 45,044 people (88.8%) were examined out of a total population in the endemic area of 50,754 and 4,029 (7.94%) of these were found to have the disease.

The number of infected people who accepted treatment varied between foci in 1978 (Martínez Reynoso, 1979a). In the Soconusco focus 83.2% of infected people accepted treatment, compared with 71.9% in the Chamula focus and 64.8% in the Oaxaca focus. Although the total numbers of

people with the disease had not diminished over the preceding seven years, the number of nodules detected was less. In 1974, 1,654 nodules were surgically removed from patients in the Oaxaca focus, whereas in 1978 the number removed had fallen to 779.

The October 1987 figures from the Campaign in Oaxaca estimated that there were approximately 1500 people suffering from onchocerciasis out of a population of 40,000 in the endemic area (S.Torres, pers.comm.).

In 1987 Chávez Núñez (1987) stated that the Campaign then consisted of forty brigades who visited 1,020 endemic localities in the three foci. The same treatments were given and there were 21,673 registered cases, only half as many as occurred 25 years before. There had been no new cases of blindness, although 101 people were still registered as blind due to onchocerciasis. The infections were less severe giving rise to fewer nodules and reactions to DEC were milder. Also skin biopsies were producing less microfilariae. However it was still necessary to continue the treatments on a regular basis or the level of disease would again rise. He also emphasized the importance of ivermectin as a possible replacement for DEC and the desirability of vector control.

Salazar Mallén (1977) stated that the number of people with the disease is greatly underestimated in Mexico. Figures on prevalence of the disease are often based on presence of palpable nodules, which are now smaller and encountered less frequently due to the intensive nodulectomy campaign. Skin snips are not always used as a diagnostic technique and a single snip may not be positive in a person with low infection. The figures only reflect the number of people who present themselves for examination. Martínez Reynoso (1979a) reached similar conclusions and also pointed out that, unless records are kept carefully, people who receive treatment may be counted more than once in the same year

and those who have become negative for onchocerciasis may remain registered as infected.

An ivermectin trial was started in 1987 in the Soconusco focus in Chiapas.

1.4.3 Incrimination of S.ochraceum as the primary vector.

Since the discovery of onchocerciasis in Mexico and Guatemala there have been many investigations to establish the vectors of the disease. Hoffmann (1930, 1931) in Mexico and Strong et al (1934) in Guatemala were some of the first researchers to incriminate the vector species. They showed that development of the parasite took place in S.ochraceum, S.metallicum and S.callidum and these were probably the most important species involved.

Another important work was that of Dalmat (1955) on the blackflies of Guatemala. He noted that the distribution of the disease was more similar to that of S.ochraceum than to those of S.metallicum and S.callidum. Although S.ochraceum occurred both inside and outside the disease area, it was only found in large numbers within the foci. Despite the fact that 65% of all the blackflies caught biting man were S.metallicum and this species had a greater natural infection with filarial parasites than S.ochraceum, Dalmat suspected that the latter was a better vector. This was because S.ochraceum was found to be largely anthropophilic whereas S.metallicum and S.callidum were found to be principally zoophilic and therefore many developing filarial parasites, which are indistinguishable from O.volvulus, were likely to be of animal origin. S.ochraceum proved to be an aggressive, persistent biter on humans but the other two species could be easily disturbed. S.ochraceum also tended to bite above the waist where there was the highest level of microfilariae while S.metallicum and S.callidum preferentially bit below the waist. Dalmat

concluded that S.ochraceum was the most important vector and transmission principally took place in the dry season when its biting numbers were high. At this time there was a large human reservoir of the parasite as the people were in the fields harvesting coffee.

Gibson and Dalmat (1952) noted that in the Huehuetenango focus of Guatemala there were areas within the focus where S.ochraceum was completely absent. Here the main man-biting species was S.veracruzanum Vargas, Martínez and Díaz and in other areas S.exiguum (probably S.gonzalezi Vargas and Díaz) and S.haematopotum Malloch were the dominant man-biters. All these species were shown to support O.volvulus. Davies (1974) stated that in certain areas S.gonzalezi and S.haematopotum may sometimes outnumber other species biting in Mexico.

Dalmat and Gibson (1952) and Dalmat (1955) also carried out experiments on the longevity and dispersal of the suspected vectors. They found that S.ochraceum, S.metallicum and S.callidum were capable of surviving at least 62 days in the wild and could travel a distance of at least 10 km from the release site. For a species to be a good vector it is important that its life is sufficiently long to allow the development of the parasite to an infective stage and also that it has the ability to disperse so that it can find blood meals and spread the parasite.

De Leon and Duke (1966) investigated the abilities of S.ochraceum, S.metallicum and S.callidum to take up and support the parasite. They found that S.ochraceum took up very high numbers of microfilariae suggesting that the microfilariae might actually be attracted towards the fly as well as being passively ingested. Although S.ochraceum ingested large numbers of microfilariae only a small percentage of these managed to cross the midgut wall and only rarely did the presence of high numbers of microfilariae lead to death of the fly. Those microfilariae

which succeeded in crossing the midgut wall developed synchronously to infective larvae in 7 to 8 days. S.metallicum was found to take up less larvae but a much higher percentage crossed into the thorax causing death of the fly if the initial uptake had been large. Larvae were found to develop to the infective stage in this species but in smaller numbers than in S.ochraceum. Many larvae developing in S.metallicum were found to be stunted or malformed and development was asynchronous. S.callidum was found to be able to survive larger uptakes of microfilariae than S.metallicum and development took place synchronously in 7 to 8 days.

Similar results were found by Bain et al (1974), Omar and Garms (1975, 1977), Collins (1979b) and Ito et al (1980). Omar and Garms (1975, 1977) noted that only 2.6% of microfilariae which S.ochraceum took up managed to cross into the thorax, the remainder died and disintegrated in the stomach. This species was found to have a very well-developed cibarial armature in the buccopharyngeal apparatus which inflicted lethal damage on the majority of the microfilariae. This cibarial armature was lacking in S.metallicum and, consequently, if numerous microfilariae were taken up during a blood meal many penetrated the stomach wall and were found in various parts of the body often killing the fly.

Garms (1975) looked at the natural infection of vector species in Guatemala. He found thoracic infections in S.ochraceum, S.metallicum, S.callidum and S.gonzalezi but there was only a good correlation between rates of infection in S.ochraceum and the occurrence of the disease in the human population. S.metallicum was found to contain the infective stage of a different filarial species and also contained low rates of first and second stage larvae both inside and outside the focus. It is therefore probable that many infections seen in S.metallicum were of animal origin. Up to 15% of parous S.ochraceum were infected with

O.volvulus but none of these infections occurred outside the disease foci.

Collins (1979a) found that S.ochraceum was the predominant biting species throughout the year at the sites where he worked in Guatemala. He concluded that in order to maintain transmission it was necessary for S.ochraceum to bite in large numbers as the natural infection rate of this species is low despite it being the best vector. Ochoa (1982) found S.ochraceum to be the predominant man-biter at two sites in Guatemala with S.metallicum being the most common at the other. However S.ochraceum was the only species to be found harbouring the infective stage of O.volvulus and although S.metallicum and S.callidum were found to contain first and second stage larvae it was not known whether they were of human origin. Hashiguchi et al (1982) caught over 8,000 flies which were attracted to cow baits in Guatemala. 80% of the attracted flies were S.metallicum and 8% S.callidum but no S.ochraceum were captured although they were common in these areas.

From these and other studies (e.g. Garms and Ochoa, 1979; Ortega and Oliver, 1984, 1985) it is apparent that S.ochraceum is the most important vector of onchocerciasis in Mexico and Guatemala. This is due to its distribution in the foci, its large biting populations, its marked anthropophilic preferences, its habit of biting above the waist and its ability to ingest large numbers of microfilariae without damage to itself, and so allow a proportion of them to develop synchronously to infective larvae. S.metallicum and S.callidum have been found to be secondary vectors, although the former is more important due to its wide distribution and great abundance. S.veracruzanum, S.haematopotum and S.gonzalezi are seen as potential vectors in limited areas.

1.4.4. The history of vector control campaigns.

In 1931, at the same time as the health Campaign was started, an anti-larval service was begun. This was an erratic procedure and consisted of twice monthly removal of vegetation from streams in coffee plantations (fincas) and small settlements to reduce the number of larval attachment sites. However, in 1935 and 1936 Parra Sevilla and Díaz Nájera showed that this had little effect on S.ochraceum populations and experimented with various plant extracts and chemicals to kill the larvae. In 1937 creosote emulsion was recommended as a larvicide and this was administered every 20 days by the locals who were supervised by the Campaign. However in 1940 this stopped due to opposition by the finca owners and some officials. In 1949 gamexane was used and in 1952 DDT was tried for the first time. (Ortiz Mariotte, 1963).

Between November 1954 and December 1958 a pilot scheme using DDT was carried out in the Rio Despoblado Valley between Huixtla, Motozintla and Comaltitlan. Approximately 500 streams in an area of 180 km2 between the altitudes of 700m and 1200m were treated every 15 days with DDT. It was chosen as a cheap and easily transportable pesticide and applied as a 50% wettable powder at 5g/1000l of water. Treatment points were every 300m to 500m in the streams. After two applications there were few eggs, larvae or pupae and the numbers remained low after 2 months without further treatment. However, although the numbers of larvae were reduced the numbers of adult flies were still elevated after larviciding. It was not known whether they were coming from outside the treatment area or from streams within the area which were inaccessible or considered too large to support the vectors. In 1957 the treatment area was extended to the south and east to try to prevent flies invading the central area. After 2 years there was a notable diminution of flies in the central area and after 3 years the numbers of flies were lower at the edges of the

area too. This suggests that flies had been invading from outside the area (Chávez Núñez, 1963b).

In 1966 a routine larviciding scheme began again in the same area. DDT was applied three times a month to streams and fogging was also carried out with a 25% DDT emulsion every 15 days. This was discontinued after 13 months but there is a lack of data on the effect it had on larval and adult populations (Davies, 1968). In 1965 there was a control scheme in Oaxaca using DDT but again there is a lack of information available on its effect on Simulium populations. In 1968 Davies made recommendations for control schemes in the onchocerciasis foci in Mexico. This resulted in a further control programme in Chiapas from 1969 to 1977 but the results are not well documented (Martínez Reynoso, 1979b).

In 1983 Gaugler et al experimented with the bacterium, Bacillus thuringiensis israelensis (B.t.i.), and the nematode, Steinernema feltiae, as larvicides. B.t.i. was found to be highly toxic but only carried for a short distance in the streams. S.feltiae was found to be ineffective as a control agent.

Simulium control programmes pose many problems in Mexico due to the nature of the terrain and the breeding habits of the vectors. The land in the endemic areas is steep, rugged and for the most part inaccessible by vehicle. S.ochraceum, the principal vector, develops in water ranging from minute trickles to medium-sized streams. The larger streams are easier to locate and treat as insecticide carry is better. The small trickles may be completely hidden by vegetation and only flow at certain times of the year. Even if they are discovered the length of insecticide carry is short necessitating several treatment points. The number of streams may be as many as 15,000 even in the smallest focus (Davies, 1974). The majority of these streams would have to

be visited on foot and therefore large numbers of workers would be needed to implement control.

1.5 The identification of Simulium sibling species and its relevance to the understanding and control of onchocerciasis.

1.5.1 The importance of Simulium species complexes.

Mayr (1942) defined sibling species as "morphologically similar or identical species which are reproductively isolated". A group of these species is known as a species complex. Many insect vectors, such as Anopheles gambiae Giles (Diptera : Culicidae), and A.maculipennis Meigen have proved to be species complexes (White, 1980). Although the different members of the complex may be identical, or virtually identical, in appearance, they may be very different ecologically and have different feeding preferences and vectorial capacities. A variety of techniques have been used to separate individual members of species complexes. These include cytotaxonomy, enzyme electrophoresis, morphometrics, cuticular hydrocarbon analysis and most recently DNA probes. Townson et al (1987) and Rothfels (1987) have recently reviewed the use of some of these methods in distinguishing members of simuliid species complexes. For this study cytotaxonomic and cuticular hydrocarbon techniques were used to separate or try to separate different siblings of the S.ochraceum and S.metallicum complexes. These techniques and their significance are discussed in the following sections.

1.5.2 The basis of cytotaxonomy.

The Diptera are particularly suited to cytotaxonomic study as some of their tissues contain giant chromosomes. These polytene chromosomes may be stained to reveal a series of chromatic and achromatic bands whose width and appearance are consistent amongst members of the same species. It is therefore possible to map the positions of these bands for the entire chromosome complement and use it as a key to identify species.

Rothfels (1956) showed that differences in the chromosome pattern of Simulium larvae could distinguish between biologically distinct sibling species that were considered morphologically identical. Chromosomal rearrangements are involved in nearly all speciation events in the Simuliidae and therefore polytene banding analysis is a valuable technique in distinguishing sibling species (Rothfels, 1980). Members of species complexes which are separable by their chromosomes and occur sympatrically are known as cytospecies. When distinct populations only occur allopatrically it is often impossible to confirm whether they are true species and they are therefore known as cytotypes (Rothfels, 1987).

In Simulium species the best polytene chromosomes are found in the larval salivary glands. In this tissue the three pairs of homologous chromosomes are intimately synapsed and appear as three entities, the longest known as chromosome I and the shortest as chromosome III. The chromosomes have median or submedian centromeres and each of the six arms may be recognized by their relative lengths and inclusion of landmarks, such as a characteristic puff known as the Ring of Balbiani. For the purposes of cytotaxonomy the chromosomes are divided sequentially into 100 sections, with section 1 at the beginning of the short arm of chromosome I (Is) and section 100 at the end of the long arm of chromosome III (IIIl). The break points of

inversions can therefore be recorded by their section limits.

Homozygous inversions will appear as reversed banding sequences and heterozygous inversions as loops. For populations to be considered as distinct from each other they must contain differences in one or more of the following : (1) interspecific (fixed) autosomal inversions, (2) X and/or Y chromosome inversions, or (3) the sets of intraspecic (polymorphic) inversions. Populations can normally be distinguished from each other by the presence or absence of certain interspecific and/or sex-linked inversions. However, in rare cases such as S.ornatipes Skuse, where there are no fixed or sex-linked differences between siblings, only the sets of polymorphic inversions can be used to separate them (Bedo 1979a, 1979b). When these siblings occur sympatrically, inversions will display heterozygote deficiency and linkage disequilibria will occur between inversions. These differences can be detected by divergences from the expected Hardy-Weinberg predictions, which assume random assortment in the absence of selection. The probability of ratios diverging from the Hardy-Weinberg equilibrium can be estimated by the chi-square test.

Sex in Simulium species is determined by the Y-chromosome which is a locus that may occur on any of the six arms. Its position may shift from one region to another in different members of a species complex (Rothfels, 1980).

1.5.3. The application of cytotaxonomy in the S.damnosum species complex.

Cytotaxonomy has been used to identify the different members of the S.damnosum species complex which is responsible for transmitting onchocerciasis in West Africa. This is the most widely studied of the simuliid vector species complexes and was first discovered by chromosomal

analysis in 1966 (Dunbar, 1966). Subsequently eight cytotypes were formally described and named by Vajime and Dunbar (1975). S.damnosum has a wide distribution over east and west Africa and to date 34 forms have been described from this region (Meredith, 1987), as well as a cytospecies which has been described recently from the Yemen (Garms et al, 1988).

Of the eight cytospecies originally described six occur within the main endemic onchocerciasis zone of West Africa. These are S.damnosum s.s., S.sirbanum Vajime and Dunbar, S.sanctipauli Vajime and Dunbar, S.soubrense Vajime and Dunbar, S.squamosum Vajime and Dunbar and S.yahense Vajime and Dunbar. They have differing ecologies and abilities to transmit the disease. The clinical manifestations of onchocerciasis in West Africa can be split into savanna and forest forms. The savanna form is the more serious due to the predominance of severe eye lesions and is mainly transmitted by S.damnosum s.s. and S.sirbanum. The milder forest form of the disease is transmitted principally by the remaining four (WHO, 1985).

It is important to understand the relative roles of these cytospecies in the transmission of onchocerciasis to enable the OCP to be run effectively. Since this programme began, the major problems have been reinvasion of flies from outside the control area and the development of resistance to the larvicide temephos, in some areas. Cytotaxonomy has been used to ascertain which cytospecies were causing these problems. The majority of reinvading flies were found to be the savanna species, S.damnosum s.s. and S.sirbanum, with some S.squamosum (Garms et al, 1979; Cheke and Garms, 1983). As the first two of these species are the most important vectors of the disease, their control was essential if they were not to jeopardize the success of the programme. The control area was therefore extended to encompass the breeding sites of the reinvading flies.

Insecticide resistance was first detected in 1980 in S.soubrense and S.sanctipauli (Guillet et al, 1980). These are less important vectors and hence the consequences of their reduced susceptibility to insecticides are less severe. However, resistance now occurs in many of the cytospecies including S.damnosum s.s. and S.sirbanum and current control strategy now requires knowledge of the cytospecies present, their resistance status and vectorial abilities before decisions on larviciding strategy are taken (Kurtak et al, 1987).

1.5.4 The S.ochraceum and S.metallicum species complexes.

S.ochraceum has been recorded from Bolivia, Ecuador, Colombia, Guatemala, Jamaica, Mexico and Panama (W.H.O., 1971), but it is only known to be a vector of onchocerciasis in Mexico and Guatemala. Hirai et al (in preparation) made a cytological analysis of this species from Mexico and Guatemala and found three cytotypes, A, B and C. Cytotype A was found both within and outside the onchocerciasis foci in Guatemala and within the Soconusco focus in Mexico. Cytotype B was found within the Oaxaca focus in Mexico and cytotype C was found outside the foci in Guatemala. Cytotype C occurred in a savanna-like area, which differed from the habitat in the foci, and was said to have a low anthropophilic tendency. From their study it is apparent that the different S.ochraceum cytotypes may have varying ecologies and biting preferences. As this species is considered to be the primary vector in these countries it is important to know whether there are differing vectorial capacities between the cytotypes in order to better understand the transmission of the disease.

S.metallicum is found in Belize, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Panama, Trinidad, Venezuela and El Salvador (W.H.O., 1971; Wilton and Collins, 1978). It is

a secondary vector of onchocerciasis in Mexico and Guatemala but the primary vector in the northern Venezuelan foci (Peñalver, 1961; Lewis and Ibáñez de Aldecoa, 1962 and Duke, 1970). The S.metallicum in Venezuela differs from the S.metallicum in Mexico and Guatemala in its stream habitat, biting preferences and vectorial capacity.

In Venezuela S.metallicum tends to breed in small streams (Lewis and Ibáñez de Aldecoa, 1962) whereas in Guatemala it tends to breed in medium to large streams (Dalmat, 1955). It is primarily anthropophilic in Venezuela and the bite is initially painless to humans whereas it is far more zoophilic in Guatemala and its bite is painful. Although in both areas S.metallicum tends to bite low on the body, it bites more consistently low in Venezuela than in Guatemala (Duke, 1970). Development of O.volvulus is asynchronous in experimental infections but few deformed and stunted larvae were seen in Venezuelan S.metallicum (Takaoka et al, 1984) compared with Guatemalan (De Léon and Duke, 1966). The natural O.volvulus infection rate is much higher in Venezuela than in Guatemala.

Cytotaxonomy has recently shown that S.metallicum is a species complex (Hirai, 1983 and 1985; Conn, 1988). When cytotaxonomic analysis was carried out on Venezuelan S.metallicum specimens, cytotypes D and E were found which are completely different from those occurring in Mexico and Guatemala (Conn, 1988). It is apparent that different S.metallicum cytotypes can have varying vectorial capacities and hence it is possible that the cytotypes occurring in Mexico may have differing abilities to transmit the parasite.

1.5.5. The basis of cuticular hydrocarbon analysis for species separations.

Insects have a protective layer of cuticular waxes which contains hydrocarbons and these may be extracted by immersing the insect in a solvent. The extract can then be run through a gas liquid chromatograph to give a peak profile of the soluble cuticular constituents. The area under each peak is proportional to the amount of that constituent in the extract. Lockey (1978) succeeded in discriminating between two closely-related species of tenebrionid beetles by differences in their cuticular hydrocarbon profiles.

Cuticular hydrocarbon analysis by gas liquid chromatography was first used to discriminate between members of a Simulium complex by Carlson and Walsh in 1981. They succeeded in separating adult S.sirbanum and S.squamosum, members of the S.damnosum complex, by the relative proportions of five of their cuticular hydrocarbon peaks. Subsequently, Phillips et al (1985) used a modified technique and managed to distinguish between four members of the S.damnosum complex, of which two could not previously be distinguished as adults.

The differences in hydrocarbons may be qualitative, i.e. where a particular peak occurs exclusively in one cytospecies, or quantitative, where the relative proportions of a combination of peaks is used to distinguish between cytospecies (Phillips and Milligan, 1986). This technique has also been used to separate other insect vectors, such as members of the Anopheles culicifacies Giles species complex and females of the Psychodopygus squamiventris series. (Milligan et al, 1986; Ryan et al, 1986).

One of the advantages of cuticular hydrocarbon analysis is that it can be used on Simulium adults, whereas

cytotaxonomy is largely confined to Simulium larvae. Phillips et al (1985) have also succeeded in obtaining cuticular hydrocarbon traces from Simulium larvae and pupae, where the profiles were identical to the adults but the peaks had reduced areas. Another advantage of this technique is that desiccated specimens can be used and reasonable traces have been obtained from museum specimens collected in the 1930s (Phillips and Milligan, 1986).

1.6 Aims.

There has been considerable research involving the vectors of onchocerciasis in Mexico and Guatemala (see section 1.3.3) but until recently, they had always been considered as discrete species. S.metallicum has been shown to be a species complex with members that have differing abilities to transmit onchocerciasis. Similarly, S.ochraceum has been shown to be a species complex, but it is not known whether the different members have varying vectorial capacities. It is important to investigate the distribution and vectorial capacities of members of these complexes so that the epidemiology of the disease may be better understood and control programmes may be planned effectively, as is the case with the S.damnosum complex in Africa.

The initial research on these vector complexes principally involved collections from outside Mexico and therefore little was known of the complexes occurring inside Mexico. The first aim of this study was cytotaxonomic: to identify the members of the S.ochraceum and S.metallicum species complexes occurring in Mexico, using larval cytotaxonomy, and to determine the distributions of the siblings within the different onchocerciasis foci (Chapter 2).

One of the problems of cytotaxonomy is that it can only be performed on the larvae in the majority of Simulium species. The biting females, therefore, cannot be directly

identified. This is a major difficulty when trying to assess the vectorial capacities of the different cytotypes. The second aim was to investigate the possibility of using polytene chromosomes from adult S.ochraceum (Chapter 3).

Before S.metallicum specimens were examined cytotaxonomically, morphological differences had already been found among members of this species. Okazawa and Onishi (1980) had divided it into two species based principally on the head patterns and body colouration of the larvae. The third aim was to examine these characters in S.metallicum larvae in relation to cytotaxonomic identifications in order to establish whether there were morphological differences between the cytotypes. (Chapter 4).

Cuticular hydrocarbon analysis is a method which has been used to distinguish between adult members of some vector complexes (see section 1.5.5). It can therefore be used to establish the identity of biting females. This technique had not been attempted on members of the S.ochraceum and S.metallicum species complexes. The fourth aim was therefore to investigate the potential of this method to discriminate between members of these species complexes (Chapter 5).

There are a large number of environmental factors which may affect the distribution of Simulium larvae and these have been reviewed by Ross and Merritt (1987). Different members of species complexes have also been found to be associated with different stream characteristics (Adler, 1987). An extensive study of the factors associated with the distribution of Guatemalan Simulium species was made by Dalmat (1955), but he assumed all the species to be discrete and not members of species complexes. The fifth aim was to ascertain whether the different cytospecies of S.ochraceum and S.metallicum were associated with different stream characteristics (Chapter 6).

It is outside the scope of this study to directly assess the vector efficiency of the different cytotypes, but it was possible to make a comparison between the geographical distribution of the cytotypes and available epidemiological information to see if any tentative relationships could be found. The sixth and final aim was therefore to examine the relationship between the distribution of members of the Simulium species complexes and onchocerciasis in Mexico (Chapter 7).

Chapter 2.

Distribution of members of the S.ochraceum and S.metallicum species complexes using larval cytotaxonomy as a distinguishing technique.

2.1 Introduction.

Previous studies of the S.ochraceum and S.metallicum species complexes have been mainly based on specimens collected outside Mexico. Hirai and Uemoto (1983) first found evidence that S.ochraceum was a species complex by analysis of polytene chromosomes from Guatemalan specimens. Subsequently, Hirai et al (in preparation) have described three cytotypes, A, B and C from Guatemala and Mexico. The cytology of the S.metallicum complex was first studied by Mantel (1982) using samples from Colombia and Costa Rica where two distinct populations were found. Hirai (1983) divided S.metallicum into cytotypes A and B based on the position of the nucleolar organizer in specimens from Guatemala. Subsequently specimens from Colombia, Costa Rica, Guatemala, Mexico, Panama and Venezuela have been examined and divided into eleven cytotypes (Conn, 1988). Conn found cytotypes A, B, H and I in Guatemala and cytotype A in Mexico, all of which were considered to be distinct cytospecies.

This chapter describes the results of studies to ascertain which cytotypes were present in each of the Mexican onchocerciasis foci, for the reasons already discussed in section 1.5.

2.2 Materials and methods.

2.2.1 Collection of specimens.

Larval collections were made :

(i) In the Soconusco focus in June, July and August 1985 and in January, February and October 1987. Additionally, samples were collected in September and October 1985 and January 1986 by Mexican collaborators [see Appendix I(i) for locations of collection sites].

(ii) In the Chamula focus in February and November 1987 [see Appendix I(ii) for locations of collection sites].

(iii) In the Oaxaca focus in October 1987 [see Appendix I(iii) for locations of collection sites].

Collections were made in stream sites which varied ecologically to investigate whether the different cytotypes occurred under different environmental conditions. The ecological parameters recorded are described in Chapter 6. A 20 to 30 minute search of each stream was made for larvae and pupae. The larvae were found on pieces of vegetation and stones and placed immediately in cold, freshly-mixed Carnoy's fixative (3 parts absolute ethanol : 1 part glacial acetic acid). The fixative was changed once after 2 minutes and then again after 24 hours. Tubes containing larvae were placed in an ice box and subsequently in a refrigerator at 4 ^{o}C for later chromosomal analysis.

Some of the pupae encountered were also placed in Carnoy's fixative but the majority were placed individually in emergence tubes, which consisted of stoppered plastic tubes with a piece of slightly damp cotton wool at the bottom. When the adults emerged they were maintained alive for at least 24 hours, to allow their cuticles to harden and then pinned in association with their pupal cases.

2.2.2 Protocol for preparing slides of larval chromosomes.

The following is a detailed protocol of the procedure used in this study for staining salivary gland chromosomes and subsequently making permanent slide preparations:

(i) Select penultimate stage larvae from the sample.
(ii) Split open the abdomens of the larvae ventrally, using fine dissecting needles and place the larvae in a petri dish of distilled water for 1 hour. Take a 10 ml glass vial with a cork stopper, fill with 1 M hydrochloric acid and place in an oven at 65 °C.

(iii) Remove the larvae from the distilled water with a pair of forceps and drag over a piece of filter paper to remove any gelatinous material which has exuded from the salivary glands.

(iv) Hydrolyse the specimens in the hot acid for 3.5 to 4 minutes.

(v) Remove the larvae from the acid and cover with Feulgen stain. Place in the dark for 1 hour.

(vi) Remove the larvae from Feulgen and place in a small vial of sulphate water (400ml distilled water : 10ml 1M HCl : 1g potassium metabisulphate). Shake well and leave for 10 minutes to wash out excess stain.

(vii) Place the larvae in tap water for at least 3 hours before dissection. Larvae may be maintained in the refrigerator in this way for 2 to 3 days.

(viii) Remove the salivary glands, with stained polytene nuclei, and the pink-stained gonads from individual larvae. Place the associated larval bodies in 80% ethanol in labelled vials for reference.

(ix) Place the salivary glands and gonads in a drop of 50% acetic acid on a clean slide and break up the glands using dissecting needles.

(x) Add a drop of lacto-propionic orcein and leave to counterstain the nuclei for 5 minutes. Wash off excess stain using more 50% acetic acid and absorb with filter paper.

(xi) Add a further drop of 50% acetic acid to the preparation and lower a coverslip onto the slide. Gently tap the coverslip using a dissecting needle and examine the slide under a high resolution light microscope. Use additional tapping to optimally spread the chromosomes. When sufficient spreading is achieved squash the coverslip flat onto the slide between layers of tissue paper. Seal the slide temporarily using rubber solution.

(xii) After examining the slides place in the freezer compartment of a refrigerator until they can be permanently fixed. To achieve this, place the slides on a metal platform over liquid nitrogen. Once the slides have been sufficiently cooled, so that the chromosome material no longer sticks to the coverslips, flip off the coverslips using the edge of a razor blade. Place The slides in absolute ethanol for 2 minutes to dehydrate the material. Place a drop of Euparal over the chromosome material and lower a new coverslip on top. Leave the slides in an oven at 65 ^{o}C to dry, before storing them for future reference.

2.2.3. Interpretation of chromosome banding patterns.

Larvae were prepared for cytotaxonomic examination using the above protocol. As the complete polytene chromosome maps for S.ochraceum and S.metallicum were not available when many of the larvae in this study were scored, the larvae were separated by the major differences between cytotypes and the polymorphic autosomal inversions were not scored. This technique was more rapid and could also be used on larvae with chromosomes of an inferior quality.

Larvae were divided into cytotypes using the features shown in the idiograms in Figures 2.1, 2.2 and 2.3, which are based on Hirai's and Conn's results and include the variations found in this study (Hirai et al, in preparation; Conn et al, in press). Some of these features are illustrated in Figure 2.4, which shows a nucleus of S.metallicum cytotype B. The chromosome arms may be distinguished from each other by various features. Chromosome arm IIs has the characteristic bands and puffs of the double bubble and Ring of Balbiani and the presence of three heavy bands near the centromere. Chromosome arm IIl has the puffed arrangement known as the parabalbiani. Chromosome arm IIIs has a "frazzled" end and contains the puffed feature known as the blister.

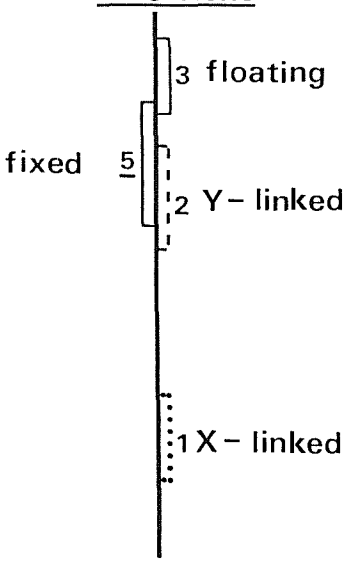
The idiograms show the positions of the fixed and sex-linked inversions which distinguish the cytotypes. The sex-linked difference in S.metallicum cytotype B occurs at the site of the nucleolar organizer, which tends to be only half expressed in males. This is indicated by the +/- label on the idiogram. In the S.ochraceum cytotypes one of the sex-linked differences is marked as 37HB. This represents a heteroband at position 37 where the band has normal expression in one member of the chromosome pair but is heavily expressed in the other (see Figure 2.5). The appearance of the centromere in chromosome I varies from standard in some of the S.metallicum cytotypes. In cytotype

Figure 2.1. Idiograms of S.metallicum cytotypes A and B.

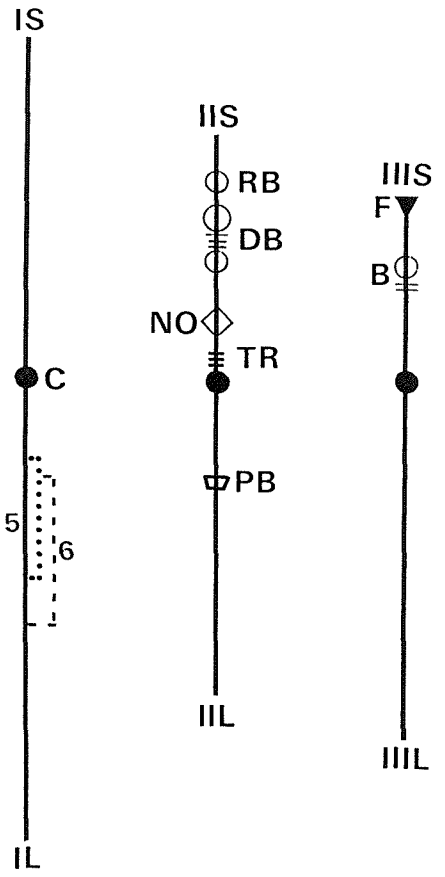
KEY

IS-IIIL - chromosome arms
C - centromere
B - blister
F - frazzle end
NO - nucleolar organizer
DB - double bubble
PB - parabalbiani
RB - ring of Balbiani

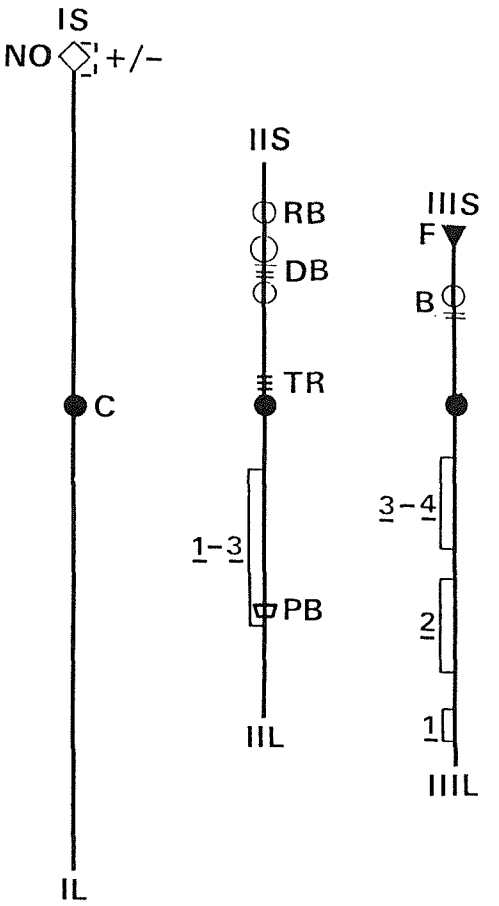
inversions:



TR - triple band



A



B

Figure 2.2. Idiograms of S.metallicum cytotypes H, I and X.

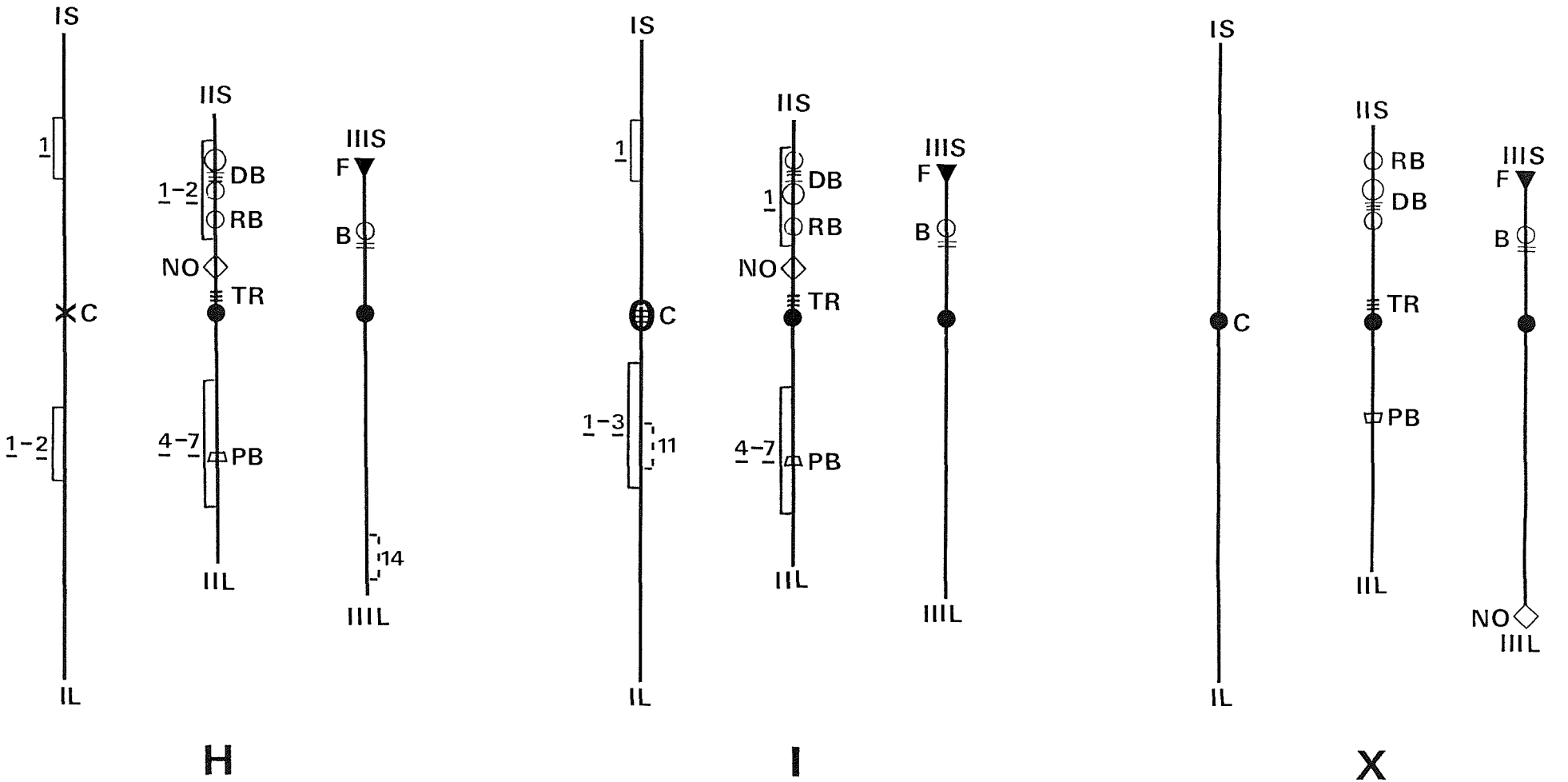


Figure 2.3. Idiograms of S.ochraceum cytotypes A, B and C.

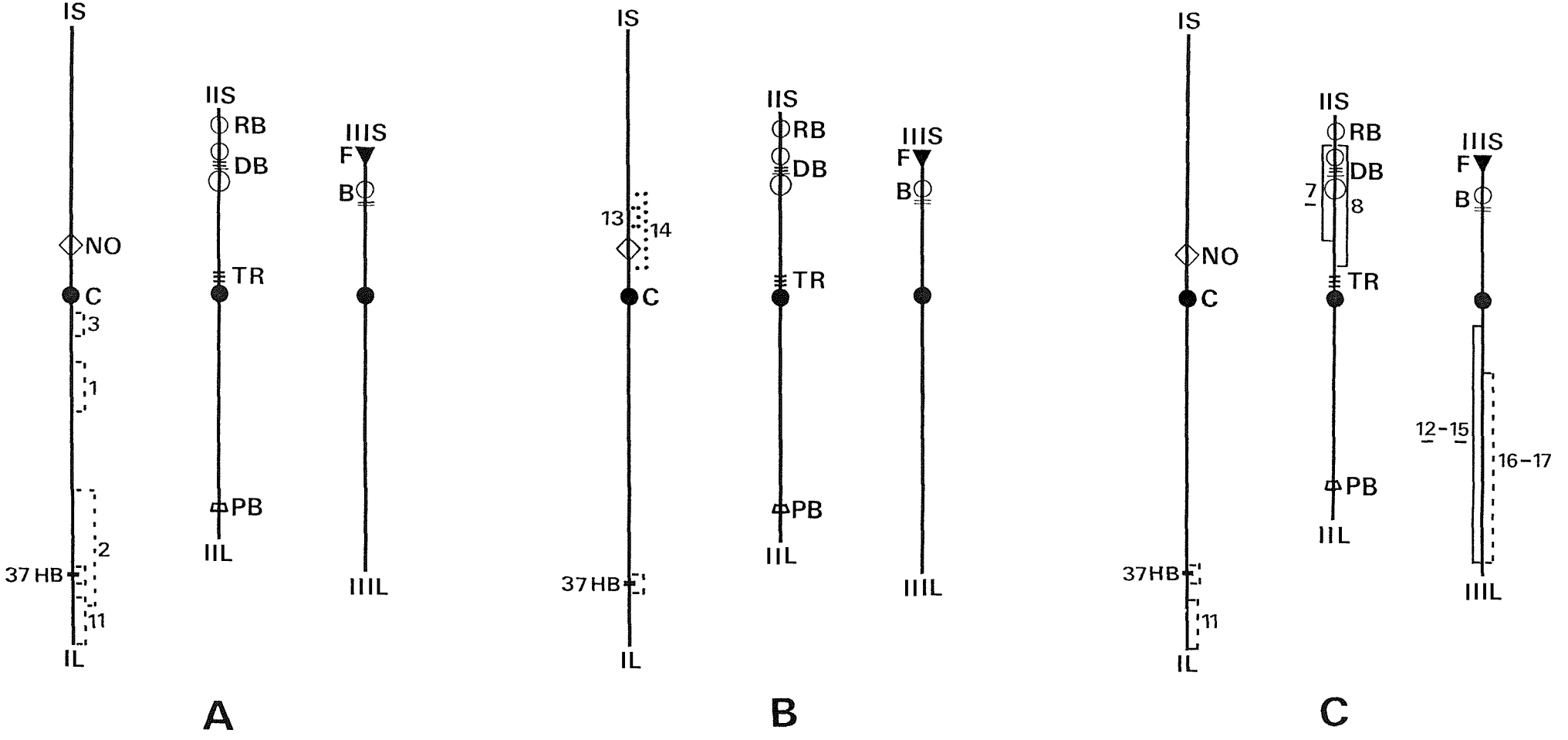


Figure 2.4. The full chromosome complement of S.metallicum cytotype B, illustrating some of the features shown on the idiogram (Figure. 2.1).

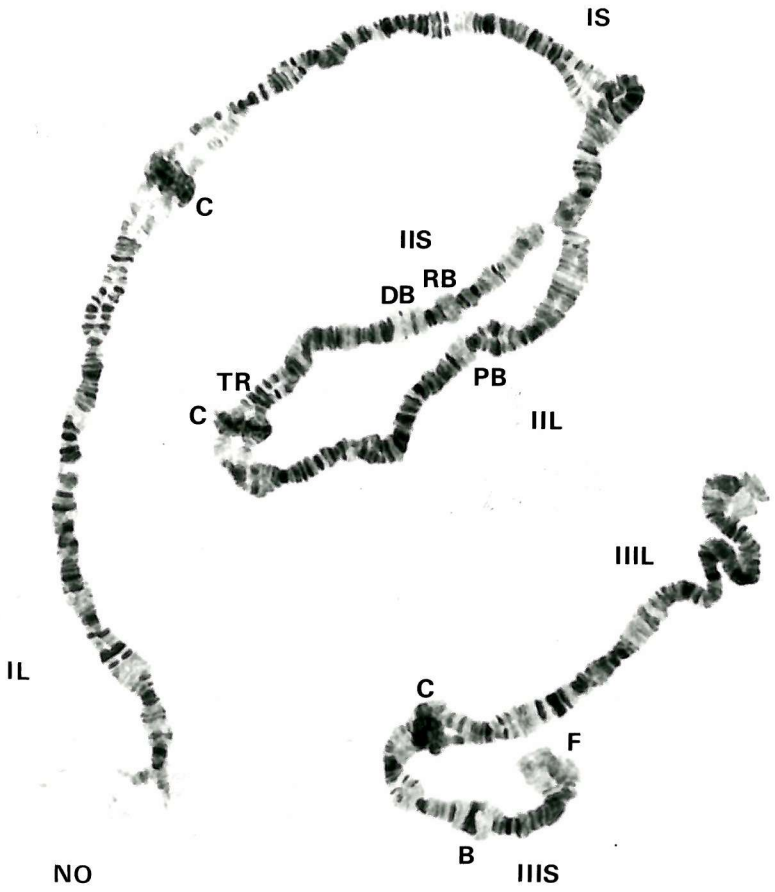
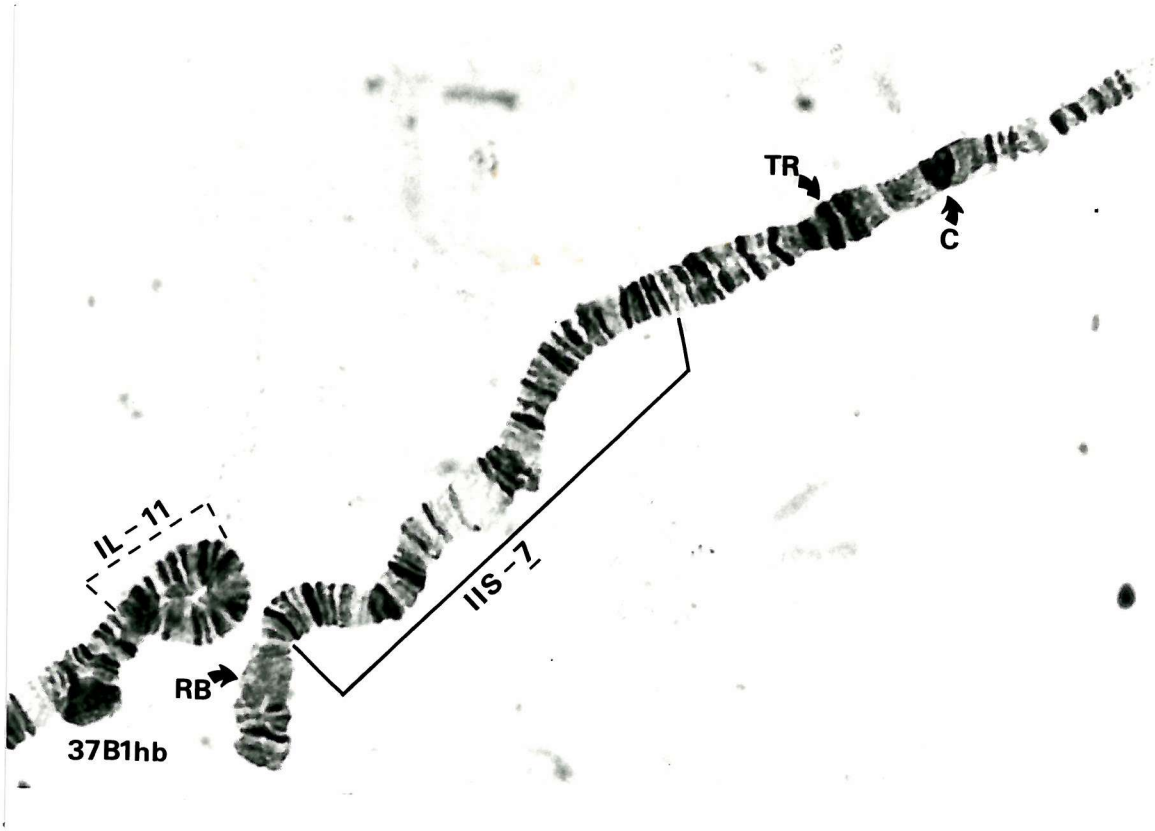


Figure 2.5. Part of the chromosome complement of S.ochraceum cytotype C showing the IIs-7 fixed inversion and the Il-11 sex-linked inversion in association with a heteroband at position 37.



H it is expanded and in cytotype I it is spindle-shaped and banded.

In addition, S.ochraceum larvae were divided into sex chromosome categories depending on the sex-linked inversions present. The frequency of the polymorphic inversion IIs-8 was also scored as it was found in conjunction with the fixed autosomal inversion IIs-7 in cytotype C (see Figure 2.3).

The frequencies of the sex-linked inversions in S.metallicum were not scored as the positions of the break points were not available when many larvae were examined. However the approximate locations of these inversions were known and corresponded to inversion loops and inverted sequences found in larvae in the present study. Once complete chromosome maps were available, sex-linked inversions were found to be in the same places in this study as Conn had found in her specimens. The entire banding sequences of some of the specimens were also checked to ensure they corresponded with Conn's maps.

2.3 Results.

A total of 529 S.ochraceum and 789 S.metallicum larvae were examined cytologically. The numbers of each cytotype identified from the three onchocerciasis foci are shown in Tables 2.1 and 2.2. Figure 2.6 summarizes the distribution of the different cytotypes of S.ochraceum and S.metallicum in each focus. The detailed results, showing the collection dates and sites, are in Appendix II for S.ochraceum and Appendix III for S.metallicum.

S.ochraceum cytotype A was found at ten sites in the Soconusco focus, cytotype B at eight sites in the Oaxaca focus and cytotype C at nine sites in the Chamula focus. No hybrids were found. Table 2.1 shows the number of specimens in each sex chromosome category for the three cytotypes. A

Table 2.1 Summary of S.ochraceum cytotaxonomic results showing the sex chromosome categories.

Soconusco Focus

S.ochraceum cytotype A Total = 394

Sex	Category	Sex-linked inversions	Numbers
Female	X0X0	none	185
Male	X0Y0	none	19
Male	X0Y2	Il-1 + Il-2 + 37HB	18
Male	X0Y4	Il-3	172

Chamula Focus

S.ochraceum cytotype C Total = 82

Sex	Category	Sex-linked inversions	Numbers
Female	X0X0	none	35
Male	X0Y1	IIIl-16,17	36
Male	X0Y2	Il-11 + 37HB	11

Oaxaca Focus

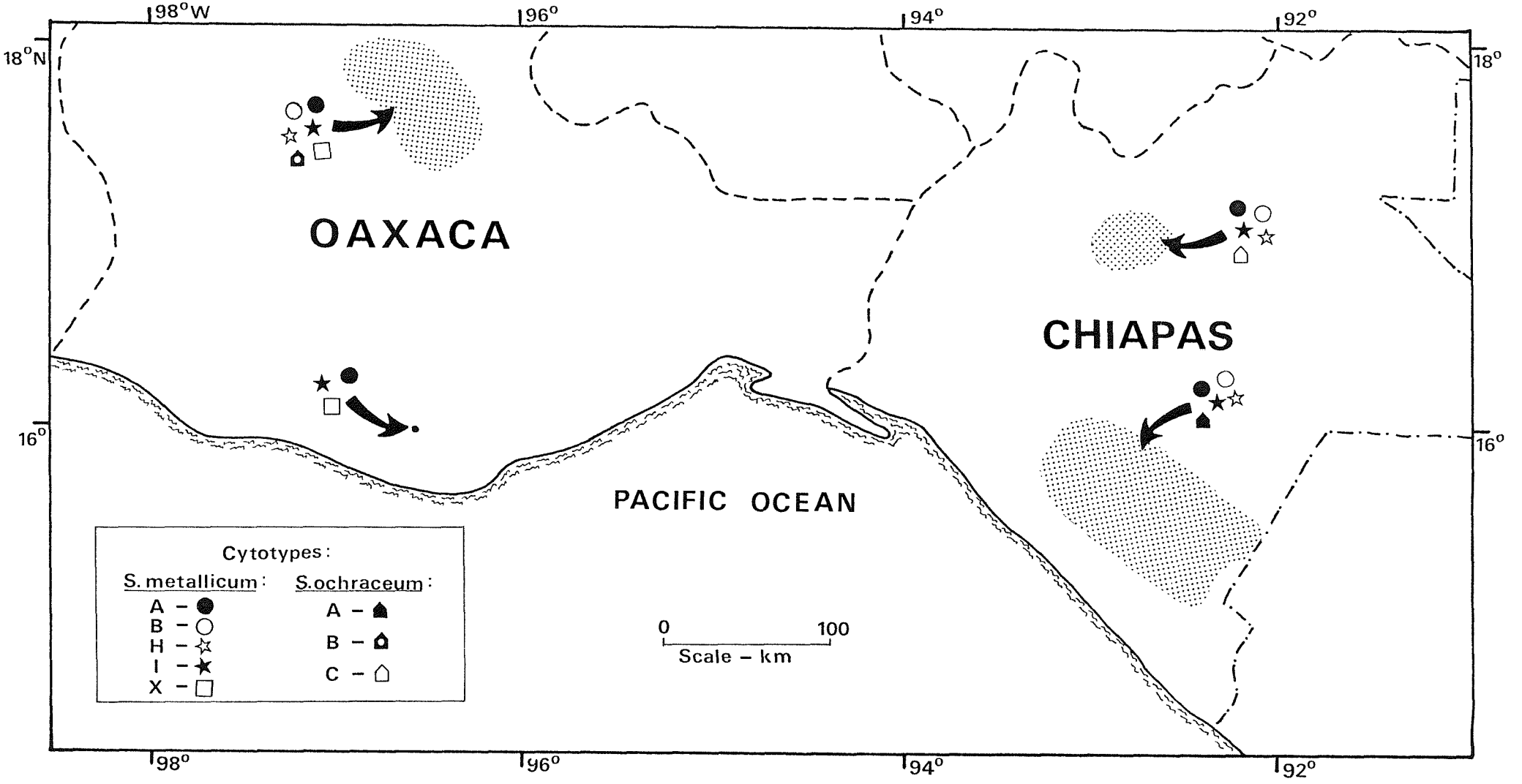
S.ochraceum cytotype B Total = 53

Sex	Category	Sex-linked inversions	Numbers
Female	X1X1	Is-13 (ii)	23
Female	X1X2	Is-13 + Is-14	2
Male	X1Y0	Is-13	25
Male	X1Y1	Is-13 (ii) + 37HB	2
Male	X2Y0	Is-14	1

Table 2.2 The numbers of different S.metallicum cytotypes identified from the three foci.

Focus	S.metallicum cytotypes :				
	A	B	H	I	X
Soconusco	263	52	37	78	0
Chamula	120	7	1	3	0
Oaxaca	41	8	5	142	32
Totals	424	67	43	223	32

Figure 2.6. Summary of the distribution of the different cytotypes of S.ochraceum and S.metallicum found in the three onchocerciasis foci and a site in south Oaxaca.



sex chromosome category occurred in cytotype C which was additional to those in Hirai et al's maps. Eleven males of cytotype C had a Il-11 heterozygous inversion in association with a heteroband at position 37 (see Figure 2.5) instead of the heterozygous sex chromosome inversions IIIl-16 and IIIl-17 (see Figure 2.3). Inversion IIs-8 was present at a frequency of 29.3% in cytotype C and four specimens of this cytotype contained B chromosomes (see Figure 2.7 and Appendix II (iv)).

S.metallicum cytotypes A, B, H and I were found in all three of the foci. Cytotypes A and I also occurred at site 54, approximately 160 km to the south of the Oaxaca focus. In addition an undescribed cytotype, labelled X, was found which did not correspond to any of the eleven described cytotypes. The most obvious difference between this and the other cytotypes was a nucleolar organizer shift to the end of IIIl (see Figure 2.8). This can be compared with Figure 2.9, which shows the nucleolar organizer in the IIs arm of cytotype A. The remainder of the inversion differences in cytotype X have not yet been fully investigated. Thirty-two of these specimens were found both within and outside the foci in Oaxaca.

No hybrids were seen between A, B, H, I and X although they frequently occurred sympatrically. Indeed at site 4d cytotypes A, B, H and I occurred simultaneously.

Sex chromosome inversions corresponded to those that Conn described, except that cytotype I males were found to be standard as well as heterozygous for Il-11.

The data in Table 2.2 do not represent a random sample, as particular larvae were selected for the study of cytotaxonomy in relation to morphology described in Chapter 4. Therefore no attempt was made to check whether or not the samples were homogenous between foci. Such an analysis

Figure 2.7. The chromosome complement of S.ochraceum cytotype C showing the IIs-7,8 inversion and a B chromosome.

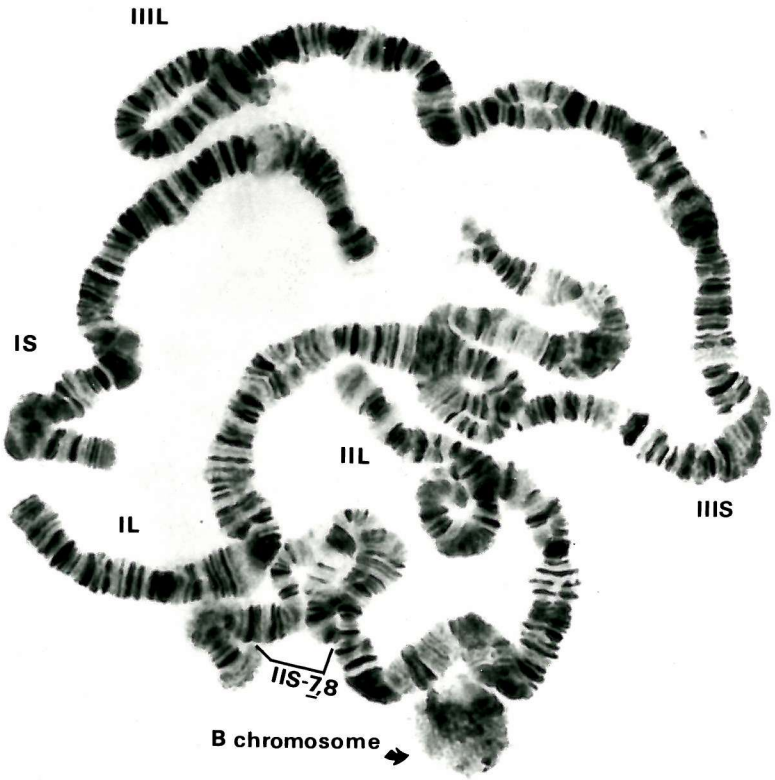


Figure 2.8. Chromosome III of S.metallicum cytotype X showing the nucleolar organizer at the end of IIIl.

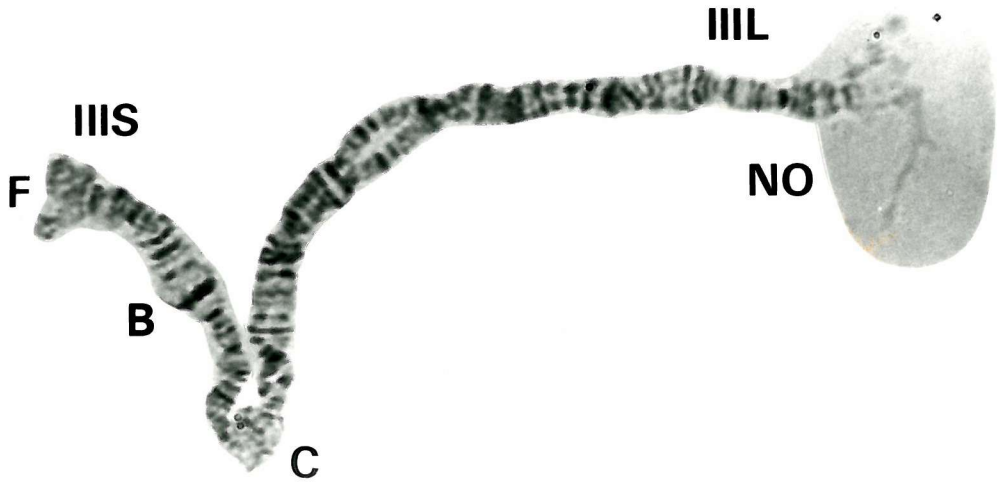
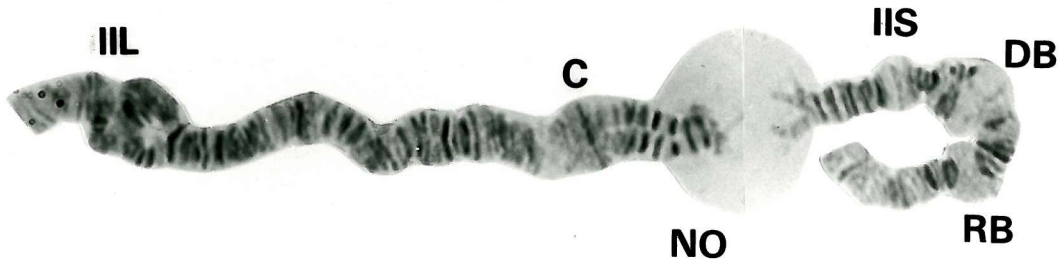


Figure 2.9. Chromosome II of S.metallicum cytotype A showing the nucleolar organizer near the centromere in IIs.



was conducted on a more comprehensive set of samples, which are unbiased, and is described in Chapter 6.

2.4 Discussion.

S.ochraceum cytotype A was found in the Soconusco focus, cytotype B in the Oaxaca focus and cytotype C in the Chamula focus. S.metallicum cytotypes A, B, H and I were all found in each of the three foci and in addition cytotypes A and I also occurred at a site 160km to the south of the Oaxaca focus. The larvae analysed corresponded to described cytotypes, except for some S.metallicum specimens found only in Oaxaca, which were named cytotype X.

Previously, Hirai et al (in preparation) also collected S.ochraceum cytotype A from one site in the Soconusco focus and cytotype B from one site in the Oaxaca focus. Larvae had not been examined cytologically from the Chamula focus before the present study, when cytotype C was found. This cytotype was described by Hirai et al from Guatemala, but was only found in an area outside the onchocerciasis foci. Cytotype A was found to occur both within and outside the foci there. In the present study, as in that of Hirai et al, none of the S.ochraceum cytotypes were found in sympatry and therefore it is difficult to assess whether they are distinct, reproductively-isolated species.

The S.ochraceum sex chromosome categories in this study corresponded to those that Hirai et al had described with the exception of some cytotype C specimens. Hirai et al found the polymorphic inversion IIs-8 to be at a frequency of 39.1%, which is similar to the 29.3% in the samples described here. In agreement with Hirai et al, B chromosomes were only found in some specimens of cytotype C and not in the other cytotypes.

As no S.ochraceum hybrids were found in the present study this adds more evidence to the case, unprovable cytotaxonomically without sympatric populations or cross-breeding experiments, that S.ochraceum from Mexico and Guatemala consists of three distinct entities which may be separate cytospecies. Other evidence, in addition to the chromosomal studies, was provided by Agatsuma (1987) who examined samples of S.ochraceum using enzyme electrophoresis. Five of his samples were from areas where cytotype A had previously been found and the remaining sample from where cytotype C occurred. There was a large genetic difference between the last sample and the former five.

S.metallicum cytotypes B, H and I were recorded for the first time in Mexico. In addition a new cytotype, named X, was found in the Oaxaca focus. All combinations of cytotypes, except for H and X, occurred simultaneously in some streams but no hybrids were seen. This is in agreement with Conn's (1988) hypothesis that cytotypes A, B, H and I are actually separate cytospecies.

Sex chromosome frequencies for S.metallicum cytotypes were not scored in this study but it was noted that some cytotype I males were standard for the inversion Il-11. Conn's nine cytotype I males from Guatemala were all heterozygous for Il-11. This may demonstrate a difference between the cytotype in Mexico and Guatemala but Conn's sample is too small to confirm this.

The significance of the distribution of the cytotypes of S.ochraceum and S.metallicum in relation to onchocerciasis will be discussed in Chapter 7.

Chapter 3.
Examination of adult polytene chromosomes as a potential method for distinguishing cytotypes.

3.1 Introduction.

One of the major problems in determining the capacity of different cytospecies to transmit onchocerciasis is the inability to obtain polytene chromosomes from the adults. Thus it has been impossible to directly incriminate cytospecies from those adults attracted to animal or human bait. It is either assumed that biting flies are the same cytospecies as larvae occurring locally or it is necessary to cytotype the larval progeny of the biting flies after they have been reared in the laboratory. However, Bedo (1976) managed to obtain readable adult chromosomes from the Malpighian tubules of some Australian species of Simulium. Subsequently, Procunier and Post (1986) obtained identifiable polytene chromosomes from a small proportion of a sample of the S.damnosum complex in Africa. Therefore the same technique was applied to S.ochraceum to find out whether suitable preparations could be obtained.

3.2 Materials and methods.

Collections of adult blackflies were made at Las Golondrinas (site 4) on 22.1.87., Morelos (site 6) on 25.1.87 and Cuauhtehmoc (site 9) on 5.2.87 (see Appendix I(i)). The catches took place in the morning using human volunteers, who had removed their shirts and rolled their trousers up to their knees, as bait. Females of S.ochraceum were allowed to feed to repletion and were then captured in individual polypropylene tubes.

The females were maintained according to the method of Figueroa et al (1977). The tubes had small perforations in

the lid to allow air flow and a 2mm circular hole made in the bottom to accommodate a tissue paper wick which was impregnated with sugar solution. A filter paper strip was lodged inside the tube as a resting surface for the fly. This strip was not permitted to touch the sugar wick. The tubes were placed horizontally in layers in plastic sandwich boxes. A layer of slightly damp cotton wool was secured to the inside of the lid of the box using aluminium foil and masking tape. This was to maintain the humidity in the box. The boxes were kept at ambient temperature unless transported to another site in which case they were placed in an ice box during the move.

Flies were sampled at 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 34, 72 and 96 hours after capture. They were immobilized using chloroform and immediately placed in a drop of isotonic saline on a microscope slide. Their abdomens were split dorsally with dissecting needles to remove any of the blood meal remaining. They were then placed in a glass tube containing freshly-mixed Carnoys fixative and stored in an ice box. The fixative was changed once after 24 hours. On return from the field they were stored in a refrigerator at 4 °C, except during transport to England.

Chromosomal preparations were made in the same manner as for the larvae (see section 2.2.2). After staining the flies with Feulgen, the Malpighian tubules, ovaries and spermathecae were dissected out and counterstained in orcein. The Malpighian tubules and nurse cells were examined for polytene chromosomes under a high resolution microscope. The stage of follicular development was scored according to Detinova's (1962) description of Anopheles maculipennis :

"Stage N - a follicle consisting of eight undifferentiated cells. The follicle is spherical and the follicular cells compose a regular cuboidal epithelium.

Stage I - one oocyte, situated in the distal portion of the follicle, is clearly visible. Above the oocyte lie seven nurse cells. The follicle either retains its spherical shape or becomes slightly oval.

Stage I/II - a crown of one or two rows of yolk granules appears round the nucleus in the oocyte protoplasm. The follicle takes on an oval shape.

Stage II - larger and more numerous yolk granules are seen in the protoplasm of the oocyte around the egg. The egg grows, becomes considerably larger than the nurse cells and takes up about half the follicle.

Stage III - the egg gradually increases its share of the follicle space from one half to three-quarters. Its nucleus is no longer visible through the mass of yolk. The follicle becomes somewhat elongated.

Stage IV - the follicle becomes longer and nurse cells occupy only the uppermost part of it. The oogonium, full of yolk, is well developed and occupies more than nine-tenths of the follicle.

Stage V - the chorion covers the whole egg. The remains of the nurse cells are found at the proximal end of the follicle. The floats appear on the egg, which is now ready for laying."

The flies were examined for the presence of follicular relicts and spermathecae were removed to establish whether sperm was present.

3.3 Results.

S.ochraceum was not found to contain polytene chromosomes in either the Malpighian tubules or the nurse cells which were in a condition suitable for cytotaxonomy. The Malpighian tubule chromosomes were more developed than those of the nurse cells. The Malpighian tubule chromosomes were either :

i) undifferentiated and appeared as dots (see Figure 3.1),
ii) partially polytenised and appeared as fine threads (see Figure 3.2),
iii) polytenised but impossible to interpret due to poor quality or inability to spread (see Figure 3.3) or
iv) degenerate (see Figure 3.4)

The results are summarized in Table 3.1. Polytenised chromosomes were found in the Malpighian tubules of S.ochraceum between 3 and 8 hours after a blood meal had been taken. After this period they degenerated. Partially polytenised chromosomes were also seen in the nurse cells.

The follicles were in stage II between 1 and 5 hours after the blood meal (see Figure 3.5). Some follicles had reached stage III 6 hours after the blood meal (see Figure 3.6) and stage IV 34 hours after the blood meal (see Figure 3.7). Flies which were dissected after 72 and 96 hours contained fully developed eggs, stage V (see Figure 3.8)

Large, deeply-stained, follicular relicts could be seen in parous flies which were killed 1 to 2 hours after a blood meal (see Figure 3.5), however these diminished in size with time and were difficult to see 8 hours after a blood meal. Sperm was also deeply-stained and was present in the spermathecae of all the flies (see Figure 3.9).

Figure 3.1. Malpighian tubule chromosomes which are undifferentiated and appear as dots.

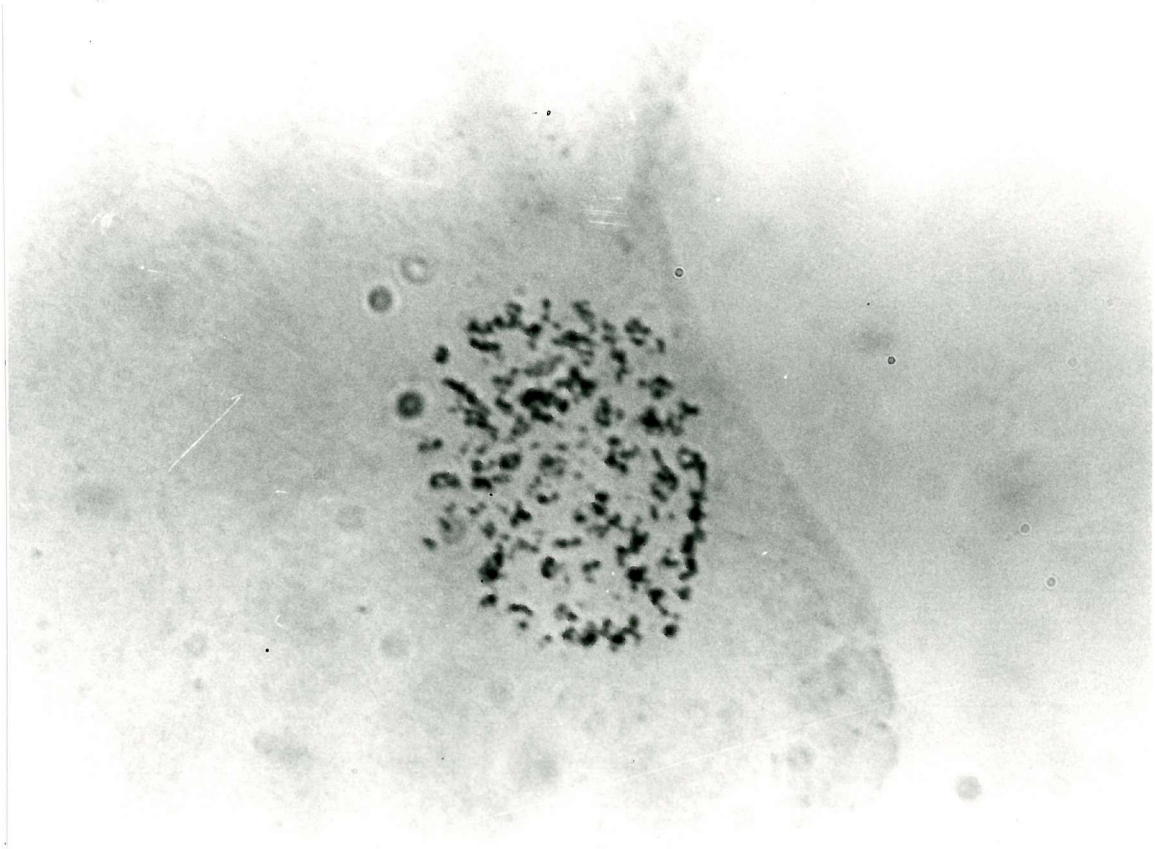


Figure 3.2. Malpighian tubule chromosomes which are partially polytenised and appear as fine threads.

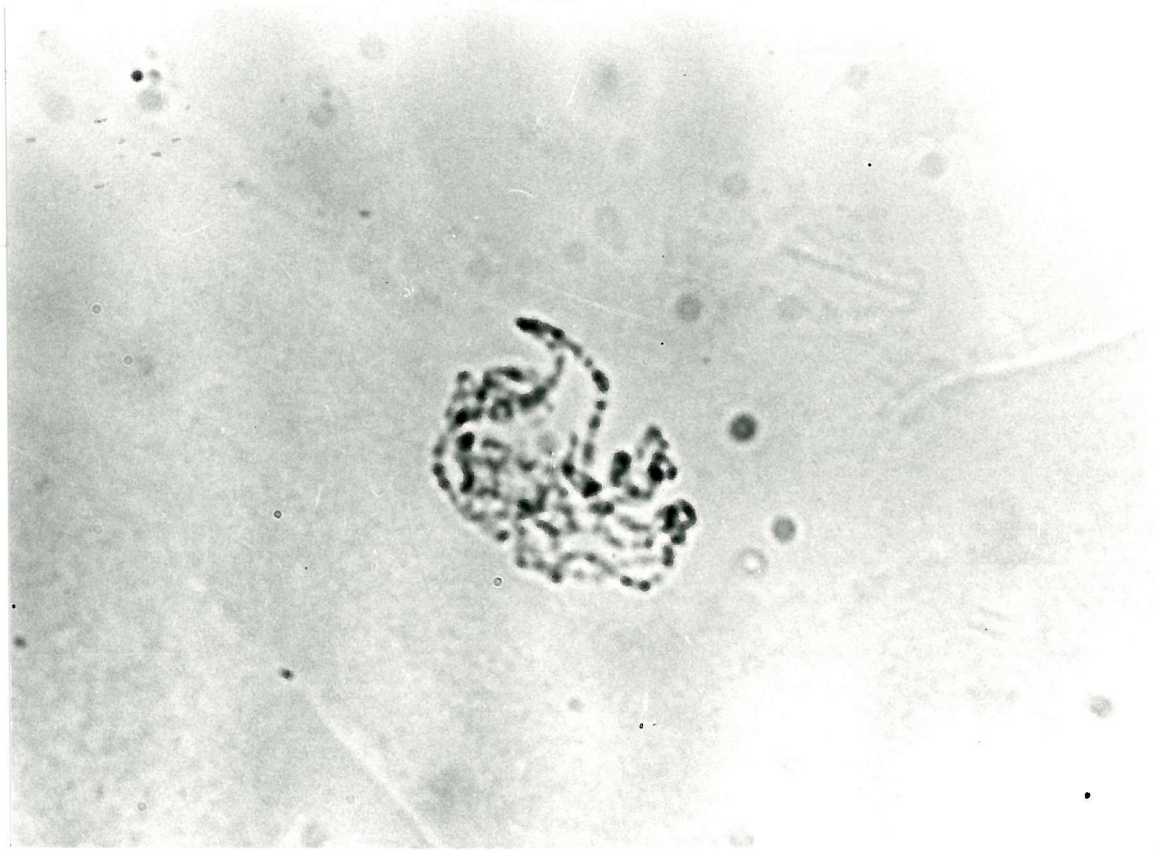


Figure 3.3. Malpighian tubule chromosomes which are polytenised but are impossible to interpret due to poor quality.

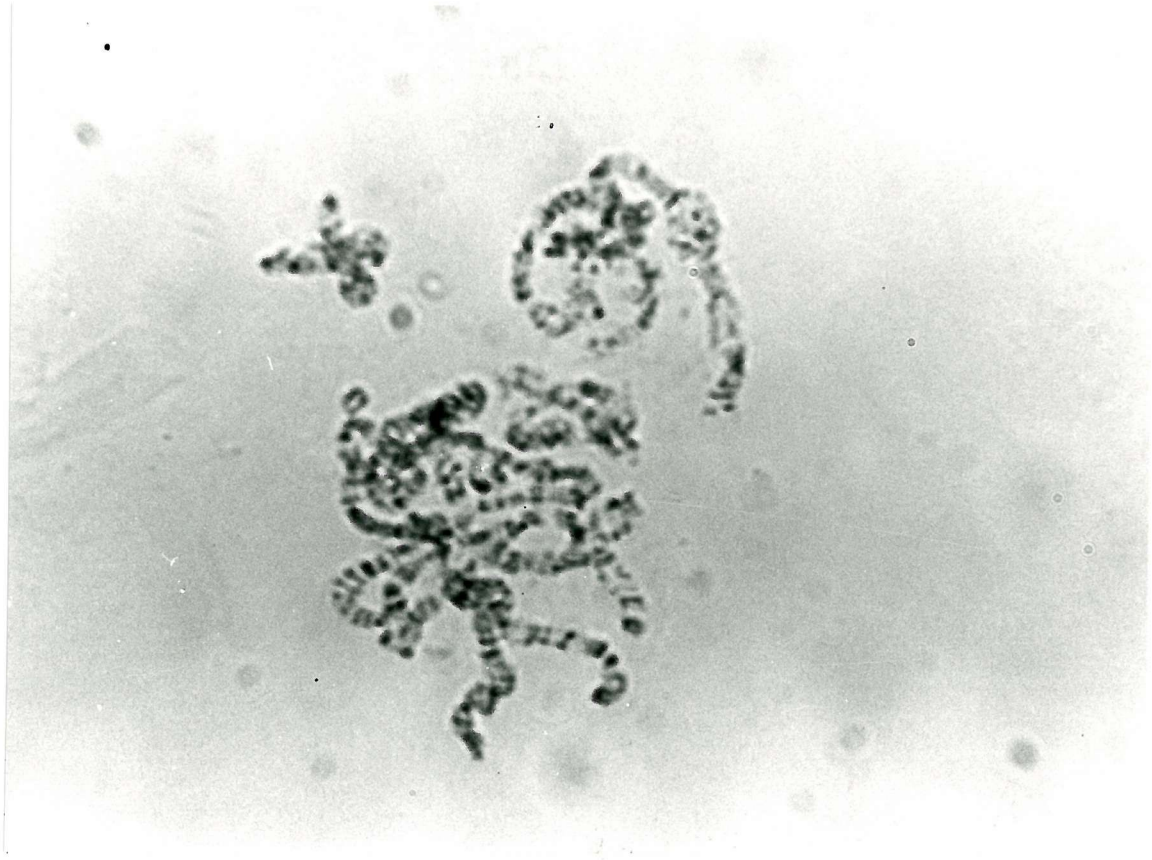


Figure 3.4. Malpighian tubule chromosomes which have degenerated.

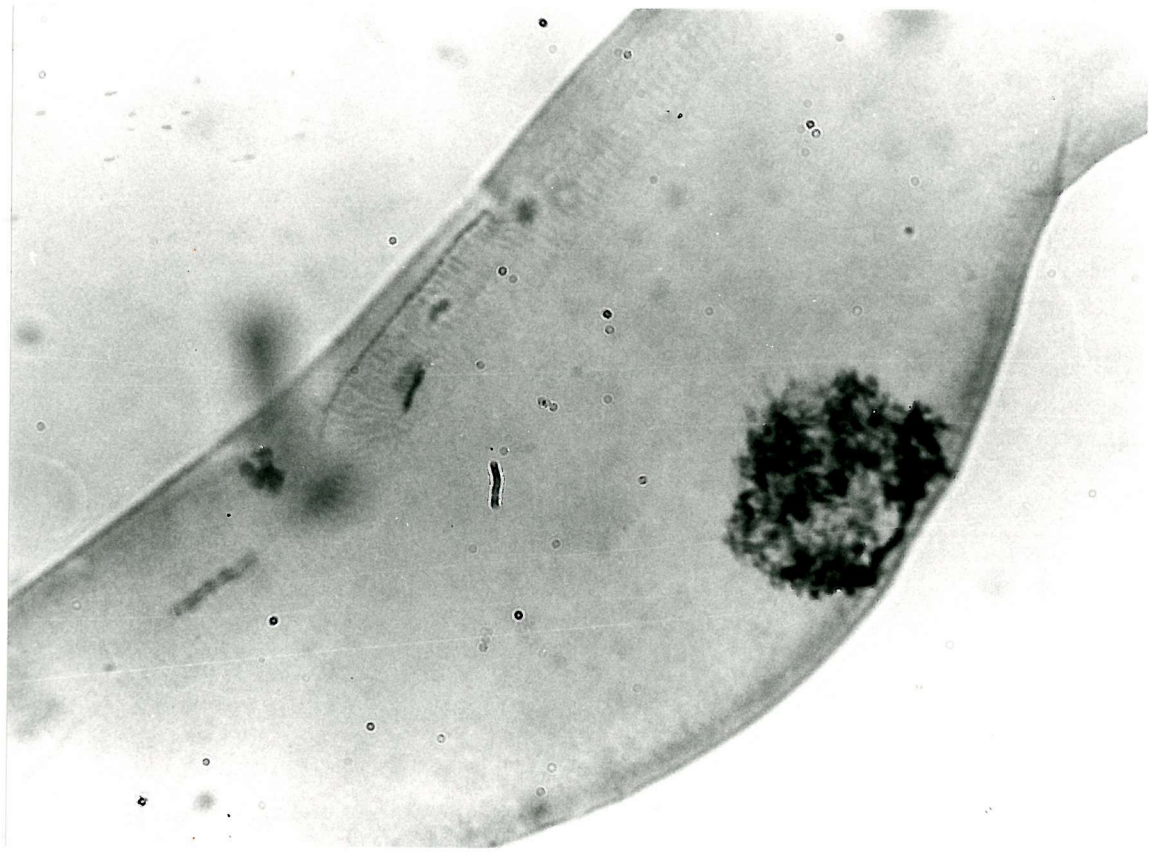


Table 3.1. The follicular stage, chromosome state and parous status of adult female S.ochraceum killed at different times after blood-feeding.

Site	Time fixed after feed (hr)	Follicle stage	Chromosome state	Number analysed	Nulliparous/ parous
6	1	II	i/ii	8	7/1
6	2	II	ii	10	7/3
4	2	II	i	9	3/6
9	2	II	ii	6	5/1
6	3	II	iii	6	2/4
4	3	II	i/ii	9	6/3
9	3	II	ii	6	6/0
6	4	II	ii	2	1/1
9	4	II	ii/iii	4	3/1
6	5	II	ii	3	3/0
4	5	II	iii	4	3/1
9	5	II	ii	6	6/0
6	6	II/III	ii	3	1/2
9	6	III	iii	4	3/1
6	7	II/III	iii	3	2/1
9	7	III	iii	3	2/1
6	8	II/III	iii	3	2/1
9	8	III	iii/iv	3	1/2
6	12	III	iv	7	-
6	24	III	iv	6	-
6	34	IV	iv	5	-
6	72	V	iv	2	-
6	96	V	iv	2	-

Figure 3.5. A stage II follicle.
(NC = nurse cell; YG = yolk granules
FR = follicular relicts)

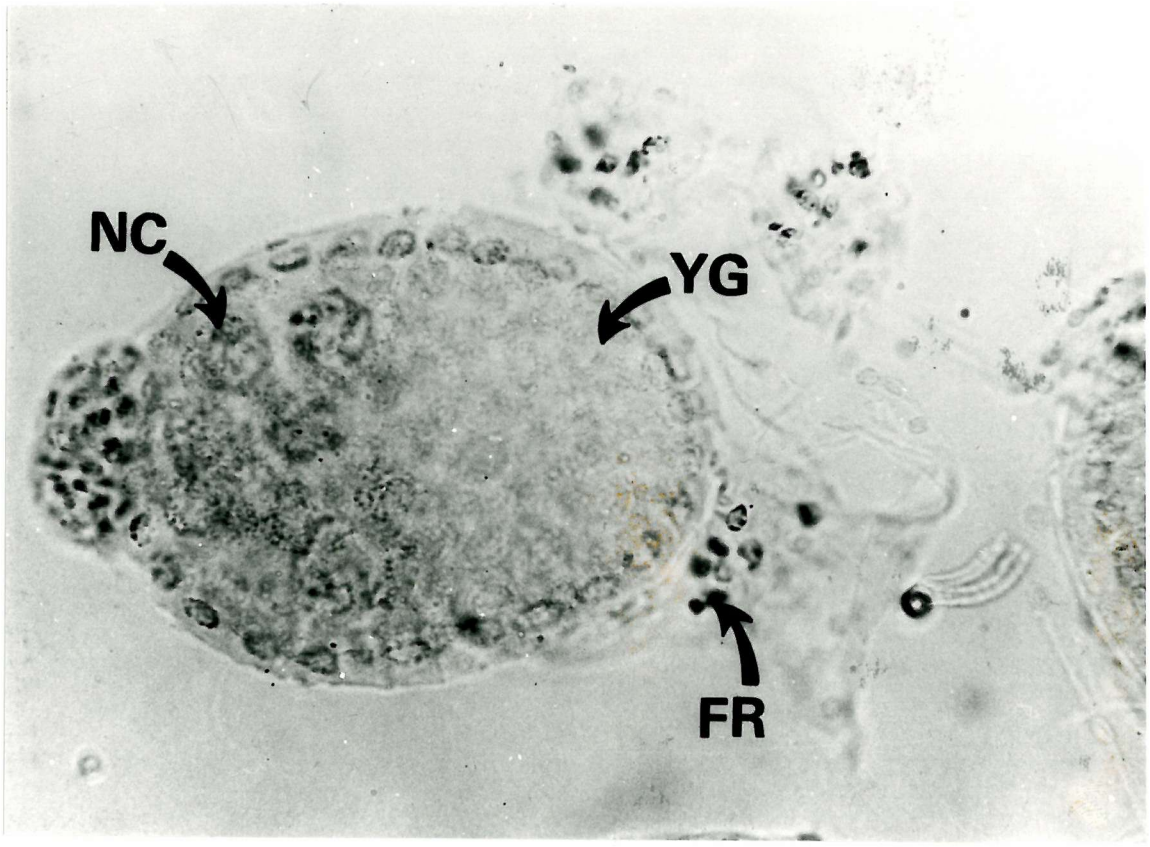


Figure 3.6. Stage III follicles.
(NC = nurse cell; Y = yolk)

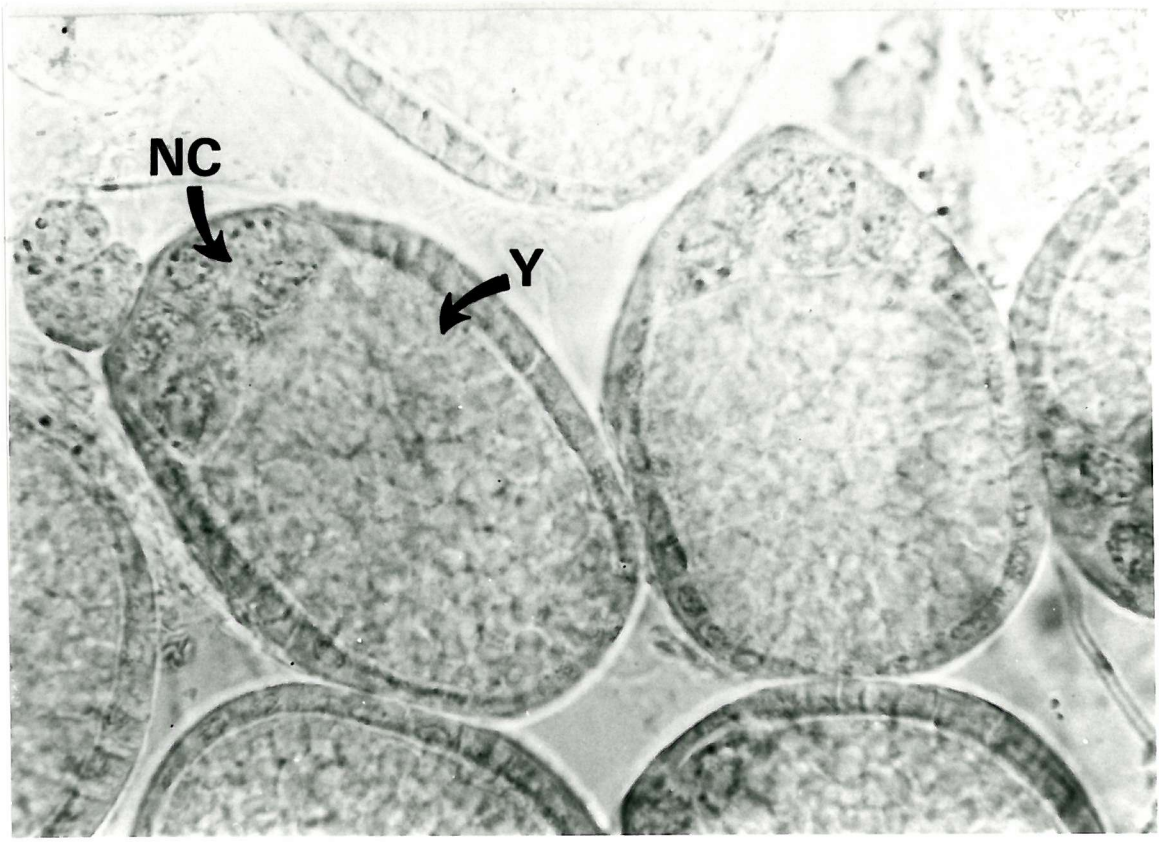


Figure 3.7. A stage IV follicle.
(NC = nurse cell; Y = yolk)

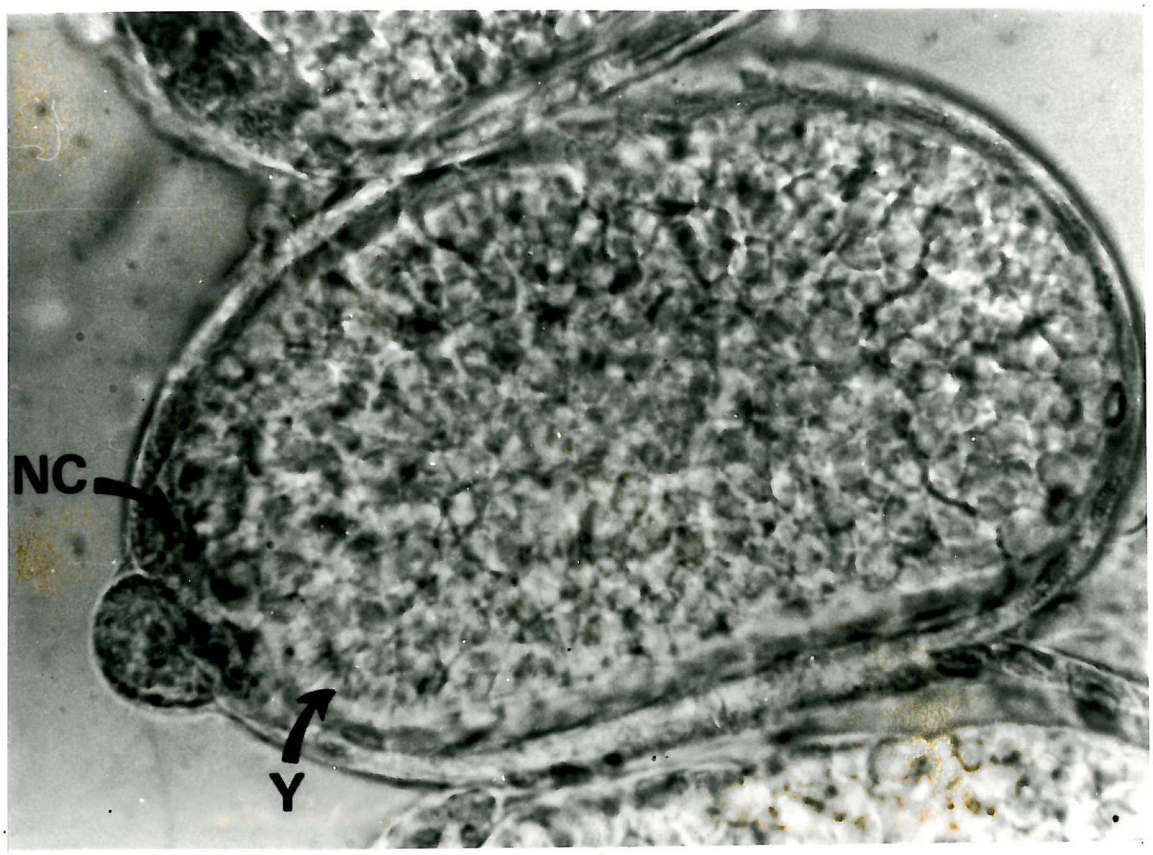


Figure 3.8. Fully developed eggs.
(E = egg; C = chorion)

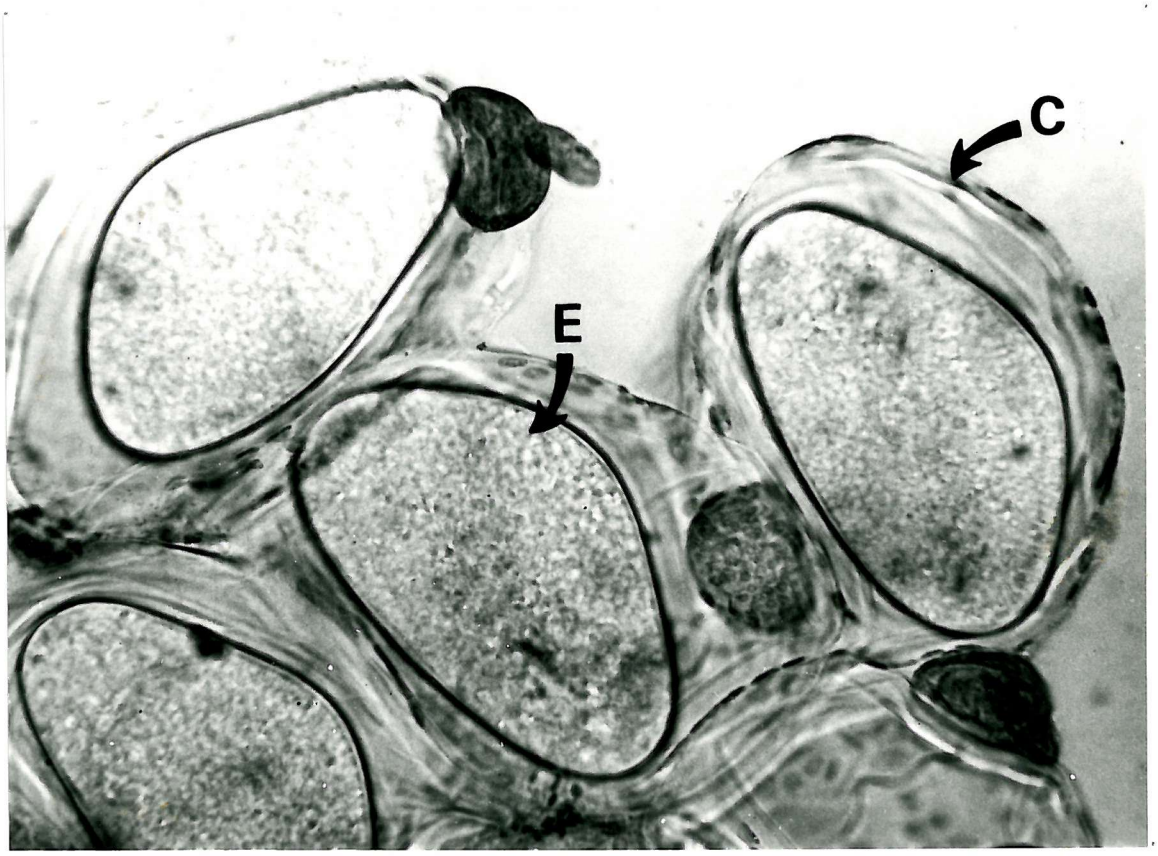
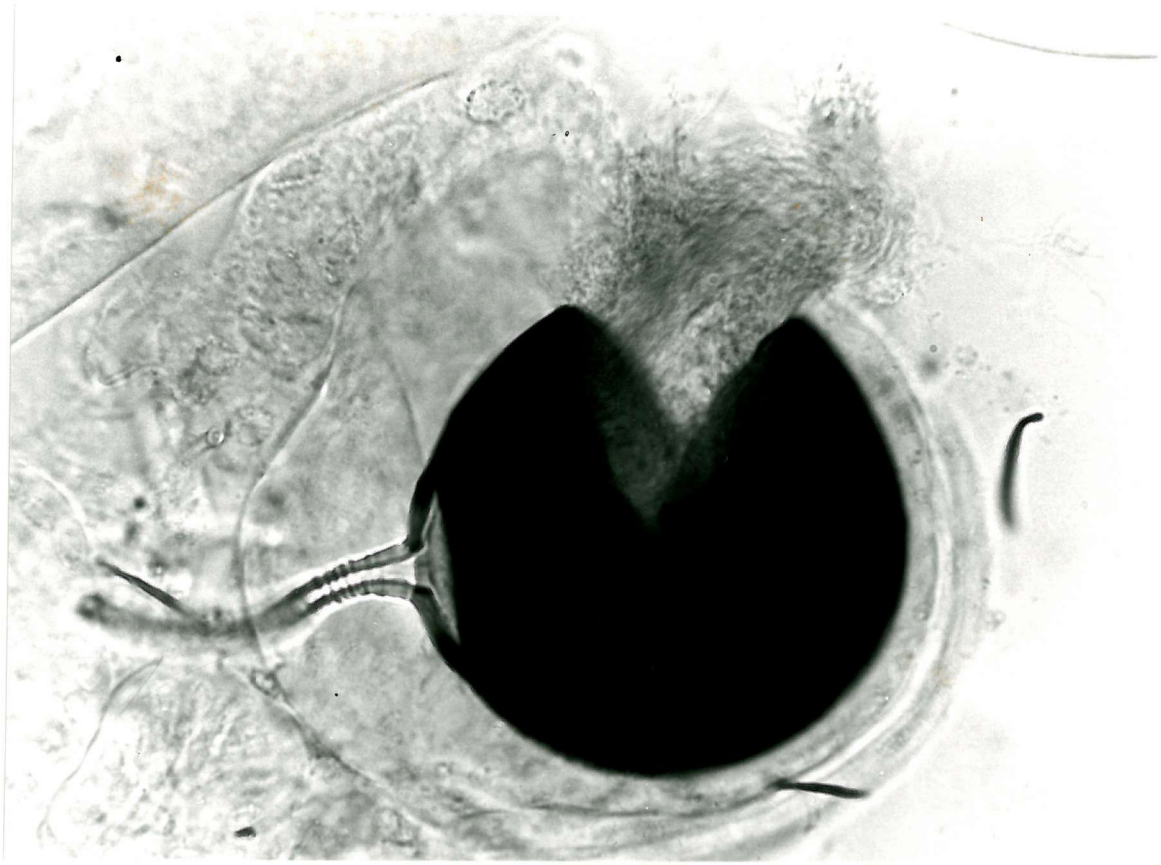


Figure 3.9. Spermatheca split open showing sperm.



3.4 Discussion.

In this experiment flies were killed at known times after feeding to repletion on human volunteers. The reason for this was that Procunier and Post (1986) found that flies with full guts generally possessed polytene chromosomes at a more advanced stage than flies with empty guts. They hypothesized that the state of the polytene chromosomes was correlated with development of the follicles and, therefore, that there was probably an optimum physiological stage of the fly for cytotaxonomy. In the present study the polytene chromosomes were in their best state between 3 and 8 hours after a blood feed, which corresponded to the follicular stages II or III. However, none of the flies contained chromosomes which were suitable for cytotaxonomic analysis.

The rate of follicular development, at least up to stage IV, appears to be slightly faster than that observed in S.ochraceum by Cupp and Collins (1979). However slightly different criteria may have been used to assess the beginning and end of each follicular stage and the developmental temperature would have been different. In their experiment flies were maintained at about 25 °C. In the present experiment flies were kept at ambient temperature which reached up to 29 °C during the day but fell to 19 °C in the early morning.

Deeply-stained follicular relicts could be seen in parous flies up to 8 hours after a blood meal. This provided a simple method of determining the parous condition of these flies as unstained follicular relicts are more difficult to see. The relicts were very large in some flies which were killed 1 to 2 hours after a blood feed implying that they had recently laid a batch of eggs. The presence of sperm in all the flies confirmed that the flies had been inseminated before taking a blood meal (Dalmat, 1955).

Chapter 4.

Differences in the larval morphology of members of the S.metallicum species complex.

4.1 Introduction.

Prior to the division of the S.metallicum complex into different members, based on cytotaxonomy, Onishi et al (1977) had already noticed morphological differences amongst the larvae from Guatemala. Okazawa and Onishi (1980) subsequently divided S.metallicum s.l. into S.metallicum s.s. and S.horacioi based mainly on larval head patterns and body colouration.

Other studies have also shown differences in S.metallicum specimens. In 1982, Petersen carried out enzyme electrophoresis on S.metallicum from Panama and the samples divided into at least two genetically distinct groups. One group had two novel alleles and bifid trichomes in the pupal stage, the other lacked these alleles and the pupae had multiple-branched trichomes. Agatsuma et al (1986) found isoenzyme differences between S.metallicum and S.horacioi.

Conn (1988) provisionally stated that S.metallicum cytotype A was S.metallicum s.s. and S.metallicum cytotype H was S.horacioi. In this chapter the results of examinations of the head patterns and body colouration of S.metallicum larvae, to see whether there were differences between the cytotypes, are described.

4.2 Materials and methods.

The head patterns and body colouration of several thousand S.metallicum larvae were examined to ascertain the range of variation in the specimens and then four major types of head pattern and four types of body colouration were selected. The head pattern was described as negative when the head spots on the cephalic apotome appeared pale against a dark background, and positive when the head spots appeared dark against a pale background. The four types of head pattern were (see Figure 4.1) :

(i) Distinct negative, where pale head spots appeared distinctly against a dark background.

(ii) Faint intermediate, where pale head spots were indistinct against a faint background.

(iii) Faint positive, where lightly pigmented head spots appeared against a faint background.

(iv) Distinct positive, where the head spots were darkly pigmented against a faint background.

The four types of body colouration selected were (see Figure 4.2) :

(1) Uniformly-coloured abdominal segments.

(2) A small colour demarcation between segments V and VI and the remainder of the abdominal segments.

(3) A distinct colour demarcation between segments V and VI and the remainder of the abdominal segments.

(4) A very distinct dark patch on the dorsal part of segments VII to IX. Segments V and VI are lighter in colour than the rest of the abdominal segments.

The S.metallicum larvae examined both morphologically and cytologically were those described in Chapter 2 (Table 2.2). They consisted of 430 larvae from fifteen sites in the Soconusco focus, 131 larvae from eleven sites in the Chamula focus and 228 larvae from seventeen sites in the Oaxaca focus. Before the specimens were hydrolysed and stained with Feulgen, the head patterns and body colouration of the larvae were scored under a dissecting

Figure 4.1. S.metallicum larval head patterns : (i) Distinct negative. (ii) Faint intermediate. (iii) Faint positive. (iv) Distinct positive.

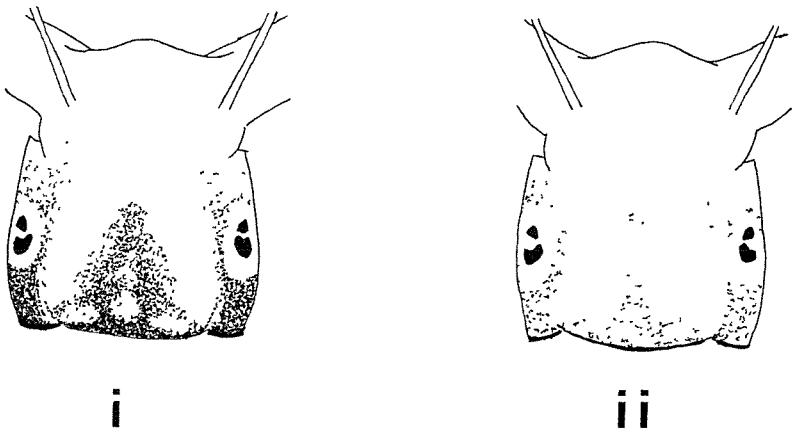
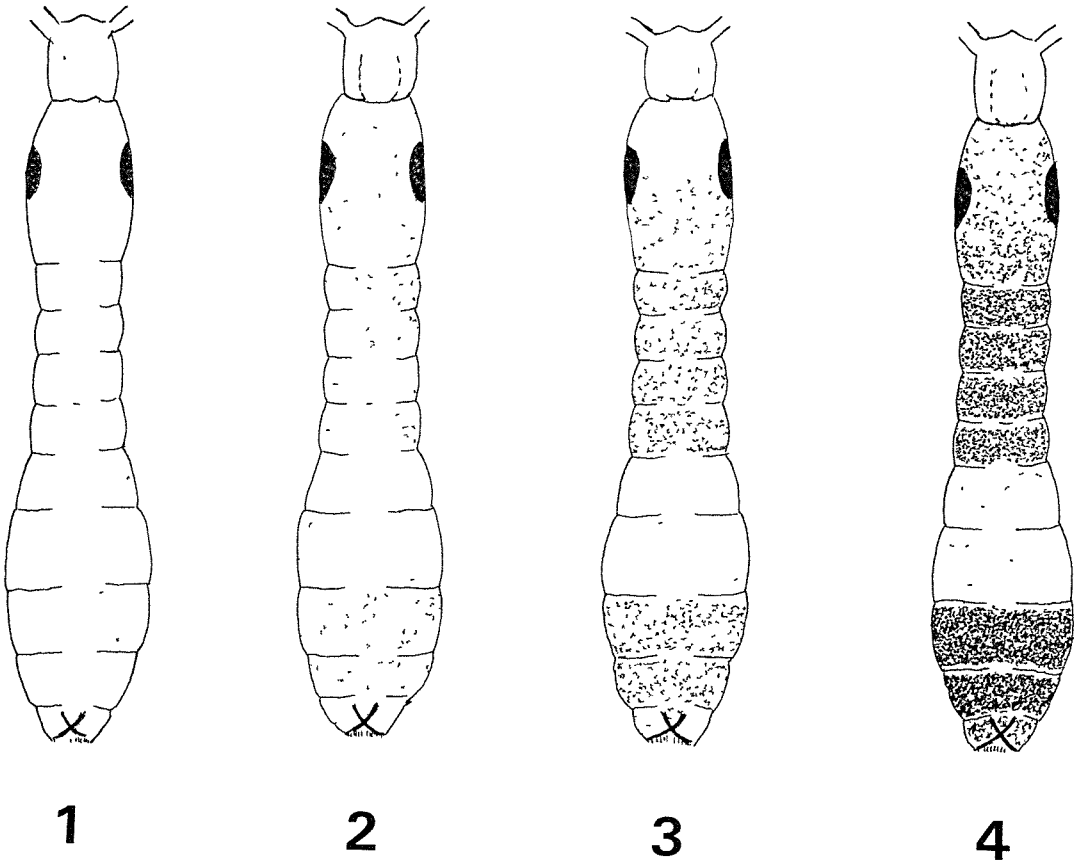


Figure 4.2. S.metallicum larval body patterns.



microscope. After this morphological examination, the chromosomes of the larvae were analysed and the specimens divided into the cytotypes A, B, H, I and X by the criteria described in Chapter 2.

4.3 Results.

After the morphological examinations, cytotaxonomic analysis showed that the sample consisted of : 424 cytotype A, 67 cytotype B, 43 cytotype H, 223 cytotype I and 32 cytotype X larvae. Distinct differences were found in the head pattern and body colouration between the different cytotypes. For the most part the larval head patterns and body colourations resembled the types shown in Figures 4.1 and 4.2, although there were minor variations in the degree of pigmentation. Some specimens had head spots of a different pattern or covering a larger area (see Figure 4.3.). However, these head patterns were still distinct positive and therefore scored as head pattern type iv. All these specimens were found to be cytotype I, in which head pattern type iv was found to be characteristic. The expression of head pattern type ii, faint intermediate, also varied slightly. In some specimens the antero-median head spot or the antero-median and the antero-lateral head spots appeared positive against a faint background (see Figure 4.4). These specimens were all found to be cytotype B or X, in which head pattern type ii is characteristic.

The results of head pattern and body colouration in relation to cytotype are presented in Figure 4.5. This figure shows that :

Cytotype A had uniformly-coloured abdominal segments and nearly always a distinct negative head pattern.
Cytotype B also had uniformly-coloured abdominal segments but its head pattern was faint and intermediate between positive and negative.

Figure 4.3. Alternative expressions of S.metallicum head pattern type iv, found in some cytotype I larvae.

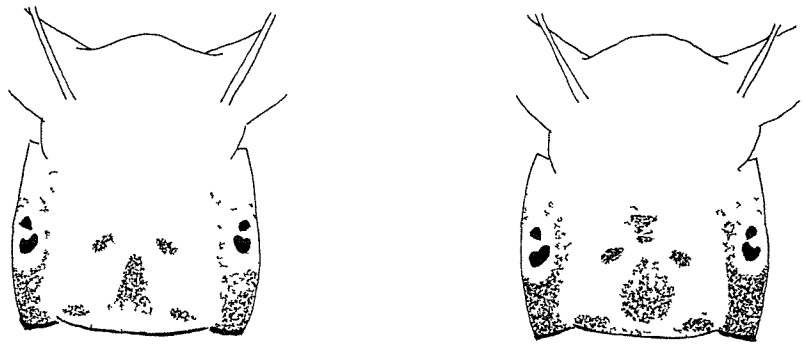


Figure 4.4. Alternative expressions of head pattern type ii, found in some cytotype B and X specimens. In these the antero-median or antero-median and antero-lateral head spots are positive.

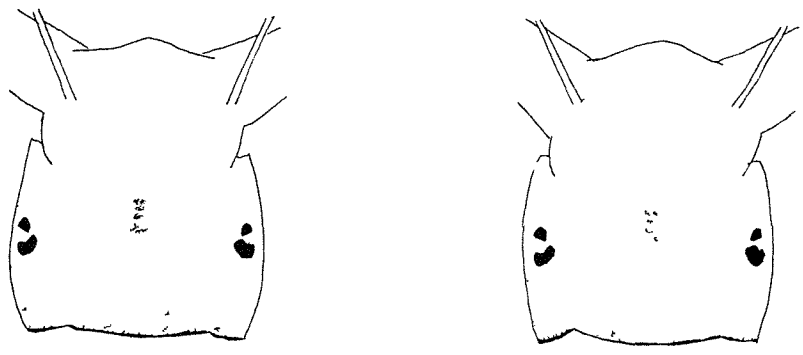
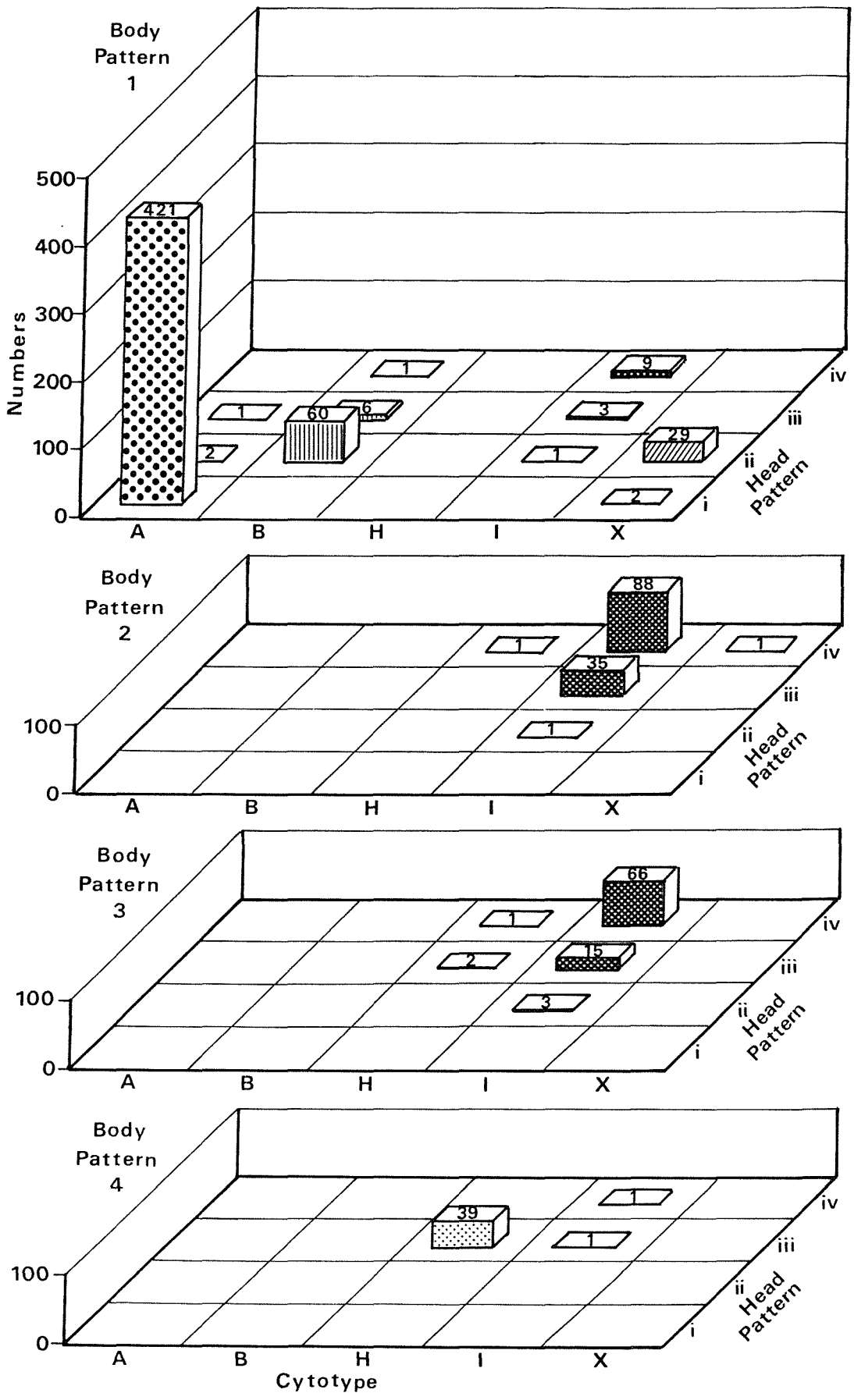


Figure 4.5. Body colouration and head patterns of S.metallicum larvae in relation to the five cytotypes A, B, H, I and X.



Cytotype H usually had a very distinct dark patch on the dorsal part of its abdominal segments VII to IX and segments V and VI were always lighter in colour than the others. The head pattern was nearly always positive and faint.

Cytotype I was more variable. The abdominal segments could be of uniform colour but in the majority of cases there was a small to distinct colour demarcation between segments V and VI and the remainder of the segments. The head pattern was nearly always positive and tended to be distinct rather than faint.

Cytotype X tended to have abdominal segments of uniform colour and a head pattern intermediate between positive and negative.

It can be seen from Figure 4.5 that when head and body pattern are considered together the probable cytotype of most larvae can be determined. Hence in most cases cytotypes A, B, H and I may be separated from each other by their morphology. However cytotype X resembles cytotype B.

4.4 Discussion.

In this study cytotype A had a negative head pattern and uniformly-coloured abdominal segments and matched the description of S.metallicum s.s.. Cytotype H had a faint positive head pattern and a distinct dark patch on its abdomen and matched the description of S.horacioi. These results are in agreement with Conn (1988).

Conn did not distinguish between cytotypes H and I stating that both had a positive head pattern and a dark patch on the abdomen. Her observations were based on 66 larvae of cytotype H from Guatemala and Panama and 18 larvae of cytotype I from Guatemala. The abdomens were only scored

for presence or absence of a dark patch and the head patterns as positive or negative. In this study, when these features were scored more critically, it was possible to distinguish between cytotypes H and I in the majority of cases. It is probable that differences could be found between Conn's H and I specimens if they were scored according to these criteria. However, all the specimens in the present study were from Mexico and it is possible that these characters vary across the geographical range of the species.

Conn examined 54 cytotype B larvae from Guatemala and scored them as having positive head patterns and uniformly-coloured abdomens. In the present study cytotype B larvae were mostly found to have faint head patterns, which were indistinct, and uniformly-coloured abdomens. It is possible that cytotype B larvae from Guatemala do vary from those in Mexico or alternatively they have been scored by slightly different criteria. However, irrespective of how the head pattern of cytotype B has been scored it is still distinct from that of cytotype A, which is negative, and so serves to separate them.

The proposed new cytoype X cannot be distinguished from cytotype B by means of head pattern and body colouration. However, this new cytotype has so far only been found in the state of Oaxaca and therefore is only likely to be confused with cytotype B specimens from there.

Larval morphology is a much easier and quicker technique for distinguishing between different cytotypes than cytotaxonomy. It can also be applied to all larval stages whereas good polytene chromosomes can only be obtained from penultimate stage larvae.

Chapter 5.

Cuticular hydrocarbon analysis as a potential method of distinguishing cytotypes.

5.1 Introduction.

Cuticular hydrocarbon analysis is a technique which has been used to separate members of several species complexes, its basis is described in section 1.5.5, but it has not previously been applied to members of the S.ochraceum or S.metallicum species complexes. The purpose of the study described in this chapter was to investigate whether different cytotypes within S.ochraceum and S.metallicum differed in their hydrocarbon complements.

5.2 Materials and methods.

5.2.1 Collection of material.

S.ochraceum and S.metallicum adult females were either caught when attracted to human bait or had emerged from pupal collections. Flies that were attracted to human bait were caught individually in hexane-rinsed glass Durham tubes which had cork stoppers. The flies were killed by leaving the tubes in the sun. Emerged flies were obtained from pupae which had been collected and pinned as in section 2.2.1. Larvae were preserved for cytotaxonomic analysis at the same sites as the pupae were collected. The collection sites, dates, methods and probable cytotypes of the flies used in the analysis are shown in Tables 5.1 and 5.2.

Table 5.1 Identity of S.ochraceum specimens used in cuticular hydrocarbon analysis.

Site*	Collection : Date	Method	Cytotype	Number
4	22.1.87	human bait	A	4
5	24.1.87	human bait	A	2
9	5.2.87	human bait	A	2
10	7.2.87	human bait	A	2
11	9.2.87	human bait	A	3
15	12.2.87	human bait	A	3
22	6.11.87	emerged	C	1
36	29.10.87	human bait	B	7 (C)**
42	24.10.87	human bait	B	1 (C)**

* see Appendix I
** (C) - contaminated traces

Table 5.2 Identity of S.metallicum specimens used in cuticular hydrocarbon analysis.

Site*	Collection: Date	Method	Probable cytotype	Number
3	20.10.87	human bait	A or B	2 (C)**
4	22.1.87	human bait	A,B,H or I	9
5	24.1.87	human bait	A	7
6i	25.6.85	emerged	H or I	1
6m	26.6.85	emerged	H or I	1
10	7.2.87	human bait	A	1
11	9.2.87	human bait	A	1
12	10.85	human bait	A	3
13	10.2.87	human bait	A	1
15a	12.2.87	human bait	A	6
17b	5.11.87	emerged	A	1
28	24.10.87	human bait	A,B or H	1 (C)**
32a	9.11.87	emerged	A or B	3
36	29.10.87	human bait	?	2 (C)**

* see Appendix I
** (C) - contaminated traces

5.2.2 Chromatographic procedure.

Gas liquid chromatography was used to separate the hydrocarbon peaks by a method similar to that of Phillips et al (1985). Flies were placed individually in clean glass vials and covered with 10ul of spectrophotometric grade hexane. The lids were placed on the vials and the hexane was left to extract the hydrocarbons for 10 minutes. The emerged flies were left on their micro-pins for this procedure, but the micro-pins were first cleaned with hexane. After the extraction period the flies were removed with forceps and returned to the labelled tubes. The vials containing the extracts were then left open so that the hexane evaporated completely. Each extract was resuspended in 2ul of hexane containing 10ppm of pentadecane, which was used as an internal standard, and left to stand for 1 minute with the lid on before the whole amount was injected onto the column. Extracts were run in a random sequence to compensate for variability in machine response on different days.

An initial attempt with a United Technologies Packard 439 gas chromatograph linked up to a LDC Milton Roy CI-10 integrator was unsuccessful in producing traces for the flies, although at least one hundred were run. It is probable that the integrator was not functioning adequately. Subsequently, the gas chromatograph used was a Hewlett Packard 5890A. This was equipped with an on-column injector and a flame ionisation detector and connected to a Hewlett Packard 5895A workstation. A 15m DB1 fused silica capillary column was used with an internal diameter of 0.32mm and a film thickness of 0.1um. Nitrogen was used as a carrier gas at 2ml/min. After experimentation the best peak separation was achieved with the following temperature programme:

(i) 2 minutes at 120 °C
(ii) a temperature rise of 7 °C/min to 225 °C
(iii) 1 minute at 225 °C

(iv) a temperature rise of 3 °C/min to 310 °C

(v) 15 minutes at 310 °C.

The detector signal was integrated by the workstation, which produced a trace of the peaks with a report of their retention times and areas.

5.2.3 Analysis.

The trace which contained the most peaks for each species was examined and the peaks were numbered sequentially according to their retention times. The peaks on all the other traces were then numbered by comparing their retention times with those on the numbered trace. Traces with less than 10 peaks were discarded. In chromatographic traces the area under the peak is proportional to the concentration of that component in the extract. The areas of all the peaks on each trace were divided by the area of the internal standard on that trace to correct for variations in the amount of sample injected and in machine response. A logarithmic transformation was then applied to the corrected peak areas to normalize the data for statistical analysis. The data for each trace was transferred onto an IBM 3090 mainframe computer and standard SAS statistical procedures were used for the analyses.

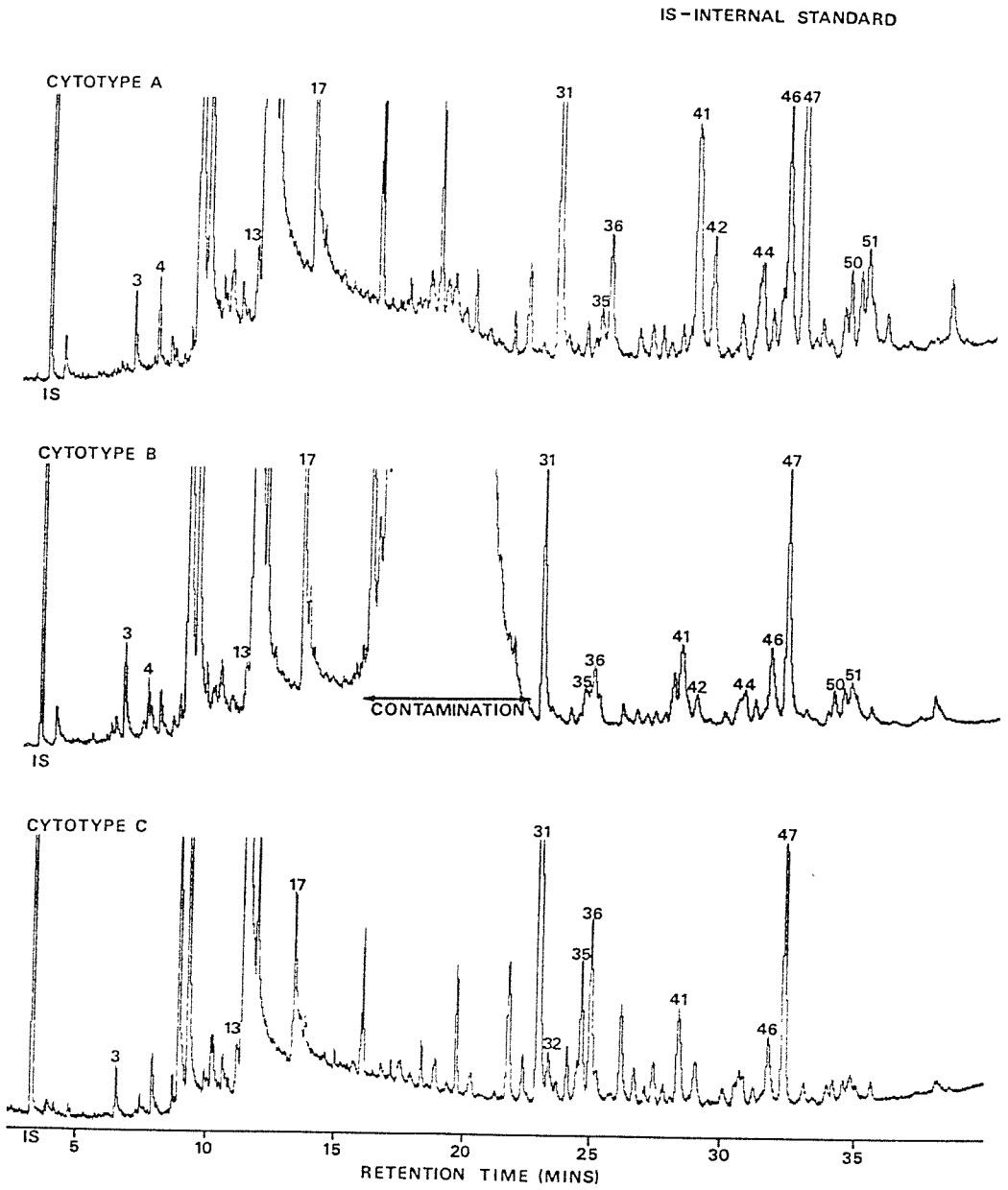
A different cytotype of S.ochraceum was found in each of the three foci according to the larval cytotaxonomy results (section 2.3). It was therefore assumed that the adults in this analysis were of known cytotype enabling principal component analysis (SAS PRINCOMP) and canonical discriminant analysis (SAS CANDISC)to be used (SAS, 1985). The former technique does not treat specimens as members of known cytotypes but groups them according to the similarities of their traces. The latter technique uses linear combinations of hydrocarbon peaks which maximize the ratio of "between-groups" to "within-groups" variance and

requires a prior knowledge of the identity of specimens (Phillips et al, 1985). The largest group of S.ochraceum adults were of cytotype A in this analysis, but only numbered 16. As a result of this only 15 peaks could be used in the discriminant analysis, because a constraint of the statistical package used is that the number of variables (peaks) must be less than or equal to n-1, where n is the number of specimens in the largest group (J.Bradshaw, pers. comm.). The peaks chosen were those which occurred most frequently together with peaks which predominately occurred in one cytotype. The same 15 peaks were used in the principal component analysis. Some of the fly extracts were found to be contaminated with a substance from the collection tubes. As the contamination was confined to a distinct region of the trace, these runs were analysed but no peaks in any of the flies were used from this part of the trace. Simplified traces of the three cytotypes showing the 15 peaks used in the analysis can be seen in Figure 5.1.

The S.metallicum cytotypes were found sympatrically (section 2.3), therefore the identity of the adults used in this analysis was less certain. In cases where a cytotype was found exclusively or almost exclusively in the collection area the specimens were presumed to be of that cytotype (see Appendix V). However, some specimens were collected in areas where more than one cytotype was common. Canonical discriminant analysis was not possible for S.metallicum as knowledge of the identity of specimens is a prerequisite for this technique. Principal component analysis and average linkage cluster analysis (SAS CLUSTER) were therefore used to see whether the identity of some of the unknowns could be established (SAS, 1985).

In total 62 peaks were used in this analysis, which had retention times of between 3 and 36 minutes. Prior to the analysis it was not known which peaks would be important in discriminating between cytotypes and therefore all the rare

Figure 5.1. Simplified traces of the three S.ochraceum cytotypes showing the fifteen peaks used in the analysis.



peaks were included. However, the two groups of large peaks which had retention times of around 9 and 11 minutes were not used as they were extremely variable (see Figure 5.3). Although the identity of the peaks was not established in this study, Phillips et al (1985) found that peaks in these positions in S.damnosum s.l. were fatty acids and were the most variable both within and between species. In addition, a few traces of S.metallicum were contaminated in the same region as the S.ochraceum traces (see Figure 5.1). No peaks from this region in any of the traces were used in the analysis.

5.3 Results.

Due to problems with machine response, traces suitable for analysis were obtained from only 25 S.ochraceum and 39 S.metallicum specimens. Examples of S.ochraceum and S.metallicum traces can be seen in Figures 5.2 and 5.3. There was a wide variation in the number of peaks on individual traces in both species. Some traces had very few peaks and their areas were small. Other traces contained many peaks which had larger areas. These differences were not due to variations in the amounts of solvent extract injected since all the peaks were divided by the area of the internal standard to exclude this source of variation. The differences may therefore have been accounted for by variations in the amounts of hydrocarbons in individual flies and differences in machine response.

The S.ochraceum used in this analysis consisted of 16 cytotype A, 8 cytotype B and 1 cytotype C specimen. When canonical discriminant analysis was carried out on these the scattergram in Figure 5.4 was obtained. Cytotype C was separated from cytotypes A and B by the first canonical axis which was highly significant by the likelihood ratio test ($p=0.0037$) (SAS, 1985). Peak 32, which only occurred in the cytotype C specimen and one cytotype B specimen, and

Figure 5.2. An example of a S.ochraceum chromatographic trace.

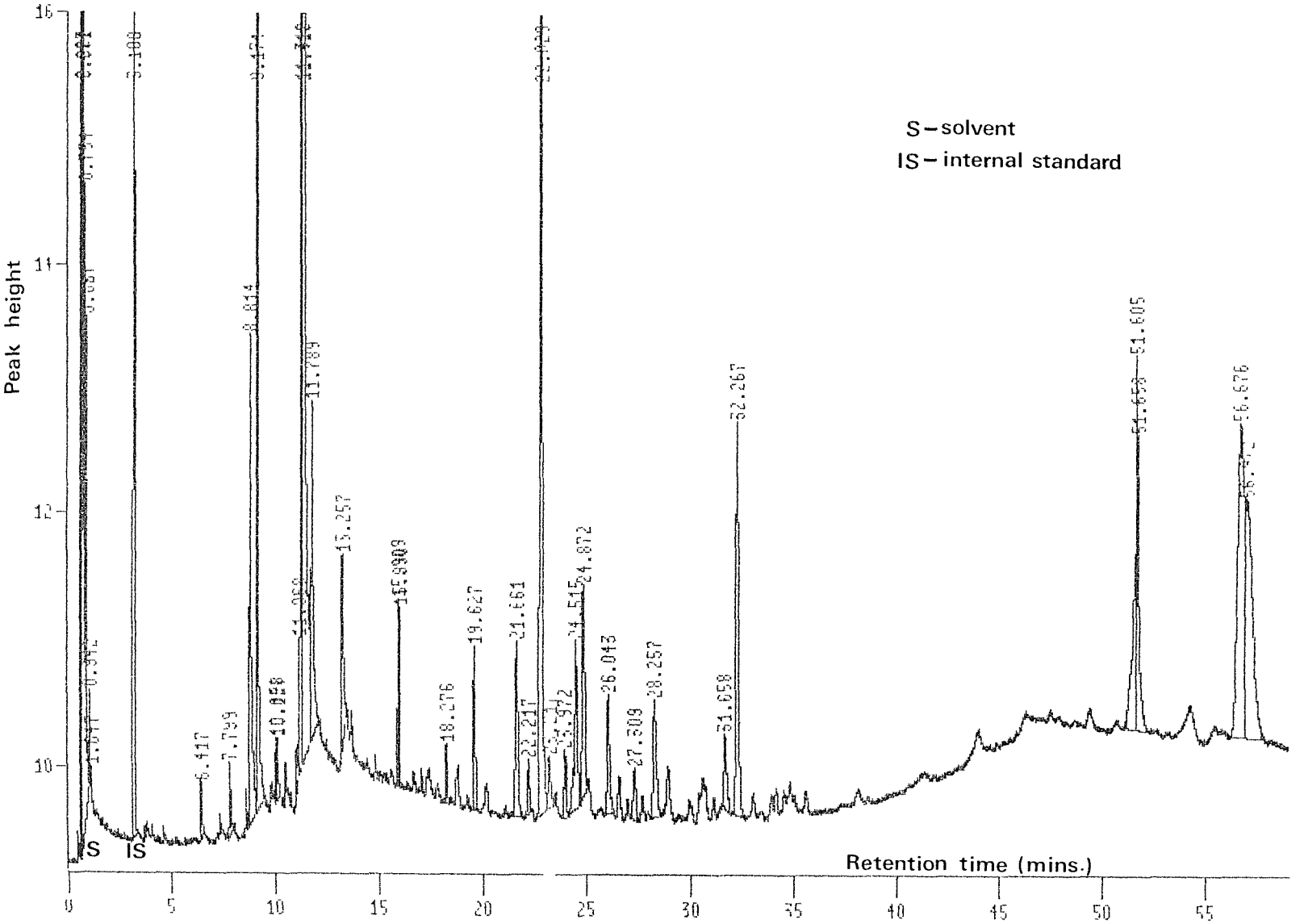


Figure 5.3. An example of a S.metallicum chromatographic trace.

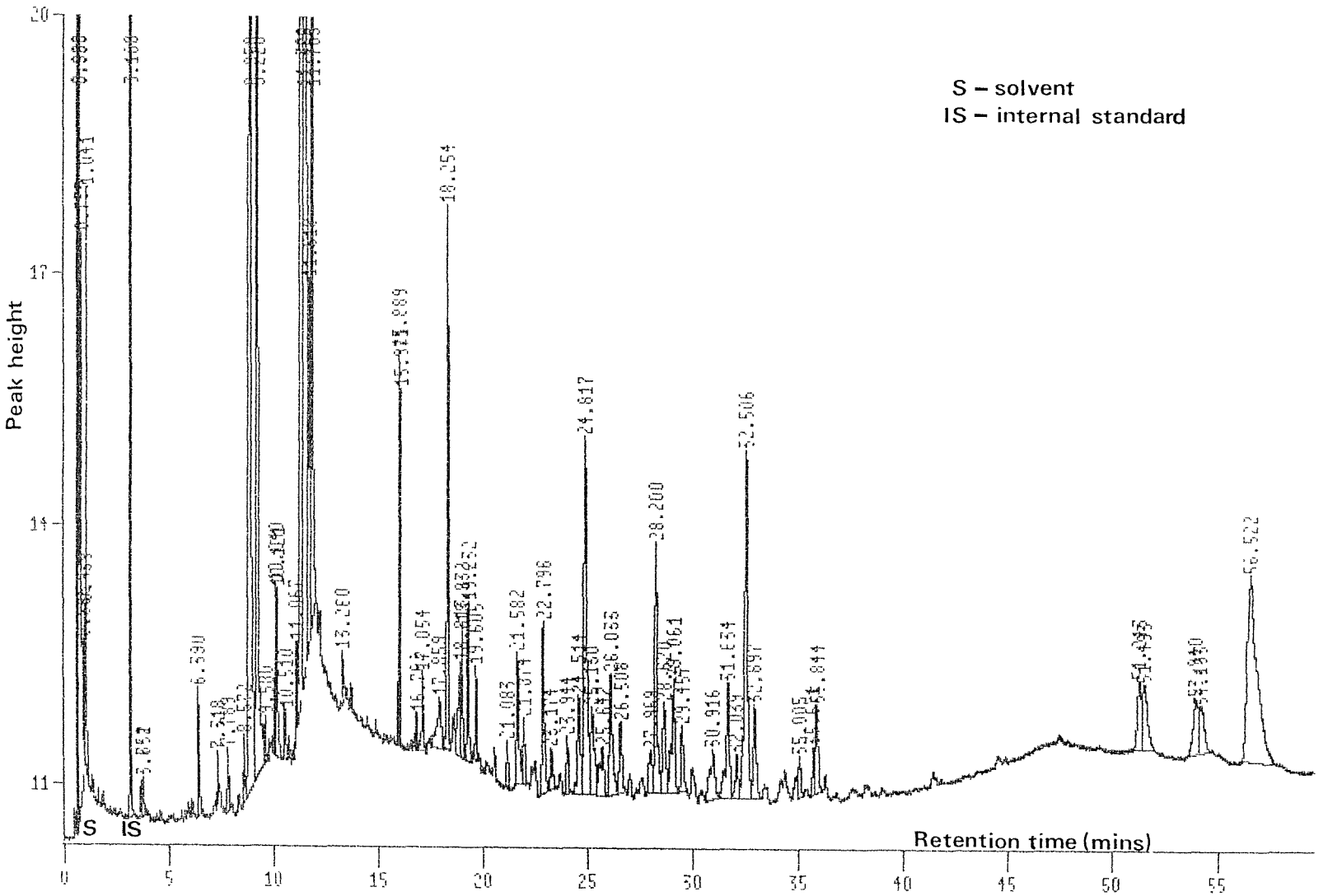
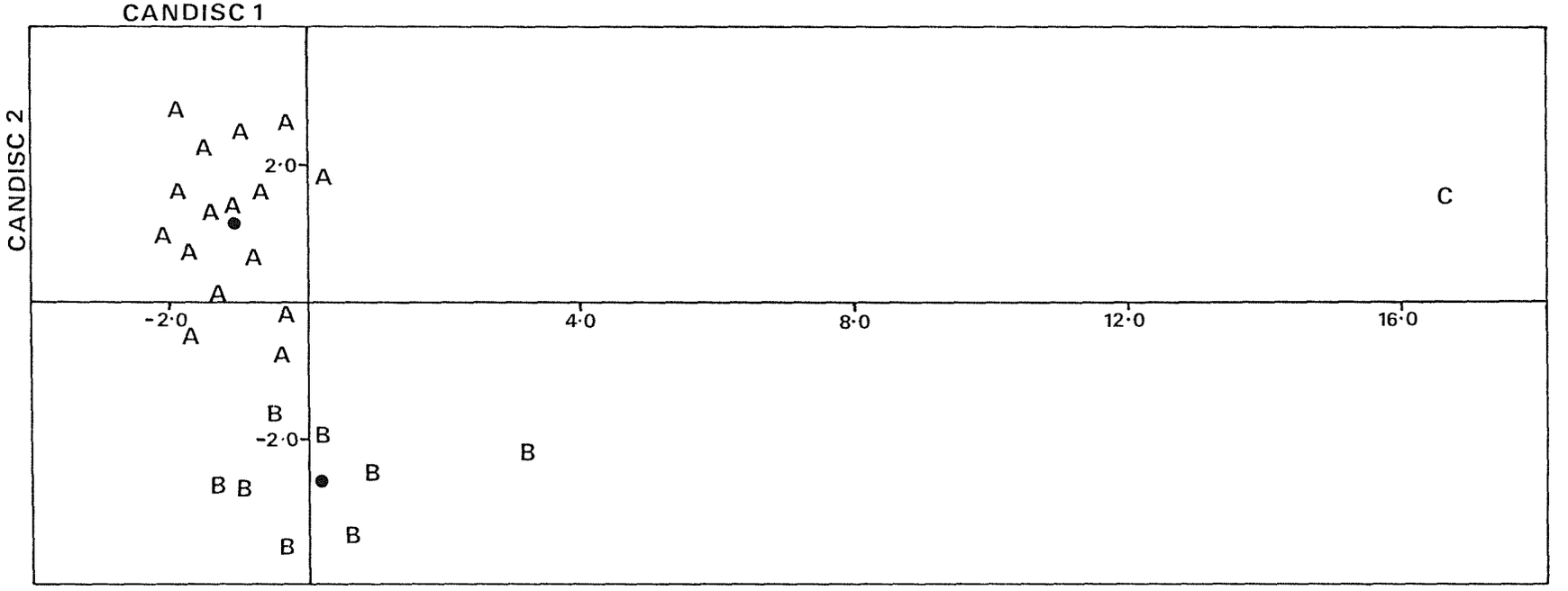


Figure 5.4. Scattergram of S.ochraceum cytotypes A, B and C using canonical discriminant analysis on hydrocarbon peaks.
(CANDISC1 = First canonical axis
CANDISC2 = Second canonical axis
● = centroid)



peak 35, which had a large area in cytotype C compared with A and B, were both important discriminators in this axis (see Figure 5.1). Cytotype A was placed in a separate cluster from cytotype B by the second canonical axis, but this was not significant by the likelihood ratio test ($p=0.1180$). When the analysis was carried out on just cytotypes A and B, the separation was still not significant ($p=0.1621$).

When principal component analysis was applied to the S.ochraceum peaks, the first principal component accounted for 52% of the variation. However, this axis did not separate cytotypes A, B and C. Examination of the traces revealed that this axis separated the specimens according to the number of peaks, those traces with few peaks being at one end and those with many at the other. The second principal component axis accounted for 18% of the variation and partially separated A from B, although there was considerable overlap. The third principal component axis accounted for 10% of the variation and separated cytotype C from A and B.

When the S.metallicum peaks were subjected to principal component analysis, the first component accounted for 40% of the variation. However, this axis separated the samples according to the quality of the traces, i.e. whether they had few or many peaks, as in S.ochraceum. The results of the cluster analysis can be seen in Table 5.3. The most significant cluster, number 38, joined a cytotype A specimen with one which was cytotype I. The other cytotype I specimen was distant, cluster number 19. The identities of the unknown specimens could not be ascertained.

5.4 Discussion.

When the traces were analysed it was found that there was a great variation in the number of peaks in individual traces. These differences may have been because flies have

Table 5.3 Average linkage cluster analysis results for S.metallicum specimens.

Number of clusters	Clusters joined		Frequency of new cluster	Normalized root-mean-square distance
38	I	A	2	0.108850
37	U*	A	2	0.119397
36	CL37	U	3	0.157204
35	A	CL36	4	0.175637
34	CL38	A	3	0.198852
33	CL35	A	5	0.198997
32	CL33	CL34	8	0.224359
31	A	U	2	0.269247
30	CL32	U	9	0.315884
29	CL30	CL31	11	0.321078
28	U	U	2	0.325626
27	U	A	2	0.341435
26	CL29	CL28	13	0.354994
25	CL26	A	14	0.375504
24	A	U	2	0.409795
23	U	U	2	0.438289
22	U	U	2	0.452787
21	U	A	2	0.454514
20	CL23	CL25	16	0.458755
19	CL20	I	17	0.489954
18	CL24	CL21	4	0.499004
17	A	A	2	0.509281
16	CL19	CL27	19	0.520355
15	CL18	U	5	0.542367
14	U	A	2	0.558846
13	CL15	CL16	24	0.594532
12	CL13	CL22	26	0.639366
11	CL12	CL17	28	0.678591
10	CL11	CL14	30	0.789847
9	CL10	A	31	0.834730
8	A	A	2	0.897346
7	CL8	A	3	0.926993
6	A	U	2	1.001030
5	CL9	CL7	34	1.026887
4	CL5	CL6	36	1.050206
3	CL4	U	37	1.580665
2	CL3	A	38	1.613094
1	CL2	A	39	2.373244

* unknown cytotype

varying amounts of hydrocarbons, possibly due to size or age. Another reason why the traces may differ in quality is that the flies may have lost hydrocarbons during storage due to exposure to a solvent. However, all the flies had been kept under the same conditions and there were variable responses in flies from the same batches. Variations in machine sensitivity may also be responsible for differences in the traces. The internal standard, pentadecane, was included to minimize this effect, however an internal standard with a higher retention time or a combination of internal standards might have been more reliable (G. Broomfield, pers. comm.).

In S.ochraceum none of the peaks used in the discriminant analysis were found to be unique in any cytotype, although peak 32 only occurred in the cytotype C specimen and one cytotype B specimen. The analysis therefore relied on quantitative rather than qualitative differences between the traces. The discrimination between the cytotypes was due to the relative proportions of the different peaks, as was found in members of the S.damnosum complex (Carlson and Walsh, 1981; Phillips et al, 1985).

The canonical discriminant analysis produced a significant separation between S.ochraceum cytotype C and cytotypes A and B. However, there was only one cytotype C specimen in the analysis, which was also the only emerged fly. Cytotypes A and B were separated into different clusters by this analysis but the results were not significant. This may have been a result of the small number of samples. The whole range of peaks could not be used in the analysis due to contamination of some of the samples. Peaks from this unused region may have been important in discriminating between cytotypes.

Principal component analysis showed that over half the variation in the specimens was a result of the differences between traces, whether they contained few or abundant

peaks, rather than differences between the cytotypes. This amount of variation, which is unlikely to be due to cytotype differences, would make it more difficult to assign a fly of unknown cytotype to one of the cytotype classes by discriminant analysis. The present analysis was based on a very small number of specimens, due to very limited numbers of cytotypes B and C being available and problems with machine response. The differences in the quality of the traces would have a less pronounced effect on the analysis if a larger number of specimens of known cytotype were analysed. More samples are needed to assess the value of this technique in separating members of the S.ochraceum complex.

It was significant that the cytotype C specimen was the most distant from the other two, as this has been found to be genetically the most different as well (see section 2.4). In the absence of the S.ochraceum cytotypes being found in sympatry, thus precluding a cytotaxonomic definition of their specific rank, this result adds more evidence to the view that cytotype C is distant from the other two cytotypes and so it may indeed be a true cytospecies.

Cluster analysis failed to separate the S.metallicum specimens of known cytotype from each other or indicate the identity of unknown specimens. However, this may be partly due to the large amount of variation in the quality of the traces which was shown by the principal component analysis. More specimens would be needed to establish the usefulness of this technique for separating members of the S.metallicum complex. Ideally, pure samples of the five cytotypes, A, B, H, I and X are needed so that the differences between groups can be assessed. If significant differences were found between groups, unknown specimens could then be assigned to one of these groups.

Chapter 6.

Environmental factors associated with the larval distribution of the different members of the S.ochraceum and S.metallicum species complexes.

6.1 Introduction.

There have been a variety of studies in Mexico and Guatemala of the distribution of Simulium larvae in relation to environmental variables. Dalmat (1955) investigated the distribution of Simulium species and found that each occurred in a characteristic range of stream sites. The parameters which were associated with the larval distribution of S.ochraceum, S.metallicum and S.callidum have also been examined in the Soconusco focus of Mexico (Freeman, 1983; Montoya and Ortega, 1981). Recently the characteristics of streams in which the anthropophilic species, particularly S.ochraceum, occur have been examined in Guatemala (Yamagata, 1984, 1986; Yamagata and Kanayama, 1985; Yamagata et al, 1984). Takaoka (1982) compared the distributions of S.horacioi and S.metallicum larvae in Guatemala.

In previous studies of the distribution of S.ochraceum and S.metallicum they were considered only as two distinct species and not as species complexes. The results of this chapter concern investigations on whether the different S.ochraceum and S.metallicum cytotypes were found in differing stream habitats. If so, such information would prove to be important if a larval control programme was to be implemented against the principal vectors.

6.2.1 Materials and methods.

The collection of specimens is described in section 2.2.1. Samples were taken at a variety of altitudes and at as many widely-distributed sites as possible. A major constraint of choosing collection sites was the inaccessibility of the terrain. Most sites were therefore located close to access roads, which were few in number. Locations of collection sites are given in Appendix I. Collection dates are given in Appendix IV (for collections used in the ecological analysis) and Appendix V (all collections). When the larval collections were made the following stream parameters were also noted :

(i) Altitude (m) was measured with an altimeter.
(ii) pH was measured in divisions of 0.3 with Duotest pH paper.
(iii) Water temperature (^{o}C) was measured with a mercury thermometer to an accuracy of 0.5 ^{o}C.
(iv) Water clarity was estimated as clear, slightly cloudy or cloudy.
(v) Shade was estimated as none, scant, partial or total.
(vi) Vegetation was scored as being in the water, at the sides or covering the stream.
(vii) The approximate size of the stream was estimated on a scale of 1 to 5:

Type 1. A very small trickle. The stream bed did not differ from the surrounding vegetation and the whole stream tended to be covered by dense vegetation. Maximum width was approximately 30cm. An example is shown in Figure 6.1.

Type 2. A small, shallow stream with a definite stream bed, having a maximum width of approximately 100cm. An example is shown in Figure 6.2.

Type 3. A medium-sized, shallow stream with a maximum width of less than 200cm. An example is shown in Figure 6.3.

Type 4. A large stream, which was deeper and 200cm or more in width. An example is shown in Figure 6.4.

Type 5. A very large stream which had distinct banks and was 500cm or more in width. An example is shown in Figure 6.5.

In the case of some streams it was difficult to decide whether they belonged to one type or another as the width was very variable. These were therefore scored as between two types e.g. 2/3, 3/4.

The samples were sorted and identified with the aid of the staff at British Museum (Natural History) using reference specimens, original descriptions and a variety of keys including Vargas et al (1946), Vargas and Díaz Nájera (1957), Dalmat (1955), Escalante (1959), Onishi et al (1977) and Okazawa and Onishi (1980). The numbers of larvae of each cytospecies of S.ochraceum and S.metallicum, together with the numbers of larvae of other Simulium species, were recorded in each sample.

The S.ochraceum larvae were assumed to be cytotype A in the Soconusco focus, cytotype C in the Chamula focus and cytotype B in the Oaxaca focus on the cytotaxonomic evidence of Chapter 2. S.metallicum larvae, which had not been cytotaxonomically examined, were divided into their most probable cytotype based on head pattern and body colouration as described in Chapter 4. Thus larger samples were analysed than those discussed in Chapter 2, which was restricted to data only on larvae identified cytologically.

Figure 6.1. Example of a type 1 stream.
(Reforma, Oaxaca - site 45
approximate width = 15cm)

Figure 6.2. Example of a type 2 stream.
(Chespal, Soconusco - site 13a
approximate width = 7.0cm)

Figure 6.3. Example of a type 3 stream.
(Pantelho, Chamula - site 32a
approximate width = 120cm)

Figure 6.4. Example of a type 4 stream.
(Varitas, Soconusco - site 7
approximate width = 200cm)

Figure 6.5. Example of a type 5 stream.
(Once de Abril, Soconusco - site 16
approximate width = 500cm)

6.2.2 Analysis of species distribution in relation to environmental variables.

Canonical correspondence analysis (CCA), from the computer programme CANOCO (Ter Braak, 1988), was used to investigate whether different species occurred in different environmental conditions. In CCA, regression and ordination have been integrated into a technique of multivariate direct gradient analysis, called canonical or constrained ordination. This technique measures the separation of species' distributions along ordination axes which are constrained to be linear combinations of environmental variables. CCA produces an ordination diagram of samples, species and environmental variables which optimally displays how community composition varies with the environment. CCA assumes species to have a unimodal distribution and the species score can be considered as the centre of the unimodal curve (Jongman et al, 1987).

The sample scores are linear combinations of environmental variables and are weighted according to the total abundance of specimens in the sample. The species scores are a weighted average of the sample scores. The weighted average of a species distribution with respect to an environmental variable is defined as the average of the values of that environmental variable at those sites at which that species occurs, the weighting of each site being proportional to species abundance. The weighted average indicates the centre of a species distribution along the environmental variable (Ter Braak, 1986).

The first canonical axis maximizes the dispersion of species scores by choosing the best weights for the environmental variables. The second and further canonical axes also maximize the dispersion of the species scores, but subject to the constraint of being uncorrelated with the previous canonical axes. The number of axes is equal to the number of environmental variables, although four are

normally extracted. The eigenvalue of each axis is a measure of how much variation in the species data is explained by that axis and therefore by the environmental variables. It has a value of between zero and one, and the higher the value the more important the axis. The eigenvalue is the ratio of the dispersion of the species scores and that of the sample scores (Ter Braak, 1988).

The six environmental variables: altitude, temperature, size, pH, shade and water clarity were used. For the purpose of the analysis the values of each of these factors were split into classes, which are shown in Appendix VI (i). The altitude, temperature and pH ranges corresponded to those used by Dalmat (1955). Any missing values were estimated from the values in similar streams in the same focus. One hundred and twenty-seven streams, which are listed in Appendix IV, were used in this analysis. Sixty-seven of these streams were from the Soconusco focus, 24 from the Chamula focus and 36 from Oaxaca. Only one sample was used from each stream. In cases where more than one sample was taken from a stream, the sample collected on the date in Appendix IV was the one used in the analysis. The numbers of each species in the samples can be seen in Appendix V. The species analysed were S.ochraceum cytotypes A, B and C, S.metallicum cytotypes A, B, H, I and X, S.callidum and S.downsi. S.paynei and S.rubicundulum were also included, but as a combination, as the early instar larvae are difficult to separate . Other species, listed in section 6.3.1, occurred too infrequently to be analysed.

6.3. Results.

The conditions recorded at each stream are shown in full in Appendix IV and the numbers of each species found in each sample are shown in Appendix V. Sites where more than one stream was sampled are shown as 1a, 1b, 1c etc.

6.3.1 Species identified.

The species identified in the samples were :

S.ochraceum Walker cytotypes A, B and C
S.metallicum Bellardi cytotypes A, B, H, I and X
S.callidum (Dyar and Shannon)
S.downsi Vargas, Martínez and Díaz,
S.paynei Vargas
S.rubicundulum Knab
Cnephia aquirrei (Dalmat)
Cnephia grenieri Vargas
S.nigricornis Dalmat
S.veracruzanum Vargas, Martínez and Díaz
S.tricornis De Léon
S.yepocapense Dalmat
S.deleoni Vargas
S.ruizi Vargas and Díaz
S.parrai Vargas, Martínez and Díaz

and some specimens probably ascribable to the following were also found :

S.anduzei Vargas and Díaz
S.d'andretta Vargas, Martínez and Díaz
S.menchacai Vargas and Díaz

The numbers of these species identified are summarized in Table 6.1. S.paynei and S.rubicundulum have been scored together as it was sometimes impossible to distinguish between the larvae of these species when the larvae were

immature. Similarly, C.aquirrei and C.grenieri have been recorded together as Cnephia spp. The composition of species varied between the three foci.

6.3.2 Species abundance.

Members of the S.metallicum species complex were the most commonly found larvae and 12,377 specimens were identified in total. S.callidum was the second most abundant species (3832 specimens) and members of the S.ochraceum species complex (2800 specimens) were the third most abundant. All the other species collected were zoophilic species and their abundances may be seen in Table 6.1.

When considering differences in species abundance between the three foci it must be noted that a different number of collections was made in each of the foci. Table 6.2 shows the numbers of streams sampled in each focus and the numbers of collections made in each focus. In order to compare the numbers and proportions of the S.ochraceum and S.metallicum cytotypes and S.callidum found in the three foci, the numbers of specimens from each focus have been divided by the number of collections in each focus (Table 6.3).

A different cytotype of S.ochraceum was found in each of the three foci. Cytotype A was the most abundant cytotype found and cytotype B the least abundant.

Collectively, members of the S.metallicum species complex were the most abundant larvae found, but when considered separately there were great differences in the numbers and proportions of the cytotypes found in each focus. Table 6.4 shows the percentage composition of S.metallicum by cytotype in each of the three foci. In the Soconusco focus cytotype A was the most abundant, cytotype I was common and cytotypes B and H much rarer. In the Chamula

Table 6.1. Total numbers of larval specimens of each species collected.

Species	Number of larvae identified : Soconusco focus	Chamula focus	Oaxaca (all)	Total
S.ochraceum : cytotype A	2445	0	0	2445
cytotype B	0	0	139	139
cytotype C	0	216	0	216
S.metallicum : cytotype A	8139	711	204	9054
cytotype B	259	19	55	333
cytotype H	445	2	54	501
cytotype I	1507	3	825	2335
cytotype X	0	0	154	154
S.callidum	3102	74	656	3832
S.downsi	429	130	0	559
S.paynei/ S.rubicundulum	710	119	290	1119
Cnephia spp.	594	0	0	594
S.nigricornis	51	0	0	51
S.veracruzanum	0	29	0	29
S.tricornis	0	2	0	2
S.vepocapense	0	1	0	1
S.deleoni	0	0	73	73
S.parrai	0	0	43	43
S.ruizi	0	0	32	32
? S.anduzei	0	0	26	26
? S.d'andretta	0	0	21	21
? S.menchacai	0	0	1	1
S. spp.	57	7	88	152

Table 6.2 Number of streams sampled in each focus and number of collections in each focus.

	Focus : Soconusco	Chamula	Oaxaca (all)	All foci
No. of streams sampled	81	25	40	146
No. of collections	168	25	40	233

Table 6.3 Number of larvae found in each focus (see Table 6.1) divided by number of collections in that focus.

Species	Focus : Soconusco	Chamula	Oaxaca (all)
S.ochraceum :			
cytotype A	14.55	-	-
cytotype B	-	-	3.48
cytotype C	-	8.64	-
S.metallicum :			
cytotype A	48.45	28.44	5.10
cytotype B	1.54	0.76	1.38
cytotype H	2.65	0.08	1.35
cytotype I	8.97	0.12	20.63
cytotype X	-	-	3.85
S.callidum	18.46	2.96	16.40

Table 6.4 S.metallicum cytotypes found within each focus shown as percentages of total S.metallicum in each focus.

S.metallicum :	Focus : Soconusco	Chamula	Oaxaca (all)
cytotype A	78.6%	96.7%	15.8%
cytotype B	2.5%	2.6%	4.2%
cytotype H	4.3%	0.3%	4.2%
cytotype I	14.6%	0.4%	63.9%
cytotype X	0	0	11.9%

focus cytotype A accounted for nearly all the specimens found and cytotypes B, H and I were rare. In Oaxaca cytotype I was by far the most abundant species, cytotypes A and X were common and cytotypes B and H were rare.

S.callidum was present in all three foci, being abundant in the Soconusco focus and Oaxaca, and rarer in the Chamula focus.

6.3.3 Results of the canonical correspondence analysis and explanations of the output from CANOCO.

The majority of the output from the CANOCO programme, which consisted of a weighted correlation matrix, variance inflation factors, species scores, sample scores, biplot scores of environmental variables, canonical coefficients, t-values of canonical coefficients and results of the Monte Carlo significance test, may be seen in Appendix VI. The significance of each of these will be discussed in turn.

The canonical correspondence analysis produced six canonical axes and the eigenvalues for the first four were as follows :

Axis	Eigenvalue
1	0.33182
2	0.10519
3	0.08824
4	0.06915
Total	0.59440

The sum of all six eigenvalues was 0.62389. The first canonical axis was therefore the most important in separating the species. The percentage variance accounted for by the first four axes of the species-environment biplot was:

Axis	% variance
1	53.2%
2	70.0%
3	84.2%
4	95.3%

The first two canonical axes therefore explained seventy percent of the variation in the distribution of the species according to these six environmental variables.

In addition to the eigenvalue, the species-environment correlation is another measure of the association between species and environment, however axes with small eigenvalues may have misleadingly high species-environment correlations (Ter Braak, 1988). The species-environment correlation is the correlation between the site scores that are weighted averages of the species scores and the site

scores that are a linear combination of the environmental variables. In the weighted correlation matrix (Appendix VI (ii)) the species environment correlations are :

Axis	Correlation
1	0.7499
2	0.4800
3	0.4447
4	0.3836

In agreement with the eigenvalues, canonical axis 1 has the highest correlation with the environmental variables and the other three axes have correlations in a decreasing order of importance.

The Variance Inflation Factor (VIF) indicates the amount of correlation between environmental variables. If the VIF is large (>20), then one variable is almost perfectly correlated with another variable and therefore has no unique contribution to the regression equation (Ter Braak, 1988). From the table in Appendix VI (iii) it can be seen that the VIFs for the six variables are all less than 2 and therefore have low correlations with each other.

The species scores, sample scores and biplot scores for environmental variables were used to plot Figures 6.6 and 6.7. The species scores and sample scores have been plotted on separate diagrams for the sake of clarity and the diagrams may be superimposed. The figures show the species distribution and the sample (site) distribution on the first two canonical axes. The positions of the arrow heads of the environmental variables are given by the biplot scores and depend on the intra-set correlations of each environmental variable with the axes (Ter Braak, 1988). Only the direction and relative lengths of the arrows convey information and therefore these arrows were plotted on the E-scale, which is ten times larger than the S-scale

on which the species scores and sample scores were plotted. The coordinates of the species scores and sample scores on the first four canonical axes with their weights are given in Appendix VI (iii,iv,v and vi). The weights are equivalent to the abundance of the specimens. The interpretation of these figures is discussed in section 6.3.4.

The canonical coefficients are the coefficients of a weighted multiple regression of the sample scores (derived from the species scores) on the standardized environmental variables. The canonical coefficients indicate which environmental factors are important in each axis and the t values are approximate indicators of the significance of these coefficients. When $t < 2.1$ in absolute value, then the variable does not contribute much to the fit of the species data in addition to the contribution of the other variables in the analysis. These variables can be deleted with little effect on the canonical eigenvalue of that axis (Ter Braak, 1988).

From the values in Appendix VI (vii) it can be seen that pH was the most important factor in the first axis with a canonical coefficient of -0.321 and a t value of -4.71. The second most important factor was altitude, and size also had an effect. The other three variables did not contribute to this axis. Temperature was by far the most important factor in the second canonical axis with a canonical coefficient of -0.440 and a t value of -5.99. Altitude, pH and possibly size also had an effect in this axis. The results for the third and fourth axes are also shown but these have not been considered due to the low eigenvalues of these axes.

The inter-set correlations of the environmental variables with the species axes also indicate the environmental factors which have most effect in each axis (see Appendix VI (ii)). The order of importance of the environmental

factors in species axis 1 was pH, altitude, size, temperature, clarity and shade. In this set of correlations temperature had a larger influence on the axis than was inferred by the canonical coefficients. This may be due to intercorrelation of temperature with other factors, although its VIF was low. In the inter-set correlations temperature was still the most important factor in species axis 2 and pH was the only other factor that could be of importance. The fraction of the total variance in the standardized environmental data that was accounted for by each species axis was as follows :

Axis	Fraction accounted for
1	0.188
2	0.022
3	0.029
4	0.012

Species axis 1 was therefore much more important than the others in separating the species.

The Monte Carlo permutation test is a statistical test which assesses whether the species are related to the supplied environmental variables. This test randomly permutates the sample numbers in the environmental data, i.e. the environmental data are randomly linked to the species data, giving rise to a "random data set". If the species are correlated with the current environmental variables, then the test statistic calculated from the data-as-observed will be larger than most of the test statistics calculated from the random data. If the observed value is among the 5% highest values, then the species are significantly related to the environmental variables. The minimum number of permutations to give a significance level of 0.01 is 99. The test may either be applied to the first eigenvalue or to the trace, the sum of all the eigenvalues. When the first axis is tested by itself the result shows

whether this axis has a significant effect on the distribution of the species. When the whole trace is used an overall test of the effect of the environmental variables on the species is given (Ter Braak, 1988). The results for both are shown in Appendix VI (iii). The first canonical axis was found to be significant to at least the 0.01 level. However, the entire trace was not significant (p=0.08).

6.3.4 Results of the canonical correspondence analysis - interpretation of the ordination diagrams.

The ordination diagrams are shown in Figures 6.6 and 6.7. The species scores and sample scores are plotted on the diagrams as points and the arrows show the relative magnitudes of the environmental factors and the directions in which they act. Altitude and size are most closely associated with the first canonical axis and the angle between the axis and these arrows is therefore small. pH is also closely associated with the first axis although it has a higher correlation with the second axis than temperature and size. Temperature is correlated with both the first and second axes. The arrows for clarity and shade are short as these factors have little effect on the distribution of the species in comparison with the other factors.

Perpendiculars may be dropped from the species scores to the environmental arrows to indicate the approximate positions of the centres of the species' distributions along the environmental variables. Figure 6.8 shows the perpendiculars for the species' distribution along the pH gradient. It can be seen from this diagram that S.metallicum cytotype X had its centre of distribution at the lowest pH and S.ochraceum cytotype A at the highest pH. The species scores are approximated in the diagram as derivations from the grand mean of each environmental variable, the grand mean being represented by the origin of

Figure 6.6. Ordination diagram showing the species scores on the first two canonical axes. (META, METB, METH, METI and METX = S.metallicum cytotypes A, B, H, I and X; DOW = S.downsi; OCHA, OCHB and OCHC = S.ochraceum cytotypes A, B and C; PAYRUB = S.paynei/S.rubicundulum). (for explanation see text)

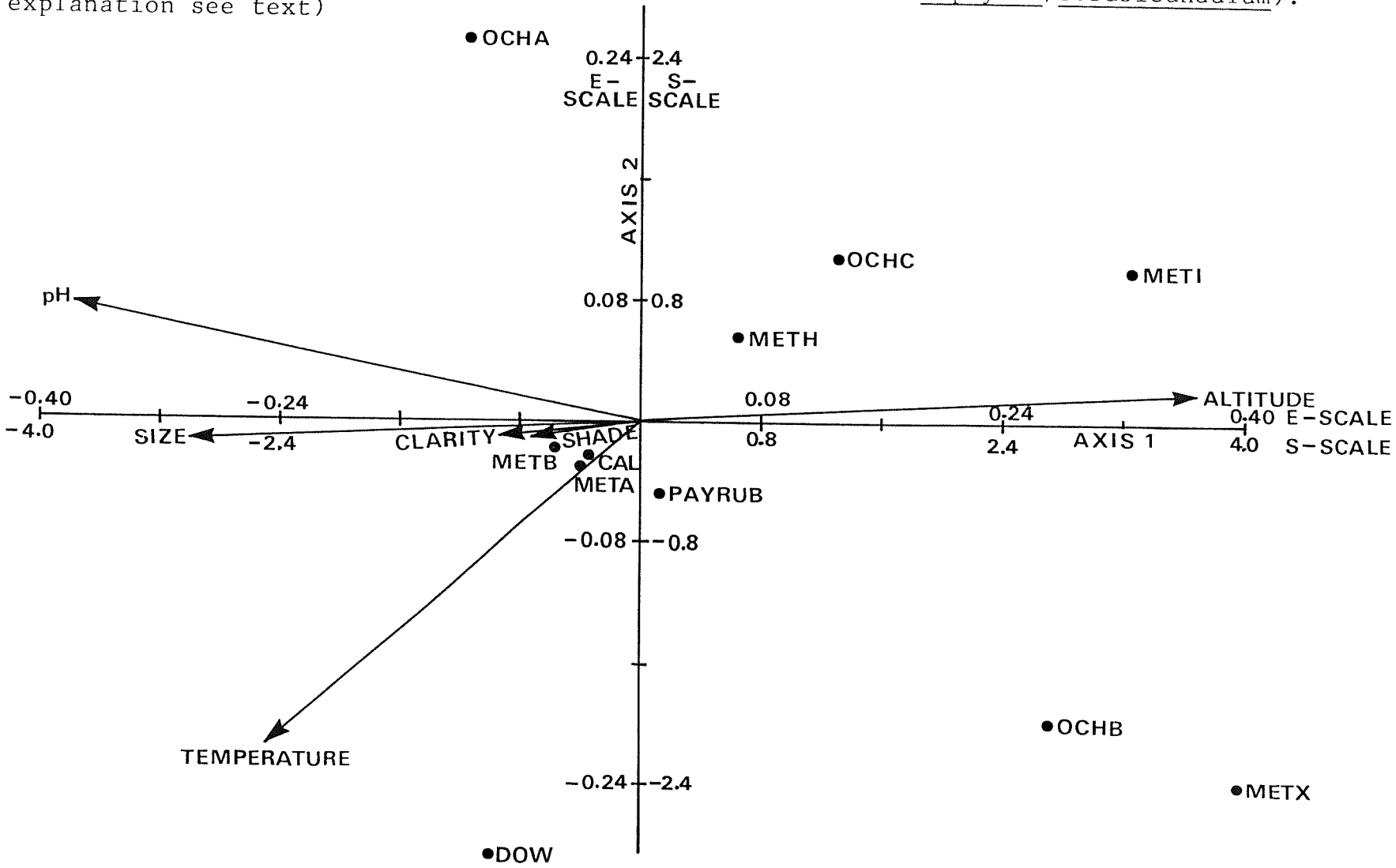
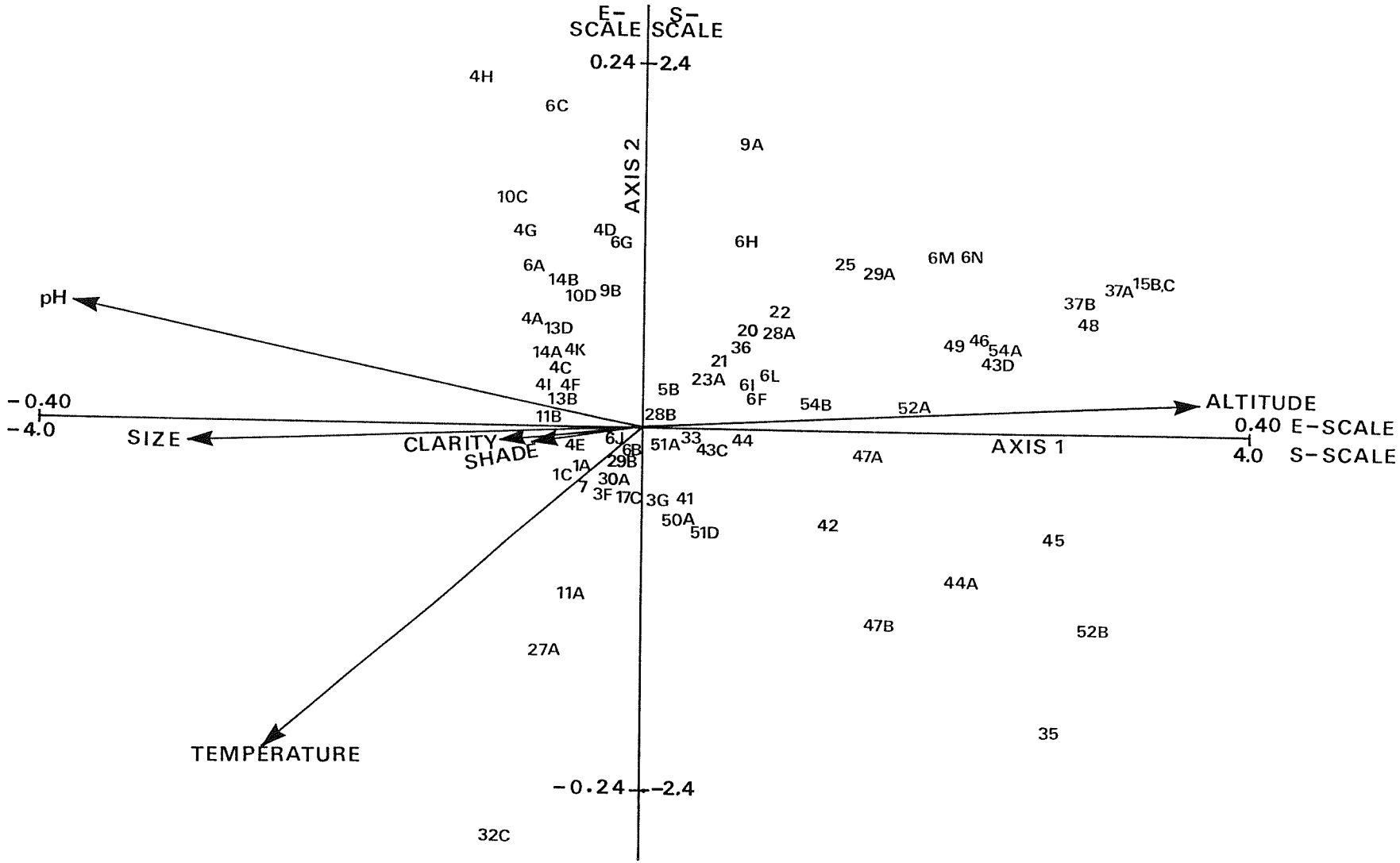


Figure 6.7. Ordination diagram showing the sample scores on the first two canonical axes. (for explanation see text).



the plot. The species score can therefore be considered as higher than average if the end point of a species lies on the same side of the origin as the head of an arrow does, and is lower than average if the origin lies between the end point and the head of the arrow (Ter Braak, 1986).

In Figure 6.9 the relative positions of the species scores can be seen on the altitude gradient. S.downsi had its species centre at the lowest altitude and S.metallicum cytotype X was found at the highest altitude. Similarly in Figures 6.10 and 6.11 the relative positions of the species scores on the size and temperature gradients are shown. It can be seen that different environmental characteristics are associated with different species.

S.ochraceum cytotype A had its centre of distribution in streams of relatively high pH, low altitude, large size and low temperature. S.ochraceum cytotype B was centred in streams of relatively low pH, high altitude, small size and low temperature. S.ochraceum cytotype C had its centre of distribution for pH, altitude and size at points intermediate between those for cytotypes A and B. It occurred at a slightly lower temperature than A and B.

S.metallicum cytotype A had its centre of distribution in streams with just above average pH, size and temperature and just below average altitude. S.metallicum cytotype B was centred in streams similar to those of cytotype A except the pH was slightly higher, the altitude slightly lower and the size slightly larger. S.metallicum cytotype H had its centre of distribution at slightly lower than average pH, size and temperature, and slightly higher than average altitude. S.metallicum cytotype I was centred at low pH, high altitude, small size and low temperature. S.metallicum cytotype X had its centre of distribution at even lower pH, higher altitude and smaller size than cytotype I, although cytotype X was found in streams of higher temperature.

Figure 6.8. Centres of species' distribution along the pH gradient.
(for abbreviations see Figure 6.6.)

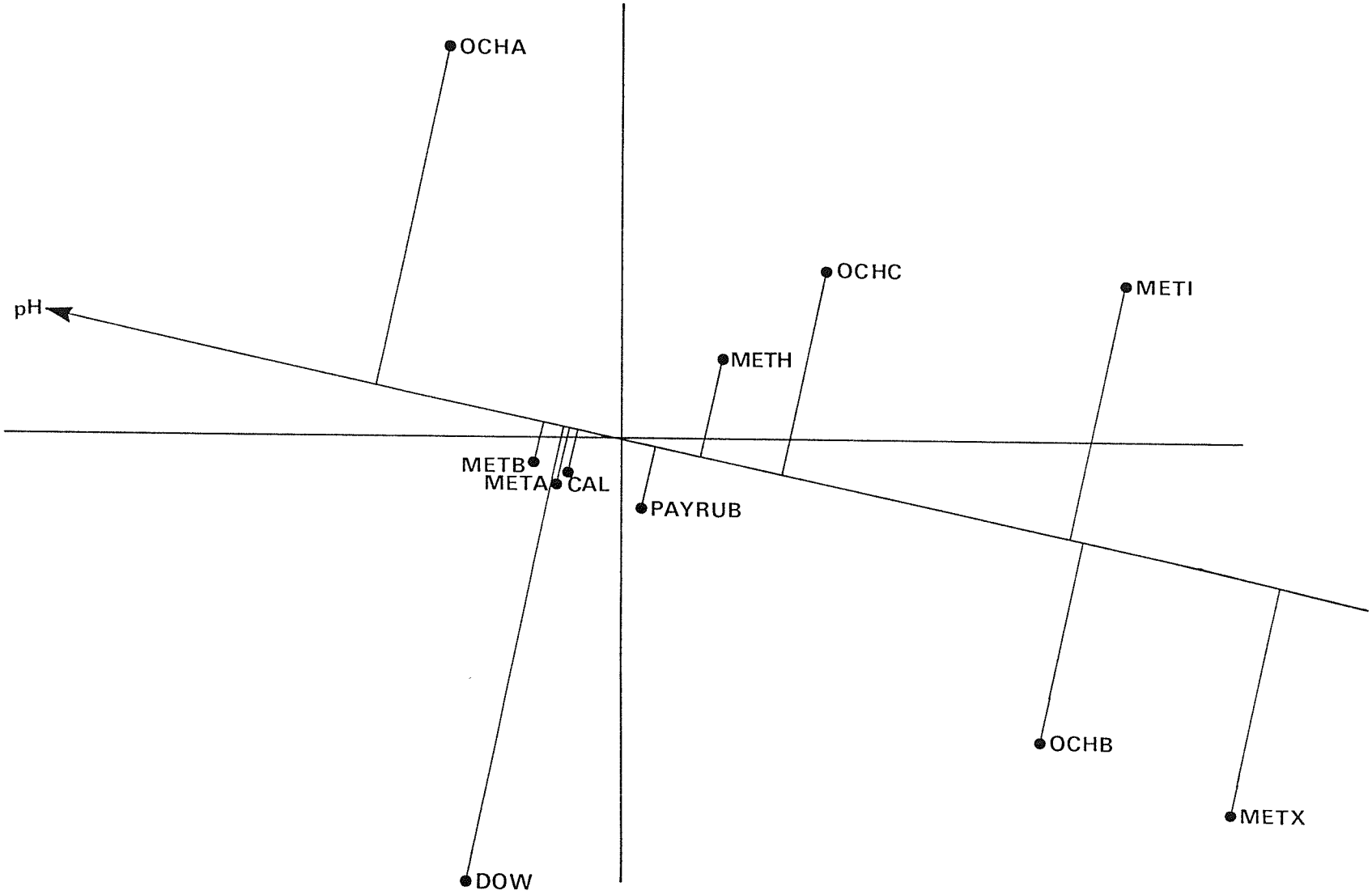


Figure 6.9. Centres of species' distribution along the altitude gradient. (for abbreviations see Figure 6.6.)

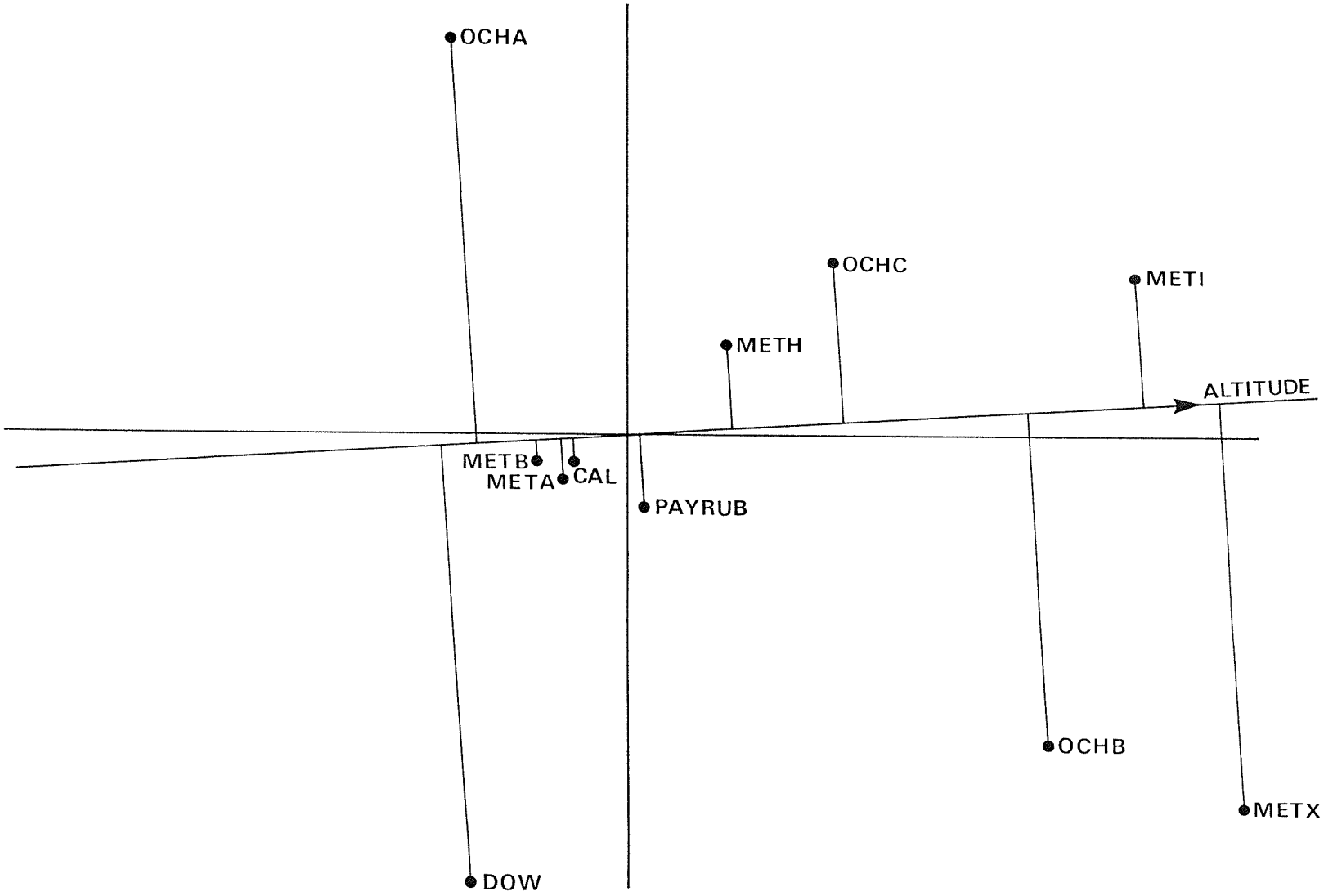


Figure 6.10. Centres of species' distribution along the size gradient.
(for abbreviations see Figure 6.6.)

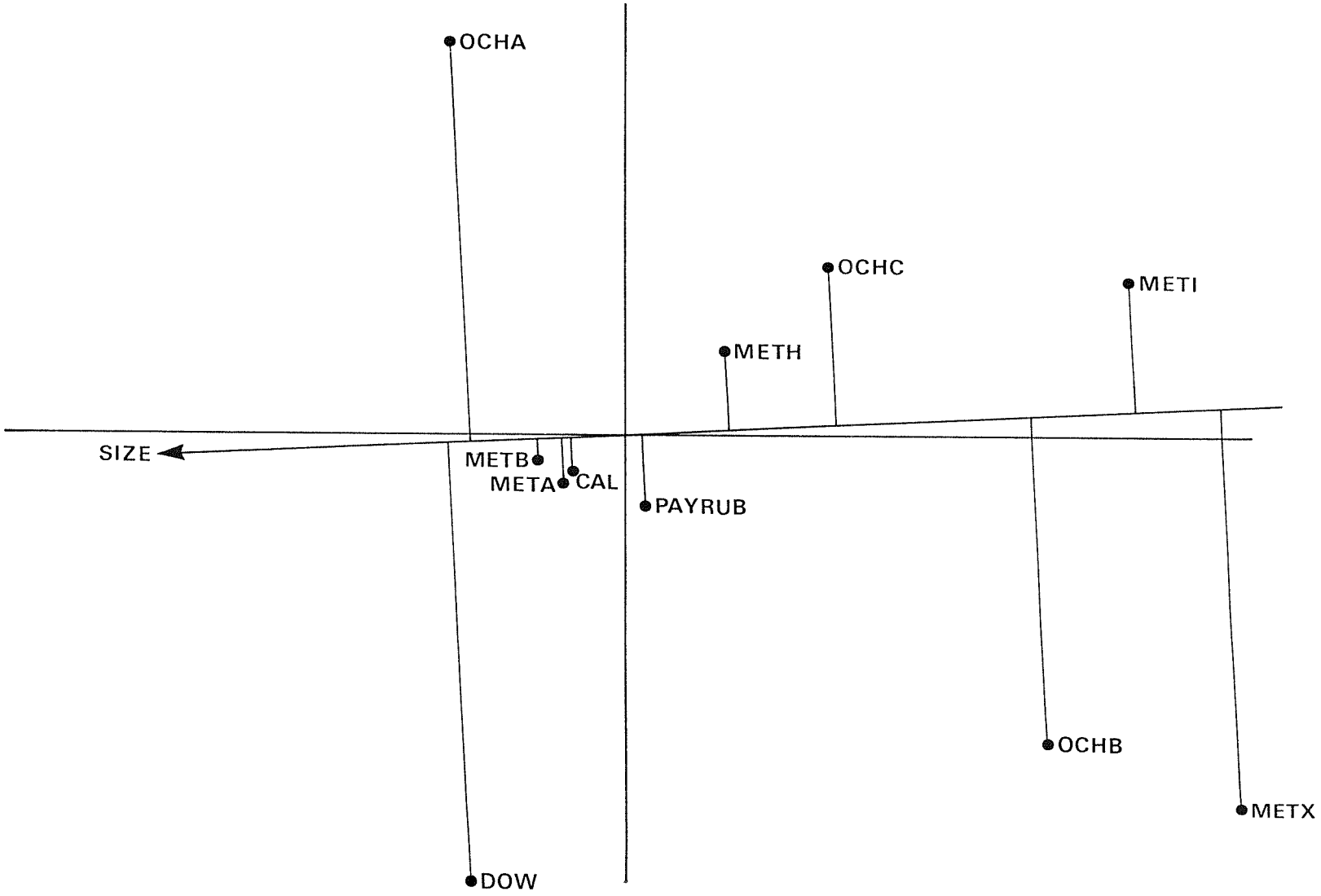
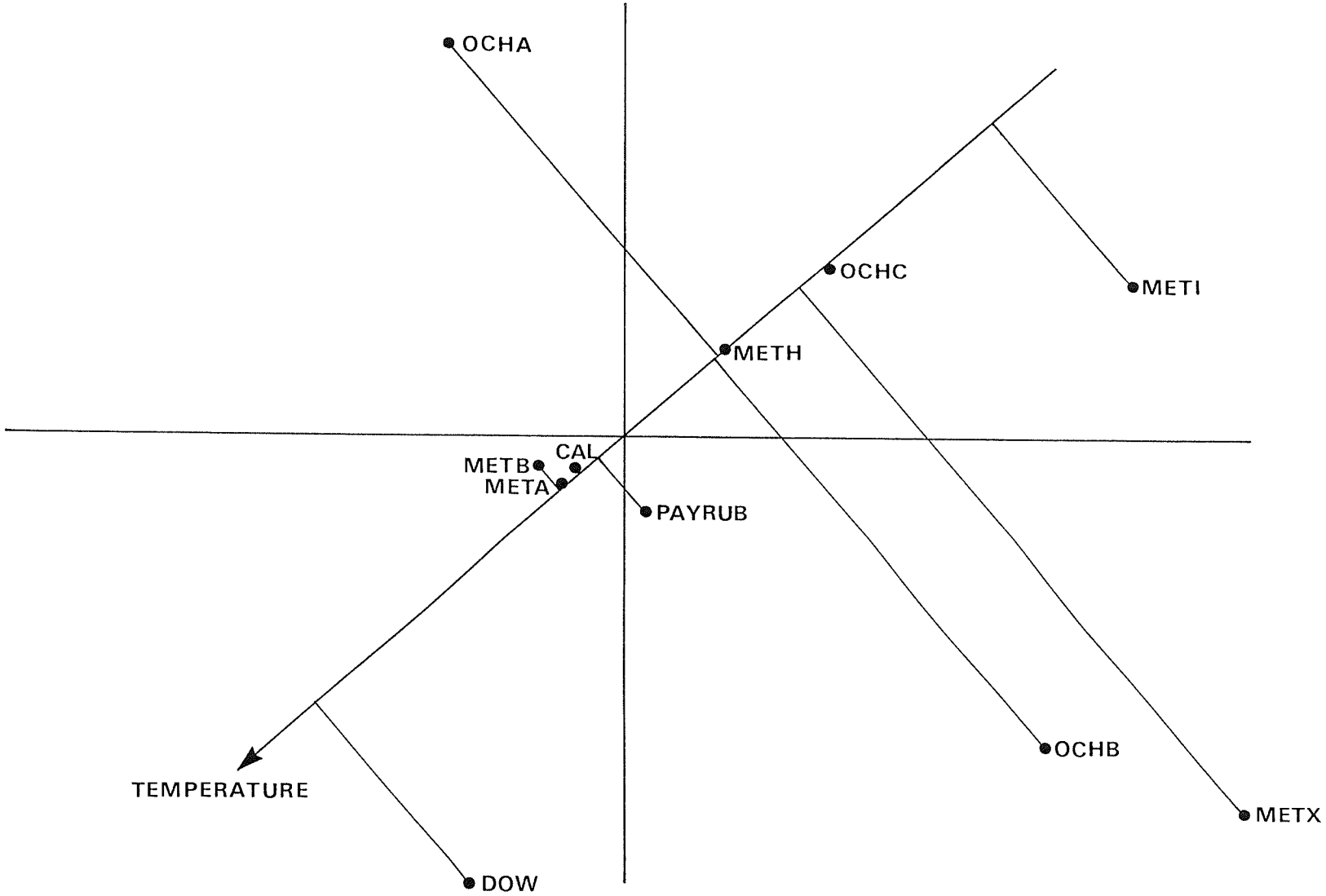


Figure 6.11. Centres of species' distribution along the temperature gradient.
(for abbreviations see Figure 6.6.)



S.callidum had its centre of distribution very close to S.metallicum cytotype A. S.paynei, which was scored jointly with S.rubicundulum, had its species score near to the origin of the plot for all the environmental variables. S.downsi was centred at slightly higher than average pH, low altitude, large size and high temperature.

In Figure 6.7 the distribution of the different sites is shown. Sites one to sixteen occur in the Soconusco focus, sites seventeen to 32 in the Chamula focus and sites 33 to 54 in Oaxaca. Some sites are not shown due to overlap but the coordinates of all sites are given in Appendix VI (iv,v,vi). It can be seen that the distribution of the sites in relation to the environmental variables was not constant between the foci. All sites in the upper left hand quarter of the diagram are in the Soconusco focus. All the Chamula sites, except one, occur in the lower left and upper right hand quarters of the diagram. The majority of sites in Oaxaca occur in the lower and upper right hand quarters.

Each site point lies at the centroid of the species points that occur at that site. Therefore it can be inferred from the diagram which species are likely to be present at that particular site. The abundance or probability of occurrence of a species decreases with distance from its location in the diagram (Ter Braak, 1986). For example, S.ochraceum cytotype B and S.metallicum cytotype X, which only occurred in Oaxaca, are situated in the lower right hand corner and the nearest site points are all in Oaxaca.
Tables 6.5, 6.6, 6.7 and 6.8 are summaries of how many streams from each focus occurred in each pH, altitude, size and temperature range respectively. Exact values for each stream are shown in Appendix IV. It can be seen from Table 6.5 that pH varied between the three foci. In the Soconusco and Chamula foci over 60% of the streams were in the pH range 6.1 to 6.5, less than 30% were in the range 5.6 to 6.0 and a few streams were more acidic. However in the

Table 6.5 pHs of the streams used in the canonical correspondence analysis.

pH range :	Number of streams in each focus : Soconusco	Chamula	Oaxaca
4.6-5.0	0	2 (8.3%)	11 (32.4%)
5.1-5.5	1 (2.1%)	2 (8.3%)	13 (38.2%)
5.6-6.0	14 (29.8%)	5 (20.9%)	10 (29.4%)
6.1-6.5	32 (68.1%)	15 (62.5%)	0

Table 6.6 Temperatures of the streams used in the canonical correspondence analysis.

Temperature range (°C):	Number of streams in each focus : Soconusco	Chamula	Oaxaca
10-11	0	0	1 (2.9%)
12-13	0	1 (4.3%)	2 (5.9%)
14-15	0	2 (8.7%)	2 (5.9%)
16-17	3 (6.2%)	4 (17.4%)	9 (26.5%)
18-19	15 (31.3%)	14 (60.9%)	11 (32.4%)
20-21	13 (27.1%)	2 (8.7%)	8 (23.5%)
22-23	6 (12.5%)	0	1 (2.9%)
> 24	11 (22.9%)	0	0

Table 6.7 Altitudes of the streams used in the canonical correspondence analysis.

Altitude range (m):	Number of streams in each focus : Soconusco	Chamula	Oaxaca
0-304	8 (11.9%)	0	0
305-609	3 (4.5%)	0	2 (5.9%)
610-914	24 (35.8%)	1 (4.2%)	3 (8.8%)
915-1219	19 (28.4%)	9 (37.5%)	12 (35.3%)
1220-1524	11 (16.4%)	13 (54.1%)	10 (29.4%)
1525-1829	2 (3.0%)	1 (4.2%)	4 (11.8%)
1830-2134	0	0	3 (8.8%)

Table 6.8 Sizes of the streams used in the canonical correspondence analysis.

Stream size : (see section 6.2.1):	Number of streams in each focus : Soconusco	Chamula	Oaxaca
1	6 (9.0%)	2 (8.3%)	3 (8.8%)
1/2	4 (5.9%)	2 (8.3%)	2 (5.9%)
2	28 (41.8%)	10 (41.7%)	22 (64.7%)
2/3	5 (7.5%)	1 (4.2%)	3 (8.8%)
3	16 (23.9%)	8 (33.3%)	2 (5.9%)
3/4	4 (5.9%)	0	0
4	3 (4.5%)	1 (4.2%)	2 (5.9%)
5	1 (1.5%)	0	0

Oaxaca focus the streams were all in the pH range 5.0 to 6.0. It can be seen from Table 6.6 that the streams in the Soconusco focus tended to be warmer, although there was overlap in stream temperatures amongst the three foci. The Soconusco focus had streams ranging from 150m to 1620m in altitude (Table 6.7). The altitudes of the streams in the Chamula focus were more restricted, between 760m and 1720m, with the majority in the range 915m to 1524m. The streams in the Oaxaca focus ranged from 440m to 1930m, although they were most prevalent between 915m and 1524m. The distribution of stream sizes did not vary greatly amongst the three foci (Table 6.8).

One stream from the Chamula focus and four from Oaxaca, which occurred at higher altitudes, were not used in the analysis. These did not contain any of the common species and are marked by an asterisk in Appendix IV.

6.4 Discussion.

The canonical correspondence analysis showed that the distribution of the different species was associated with the environmental characteristics of the streams. Four of the six environmental variables chosen: pH, altitude, size and temperature, were correlated with the first two canonical axes. These two axes explained 70% of the variation that was associated with these six factors. The two variables, clarity and shade, had little correlation with the first two axes. Altitude, pH and size were strongly correlated with the first axis and temperature was correlated with both the first and second axes. A Monte Carlo permutation test showed that the species data was significantly correlated with the first canonical axis ($p<0.01$). The species data was however only correlated to the whole trace at the 0.08 probability level.

When Dalmat (1955) made his survey of the blackflies of Guatemala he characterised the stream types and stream conditions in which each species was found. He divided the streams he encountered into five types: infant, young, adolescent, mature and old. In the present study, size one streams were approximately equivalent to infant streams, size two and three streams to young streams, size four streams to adolescent streams and size five streams to mature streams. No old streams were sampled in this study. He found that the anthropophilic species, S.ochraceum, S.metallicum and S.callidum, predominated in the infant and young streams, with both anthropophilic and zoophilic species in the adolescent streams and mainly zoophilic species in the mature streams. The majority of streams in the present study were in the infant and young categories and, in agreement with Dalmat contained mainly anthropophilic species.

Dalmat (1955) described the optimum conditions for each of the anthropophilic species but considered them to be distinct species and not species complexes. In this study, when the species were considered to be complexes, they were found to distribute differently with the environmental variables. Dalmat (1955) considered S.metallicum to be an abundant and widely distributed species which occurred in a large range of ecological conditions. In this study when the species was divided into five members of the complex, the different members were associated with different environmental characteristics.

The results of the analysis showed that S.metallicum cytotype B mainly occurred in the larger streams at the lower altitudes which had relatively high temperatures and high pHs. S.metallicum cytotype A was the most widely distributed of the cytotypes but was most abundant in streams similar to those in which cytotype B occurred. S.metallicum cytotype H had its centre of distribution in smaller streams at a higher altitude, lower temperature and

lower pH than cytotypes A and B. S.metallicum cytotype X was most abundant in the smallest streams at the highest altitudes, lowest pHs and low temperature. S.metallicum cytotype I was also most prevalent in small streams at high altitude and low pH and occurred at the lowest temperature.

Takaoka (1982) reported that S.horacioi larvae in Guatemala occurred throughout the year and tended to prefer small, permanent streams as a habitat, although they also colonized temporary streams. Very few S.horacioi larvae were found in medium-sized streams. This contrasted with S.metallicum larvae which occurred in a wide range of habitats from small trickles to large streams. Yamagata (1984) studied stream gradient and water discharge in relation to the Simulium species present and found that S.horacioi and S.metallicum occurred under different conditions. S.horacioi was most prevalent in streams with a steep gradient and small discharge, whereas S.metallicum was most prevalent in streams with a gentle gradient and large discharge. Streams with a steep gradient and small discharge tend to occur at the higher altitudes.

The S.horacioi larvae were not examined cytologically in Takaoka's and Yamagata's studies, but on the evidence of Chapter 4 they were most probably S.metallicum cytotype H or otherwise cytotype I. In this study S.metallicum cytotypes H and I tended to occur in smaller streams at higher altitudes than cytotypes A and B, which is in agreement with Takaoka's and Yamagata's findings. S.horacioi larvae were not found below 400m in altitude by Takaoka (1980). In the present study two specimens of S.metallicum cytotype H were identified at site 1a, which was at an altitude of 300m, but all the other cytotype H and I specimens occurred above 400m.

The relative abundance of the different S.metallicum cytotypes varied between the foci. Nearly all the S.metallicum specimens found in the Chamula focus were

cytotype A and it was also the commonest cytotype in the Soconusco focus accounting for nearly 80% of the specimens. However, in the Oaxaca focus, cytotype I was by far the most common accounting for over 60% of the specimens with cytotype A only consisting of 15%. Cytotype I accounted for approximately 15% of the S.metallicum in the Soconusco focus and was negligible in the Chamula focus. Cytotypes B and H occurred in low numbers in all three foci. Cytotype X was only found in Oaxaca.

There are several possibilities why the abundance and proportions of the different cytotypes varied between the foci. Firstly, it was apparent from the ordination diagram (Figure 6.7) that sites from different foci had different environmental characteristics. This was particularly noticeable in the case of pH where the majority of the Soconusco and Chamula sites were more alkaline and the Oaxaca sites were more acid. The streams also tended to be at lower altitudes in the Soconusco focus than the Chamula and Oaxaca foci, although there was overlap. It was not possible to sample streams at comparable altitudes in the three foci as the altitudes of the foci vary and the lower streams are very inaccessible in the Chamula and Oaxaca foci. However, if more streams had been sampled at lower altitudes in these foci the proportions of the different S.metallicum cytotypes may have changed, as the analysis showed that cytotypes A and B occurred at lower altitudes than H, I and X.

Secondly, the number of collections made in each focus varied. In the analysis 67 samples from the Soconusco focus, 24 from the Chamula focus, 34 from the Oaxaca focus and 2 from outside the Oaxaca focus were used. Therefore the collections in the Soconusco sampled a wider range of streams and produced a greater number of larvae.

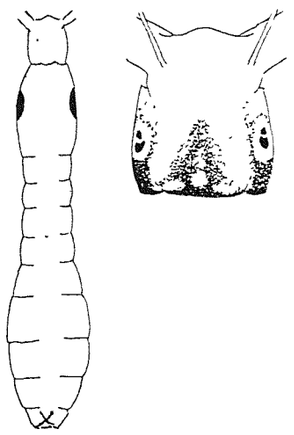
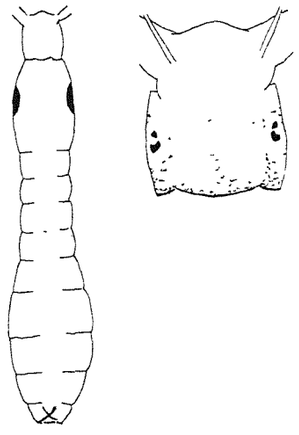
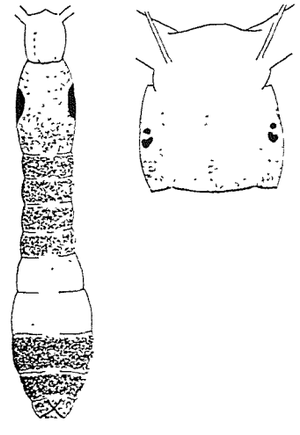
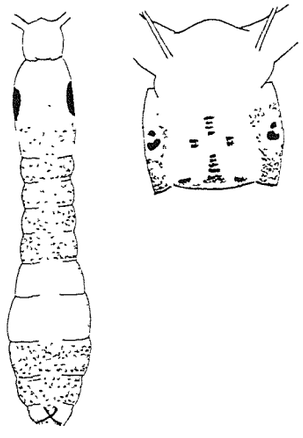
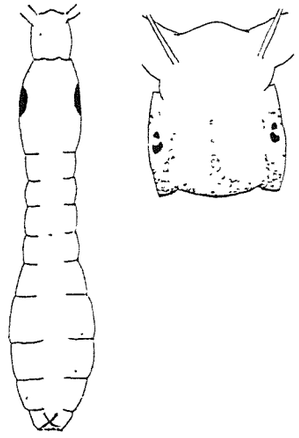
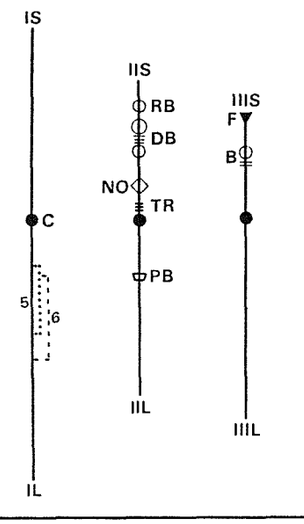
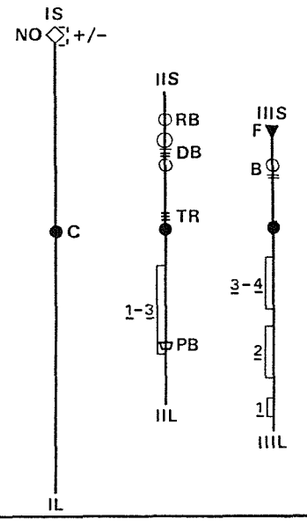
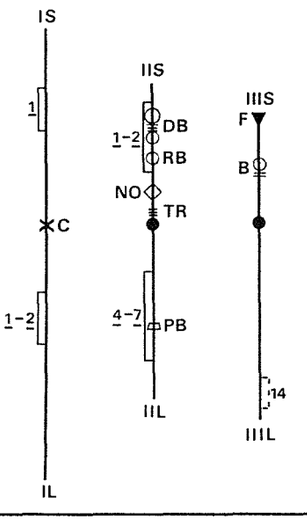
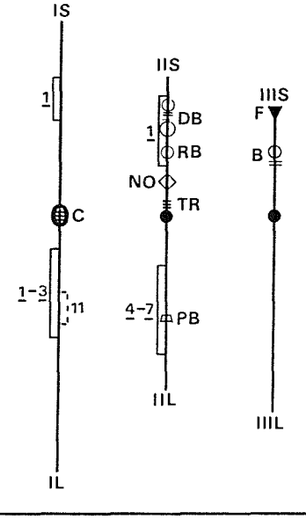
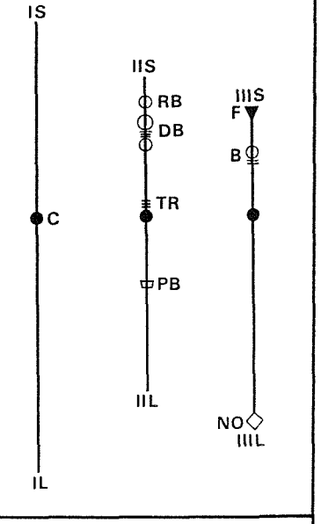
Thirdly, the collections were not all made at the same time of year or in the same year and this may have affected the

proportions of the different cytotypes sampled. In the Soconusco focus 23 of the collections were made in the rainy season of 1985 (June, July and August), 11 in the early dry season of 1987 (October and November) and 33 in the mid dry season of 1987 (January and February). In the Chamula focus, 19 of the collections were made in the early dry season of 1987 and five in the mid dry season of 1987. All the Oaxaca collections were in the early dry season of 1987. The state of the streams is dependent on the amount of rainfall and in the rainy season many temporary streams occur. These disappear in the dry season and the volume of flow decreases in the permanent streams which remain. The abundance of the different species of Simulium larvae does not remain constant throughout the year or between years but varies with the number and type of streams that are flowing (Dalmat, 1955; Okazawa, 1987; Ortega and Oliver, 1985; Takaoka, 1981, 1982).

As the abundance of larvae of each Simulium species varies throughout the year and between years with the rainfall, it would be necessary to monitor the abundance of the S.metallicum cytotypes throughout the year, and preferably for more than one year, to establish whether the percentages of the different cytotypes vary greatly between seasons and between years.

In Chapter 4 of this thesis it was found that the different S.metallicum cytotypes could be separated in the majority of cases by differences in larval head pattern and body colouration. Differences in stream preferences may also assist in identifying specimens. Pistrang and Burger (1988) studied the larval distribution of the S.tuberosum (Lundström) species complex in New Hampshire, U.S.A. and managed to distinguish some cytospecies by a combination of differences in head and body colour, and in stream characteristics. A similar classification is possible with S.metallicum. The salient taxonomic characters and ecological preferences are summarised in Table 6.9

Table 6.9 A summary of the characteristics associated with the different members of the S.metallicum species complex.

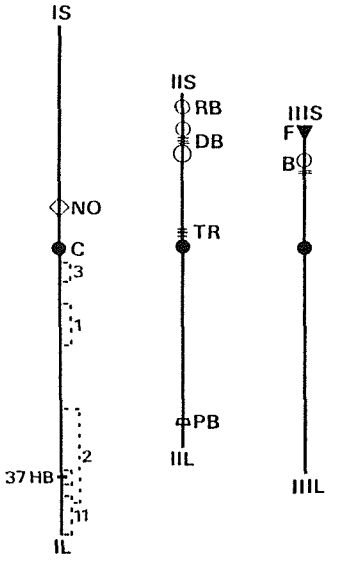
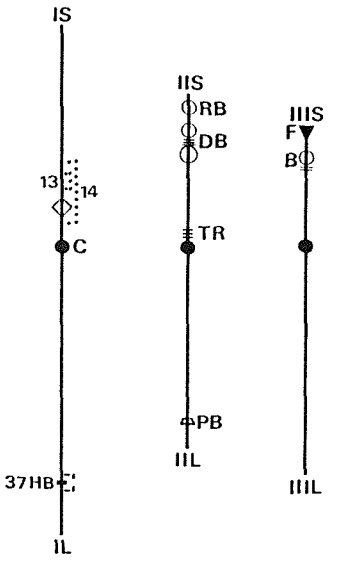
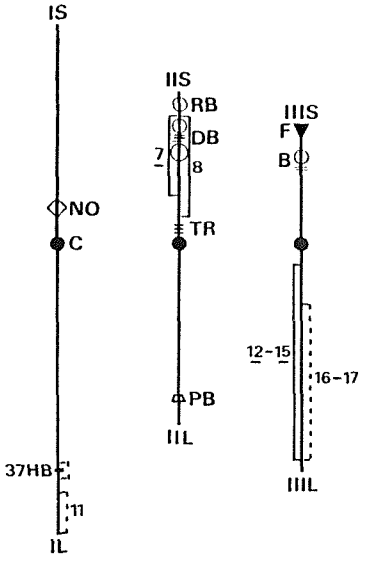
CYTOTYPE	A	B		H	I	X
LARVAL MORPHOLOGY (Ch. 4)						
CHROMOSOME IDIOGRAM (Ch. 2)						
STREAM CHARACTERISTICS:						
pH range	5.0 – 6.5	5.0 – 6.5		5.0 – 6.5	5.0 – 6.5	5.0 – 5.9
altitude range (m)	150 – 1510	270 – 1510		300 – 1330	440 – 1860	910 – 1510
size range (see 6.2.1)	1 – 4	1 – 3/4		1 – 3	1 – 4	1 – 3
temperature range (°C)	15° – 26°	16° – 26°		17° – 23°	13° – 21°	17° – 20°
DISTRIBUTION IN MEXICO (FOCI) (Ch. 2)	Soconusco Chamula Oaxaca	Soconusco Chamula Oaxaca		Soconusco Chamula Oaxaca	Soconusco Chamula Oaxaca	Oaxaca
DISTRIBUTION OUTSIDE MEXICO (Conn, 1988)	Guatemala	Guatemala		Guatemala Panama Colombia	Guatemala	–

A different cytotype of S.ochraceum was found in each of the foci and there was variation in the ecological conditions in which each cytotype prevailed. S.ochraceum cytotype A occurred in the Soconusco focus and was most prevalent in relatively large streams at low altitudes and high pH. S.ochraceum cytotype C was found in the Chamula focus and was most abundant in smaller streams at higher altitudes and lower pH than cytotype A. S.ochraceum cytotype B was collected in the Oaxaca focus and was most abundant in streams which were smaller in size, higher in altitude and lower in pH than either cytotype A or C. The average temperature of the streams in which S.ochraceum cytotypes A and B occurred was the same but cytotype C was more prevalent at slightly lower temperatures.

The abundance of S.ochraceum cytotypes varied between the foci in these collections. However, as mentioned earlier, collections were not made from comparable sites between the foci. S.ochraceum cytotypes B and C may have been more abundant at lower altitudes than were sampled or they may have been more abundant at other times of the year. More extensive studies would be needed to assess these possibilities. The differences in the cytology, range of stream conditions and geographical distribution of the larvae of the three S.ochraceum cytotypes are summarised in Table 6.10.

S.callidum was found in all three foci and had a wide distribution. It was however most abundant in streams similar to the ones in which S.metallicum cytotypes A and B occurred. S paynei and S.rubicundulum were scored together in the analysis and therefore their position on the ordination diagram is rather artificial. Two thirds of these larvae however were probably S.paynei. They occurred in a position near to the centre of the plot and therefore the values for pH, altitude, size and temperature were average for those streams sampled. S.downsi was most

Table 6.10 A summary of the characteristics associated with the different members of the S.ochraceum species complex.

CYTOTYPE		A	B	C
CHROMOSOME IDIOGRAM (Ch. 2)				
CUTICULAR HYDROCARBON ANALYSIS (Ch. 5)		Variation in peak profiles between cytotypes		
STREAM CHARACTERISTICS (Ch. 6)	pH range	5.9–6.5	5.0–5.9	5.0–6.5
	altitude range (m)	640–1510	650–1580	990–1720
	size range (see 6.2.1)	1 – 3/4	1 – 2	1–3
	temperature range (°C)	17.5°– 22.5°	16°– 22°	13°– 19°
DISTRIBUTION IN MEXICO (FOCI) (Ch. 2)		Soconusco	Oaxaca	Chamula
DISTRIBUTION OUTSIDE MEXICO (Hirai et al, in preparation)		Guatemala (inside focus)	–	Guatemala (outside focus)

prevalent in large streams at low altitude, low temperature and just above average pH.

Chapter 7.

General discussion - relationship between the distribution of members of the Simulium species complexes and onchocerciasis in Mexico.

7.1 Factors affecting the distribution of onchocerciasis in Mexico.

The distribution and prevalence of the disease in Mexico is the result of a complex set of factors and these must be considered before any relationships regarding the influence of different vector cytotypes can be drawn. Although there are three distinct foci in Mexico where the disease occurs it does not have a uniform prevalence throughout these areas. Disease levels may vary between nil and hyperendemic in different communities. In this dissertation the terms hypoendemic and hyperendemic are used in the sense of a low or high level of disease and do not have the same significance as the terms when applied to onchocerciasis in West Africa (WHO, 1987). It was impossible to obtain information on the exact levels of disease in many areas at the time of writing and therefore hypoendemic and hyperendemic are used as an indication of the prevalence of disease.

There is evidence that the distribution of onchocerciasis in Mexico has altered during the last few decades. In 1962 the disease was most prevalent in the Soconusco focus in the municipalities of Amatenango de la Frontera, Angel Albino Corzo, Comaltitlan, Escuintla, Huixtla, Mapastepec, Motozintla and Siltepec. The locations of the various municipalities are shown in Figure 7.1. The disease level was as high as 90% to 95% in some communities where the altitude and ecological conditions were favourable for large populations of biting flies (García Sánchez and Chávez Núñez, 1962). However, by 1978, the municipalities

with the highest prevalences of the disease were Acacoyagua, La Concordia, Escuintla, Huixtla, Mapastepec and Pijijiapan (Martínez Reynoso, 1979a). This resulted from the continuing trend, which was reported by García Sánchez and Chávez Núñez (1962), of people moving from hyperendemic areas in the east to the largely-uninhabited area to the west. The disease appears to spread mainly with the movement of people and not the migration of flies. There is good evidence to suggest that the flies remain near their larval sites.

Some of this evidence is derived from the results of a control campaign. When the pilot larviciding operation was carried out in the Rio Despoblado valley between 1954 and 1958 the level of adults was notably lowered in the centre despite the fact that it was only a relatively small area (see section 1.4.4). Flies appeared to invade the edge of the area from the outside but did not penetrate into the centre, suggesting that their ability to disperse was limited. Alvarez (1962) pointed out that there are very few Simulium in some places within the onchocerciasis zone and there appears to be no invasion of flies from adjacent areas where they are more abundant. Each locality has a density of flies proportional to the number of breeding sites there. Porter and Collins (1988) found a similar situation when looking at the prevalence of biting flies at four sites in Guatemala. One reason for the limited dispersal of the flies may be that the series of ridges and valleys which make up the foci act as barriers to movement.

Ortega et al (1985) have noticed a difference in the disease prevalance related to the conditions under which coffee is grown. Onchocerciasis appears to be absent in the highly cultivated coffee regions where there are large privately-owned fincas. These are situated in Union Juarez, Cacahoatan, Tapachula, Tuzantan and Huehuetan (see Figure 7.1) and have been established for 50 years or more. In contrast, coffee has been grown in the last 30 years

between Huixtla and Motozintla in small plots from which the natural vegetation has been cleared. In some of these poor communities the prevalence of onchocerciasis is 90%.

The ecological conditions in these two types of area are very different (Ortega, 1983). In the advanced fincas a relatively large area of land is modified exclusively for growing coffee. All the original vegetation is removed and replaced by rows of coffee bushes interspersed with trees planted specifically for shade. This alters the microclimatic conditions and could affect the adult flies. A high proportion of the streams are cleared of debris and clean water channels are made which limits the amount of substrate on which the simuliids can lay their eggs. On the coffee plants there is heavy use of pesticide sprays which enter the streams as run off and drift and undoubtedly affect the simuliid larvae. In the processing of coffee large amounts of coffee bean hulls are produced which ferment in water and alter the conditions of the streams. Simuliid larvae do not occur in streams where coffee hulls are found. The number of simuliid breeding sites on the finca is therefore small and the workers are only exposed to relatively low levels of biting flies.

In contrast, in the poorly-developed coffee growing regions small areas are cleared of forest and coffee bushes are planted. These areas are interspersed with patches of natural vegetation. The streams are not cleared of debris and little pesticide is used. There are abundant simuliid breeding grounds close to where the human population live and work, and biting flies occur in high numbers. The people tend to live in very poor accommodation and are frequently bitten inside their houses as well as outside (Ortega and Oliver, 1984). These observations suggest that the local ecological conditions, principally those affecting the breeding sites and the level of agricultural advancement, have a strong influence on the transmission of the disease.

Another reason why the prevalence of the disease varies is that there is an optimum altitude range where transmission occurs, which is between 500m and 1200m. In this zone the principal vectors are abundant and the developmental rate of the parasite in the fly is rapid. This rate is dependent on the ambient temperature, which varies according to altitude (Takaoka et al, 1981). Above 1800m no transmission takes place, probably due to the low numbers of vectors and the slow development of the parasite in the fly.

Hyperendemic areas of onchocerciasis tend to have biting populations of flies, principally S.ochraceum, occurring in large numbers. Although this species is the primary vector its transmission capacity is poor in relation to species such as S.damnosum in West Africa. Therefore, in general, high numbers of biting flies are needed to transmit and maintain the disease. However, this is also dependent on the reservoir of the parasite in man. Where this is large, relatively few biting flies may be necessary to maintain the disease transmission.

Onchocerciasis does not appear to be spreading to the south-east corner of the Soconusco focus although vectors occur in this region and there is movement of people between affected and unaffected areas. This may be because low levels of disease have not been detected or the vectors are unable to maintain transmission where the parasite pool is small. It has also been stated that some finca owners will not employ people who have the disease, which would reduce the potential parasite level (J.B.Davies, pers. comm.).

The levels of the parasite actually occurring in the human population now are not a natural consequence of the transmission of the disease. The reason for this is that nodulectomy and chemotherapy have been employed in the onchocerciasis foci in Mexico since the 1930s. In principle, the inhabitants of each infected community have

been treated regularly for several decades. In practice, not all communities have been visited regularly and only a proportion of the infected people seek treatment. One of the main reasons for this is the undesirable side effects of the drugs administered (see section 1.4.2).

Whether an area is free of the disease, hypoendemic or hyperendemic is therefore due to an interrelationship between the reservoir of parasites in the human population, the species and number of vectors biting and the level of treatment administered to the people.

7.2 Distribution of the different S.ochraceum and S.metallicum cytotypes in relation to the disease.

7.2.1 General comments on the abundance and proportions of the cytotypes collected.

In Chapter 6 the larval distribution of Simulium species was examined in the three foci in relation to environmental conditions. S.ochraceum was found to have a different cytotype in each focus and the ecological conditions in which each occurred varied. S.metallicum was found to consist of five cytotypes which had varying abundances and proportions between the three foci. However, the sites sampled were not directly comparable between foci and the abundances and proportions of the different cytotypes may have altered if, for example, more sites at lower altitudes had been sampled in the Chamula and Oaxaca foci. The times of year in which the samples were made also varied and this could affect the cytotypes collected and their abundance. The collections made in this study therefore were not representative of the whole year or of the complete range of habitats in each focus.

It is impossible to conclude which cytotypes may be the most important vectors solely from information on their

distribution and abundance as larvae. The abundance of a species biting man is highly dependent on its level of anthropophily and not just its local larval abundance. In areas where it occurs S.ochraceum is the predominant species biting man, although S.metallicum is frequently the most common species found in the streams nearby (Dalmat, 1955; Ortega and Oliver, 1985). However, this present study does give an indication of which cytospecies are present in each region and therefore which cytospecies may be involved in disease transmission.

7.2.2 The Soconusco Focus.

This is the largest of the three foci and the areas with the highest prevalences of the disease were stated in section 7.1. The municipalities, with their levels of disease in 1960 and 1978, are shown in Table 7.1. In this study collections were made in the municipalities of Mapastepec, Acacoyagua, Escuintla, Huixtla, Motozintla, Tuzantan, Tapachula and Union Juarez. The first four are municipalities with high disease prevalences in 1978 and the last is outside the focus.

S.ochraceum larvae were examined cytologically from all the localities in which they occurred and were found to be cytotype A (see Chapter 2). All S.ochraceum specimens collected were therefore considered to be cytotype A and were found in all the municipalities sampled except Mapastepec (see Figure 7.1). However, in this last municipality all the collections in the three communities were at altitudes below 400m, where this species is rarely found (Dalmat, 1955; Ortega et al, 1985). Despite this, Nueva Costa Rica, is known to be at least hypoendemic for the disease as this was one of the communities chosen for the 1987 ivermectin trial. This community, on account of its altitude, was selected as an area of low endemicity but

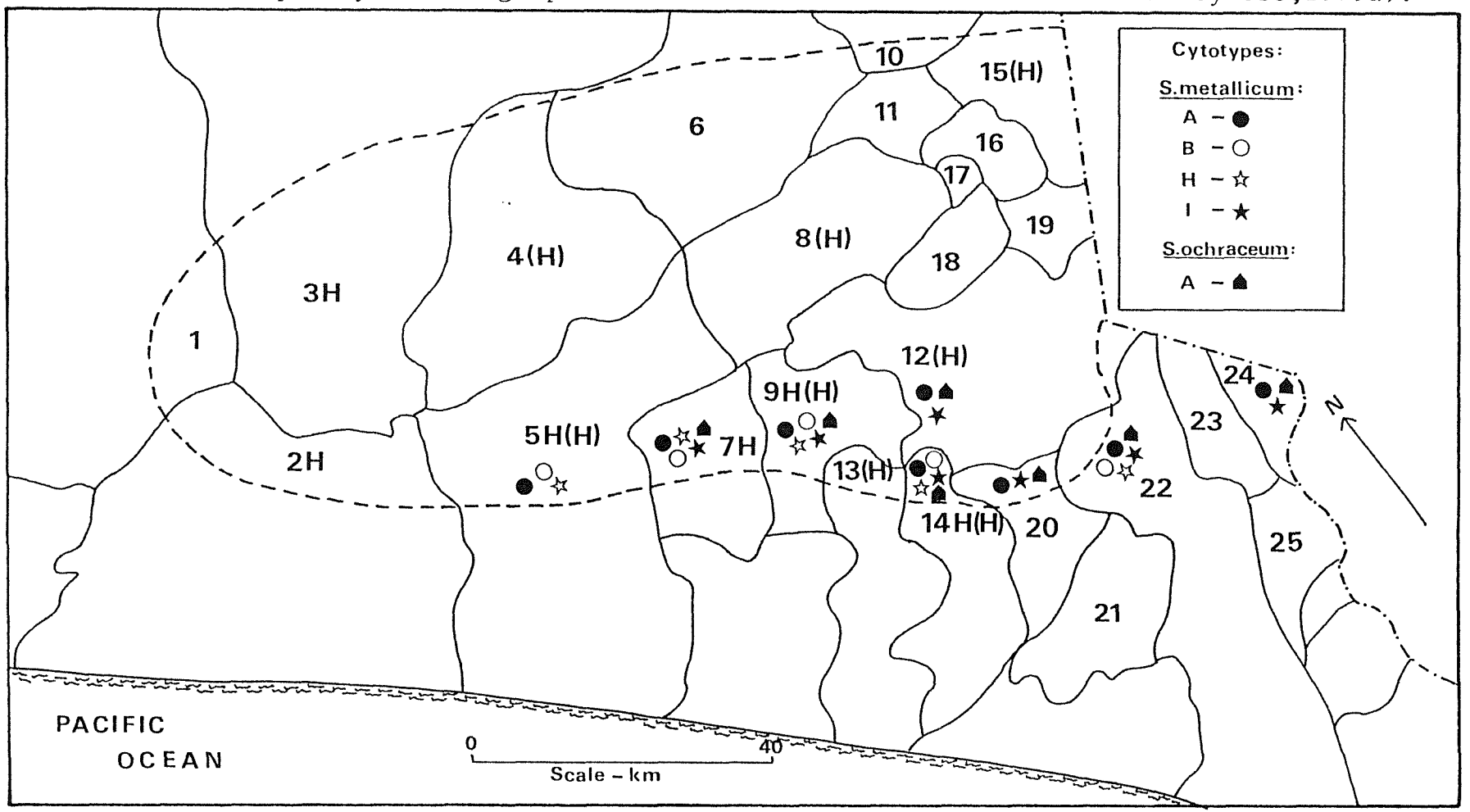
Table 7.1 Prevalence of onchocerciasis in the municipalities of the Soconusco focus.

Municipality :	Disease Prevalence : 1960*	1978**
1. Villa Corzo	-	-
2. Pijijiapan	-	34.11%
3. La Concordia	17%	18.39%
4. Angel Albino Corzo (Jaltenango La Paz)	41%	12.68%
5. Mapastepec	72%	17.54%
6. Chicomuselo	37%	7.47%
7. Acacoyagua	35%	19.00%
8. Siltepec	50%	8.99%
9. Escuintla	66%	44.72%
10. Frontera Comalapa	11%	1.43%
11. Bella Vista	12%	0.89%
12. Motozintla	48%	8.30%
13. Comaltitlan	65%	13.75%
14. Huixtla	74%	32.75%
15. Amatenango de la Frontera	25%	6.02%
16. Bejucal de Ocampo	18%	2.29%
17. La Grandeza	25%	2.00%
18. El Porvenir	33%	4.79%
19. Mazapa de Madero	18%	1.89%
20. Tuzantan	36%	7.89%
21. Huehuetan	-	3.13%
22. Tapachula	17%	1.71%
23. Cacahoatan	0%	0%
24. Union Juarez	0%	0%
25. Tuxtla Chico	0%	0%

* Morales Cisernos (1963)

** Martínez Reynoso (1979a)

Figure 7.1. Distribution of the cytotypes of S.ochraceum and S.metallicum in relation to onchocerciasis in the Soconusco focus.
(Numerals refer to municipalities in Table 7.1.)
(H) = municipality with high prevalence of disease in 1962 (García S. and Chávez N.,1962)
H = municipality with high prevalence of disease in 1978 (Martínez Reynoso,1979a).



the levels of onchocerciasis were found to be surprisingly high (R.Rivas, pers. comm.).

It is possible that transmission in this community was occurring higher up where coffee was being grown or alternatively one of the other vectors may be transmitting the disease. S.metallicum larvae were collected in all but one locality in this focus, and when analysed cytologically were found to consist of cytotypes A, B, H and I. All the larvae collected were then divided into cytotypes based on morphology, and their distribution by municipality may be seen in Figure 7.1. In Nueva Costa Rica considerable numbers of S.metallicum were biting near stream 3f at 240m, where S.metallicum cytotype A larvae were found. S.metallicum cytotype B larvae as well as S.callidum were also collected in the streams nearby. Cytotype H larvae were also found in another community in Mapastepec. It was impossible to sample sites at higher altitude within Mapastepec as there was no road access.

S.ochraceum cytotype A was however found in all the other municipalities regardless of the level of the disease. The numbers of S.ochraceum larvae found varied considerably between communities. S.metallicum cytotype A was the most widespread form of this complex and occurred at all localities where this species group was found. It was also by far the most abundant cytotype. Cytotype I was also abundant, but this did not occur in Mapastepec or the two lowest sites in Motozintla. Cytotype H occurred in all municipalities except Motozintla, Tuzantan and Union Juarez, but was much less abundant than cytotypes A and I. Cytotype B was the least common and was also absent in Motozintla, Tuzantan and Union Juarez.

In section 7.1 the tendency of flies to remain near their breeding sites was discussed. If all the cytotypes show this tendency, then the vectors in a particular locality would be the ones that bred nearby. Information on the

level of disease was only available for a few localities which were sampled. In Las Golondrinas, Acacoyagua, which is hyperendemic for onchocerciasis (M.Ortega G., pers. comm.) S.ochraceum cytotype A and S.metallicum cytotypes A, B, H and I were found. In this community S.ochraceum is the principal biting species and is undoubtedly the species transmitting the disease (Ortega et al, 1985).

Morelos, in the municipality of Huixtla, is also a hyperendemic site (R.Rivas, pers. comm.) and S. metallicum cytotypes A, H and I were found here in conjunction with S.ochraceum cytotype A, which was found in high biting numbers by Ortega and Oliver (1985). Larvae of S.ochraceum cytotype A and S.metallicum cytotypes A, B and I were found in Jalapa, Escuintla, which is hypoendemic for the disease (Beltrán et al, 1979). It is apparent that many localities have a variety of cytotypes breeding in their vicinities and therefore it would be necessary to monitor the cytospecies biting man and their natural infections with the parasite to ascertain which were transmitting the disease.

Although Union Juarez is a municipality outside the disease focus, S.ochraceum cytotype A and S.metallicum cytotypes A and I were found there. This is an area to where the disease could potentially spread but it has never been detected there.

7.2.3 The Chamula Focus.

In 1959, the disease was most prevalent in this focus in the municipalities of El Bosque, Chenalho, Larrainzar, Pantelho and Simojovel (see Figure 7.2). However, the disease levels were less than 10% in the populations despite the fact that there had been no drug therapy or nodulectomy in the region for several years (Morales Cisernos, 1963). In 1978 the disease occurred in El Bosque,

Chenalho and Pantelho, the higher prevalences being in the latter two, but in all cases less than 4% of the population (see Table 7.2). The disease was not detected in Jitotol and Larrainzar in 1978 (Martínez Reynoso, 1979a).

The Chamula focus is the smallest and least serious of the three. It was believed to have been formed in the 1930s as a result of migrant workers contracting the disease in the Soconusco focus and carrying it back when they returned. S.ochraceum and S.metallicum together with other vector species are present in the Chamula focus, but it is not known how much transmission is actually occurring here compared with the amount of disease which is contracted in Soconusco (García Sánchez and Chávez Núñez, 1962). The migrant workers are present in the Soconusco focus for the duration of the coffee harvesting season, which is also the time of year when the transmission rate is highest (Ortega et al, 1985). In the last few years there has been a trend for the people in the Chamula region to seek work in the towns rather than harvest coffee in the Soconusco focus (Paulat, 1979). The present levels of disease are highest in the municipalities of Chenalho and Pantelho, which also still produce the majority of migrant workers.
There are three possibilities as to where transmission of the disease takes place. Firstly, it may be contracted in Soconusco and no transmission may be occurring in the Chamula region. Secondly, the disease may be contracted both in the Soconusco focus and in the Chamula focus. Thirdly, transmission may only be occurring in the Chamula focus. There is not enough evidence available to choose between these possibilities but there are some facts that can be considered.

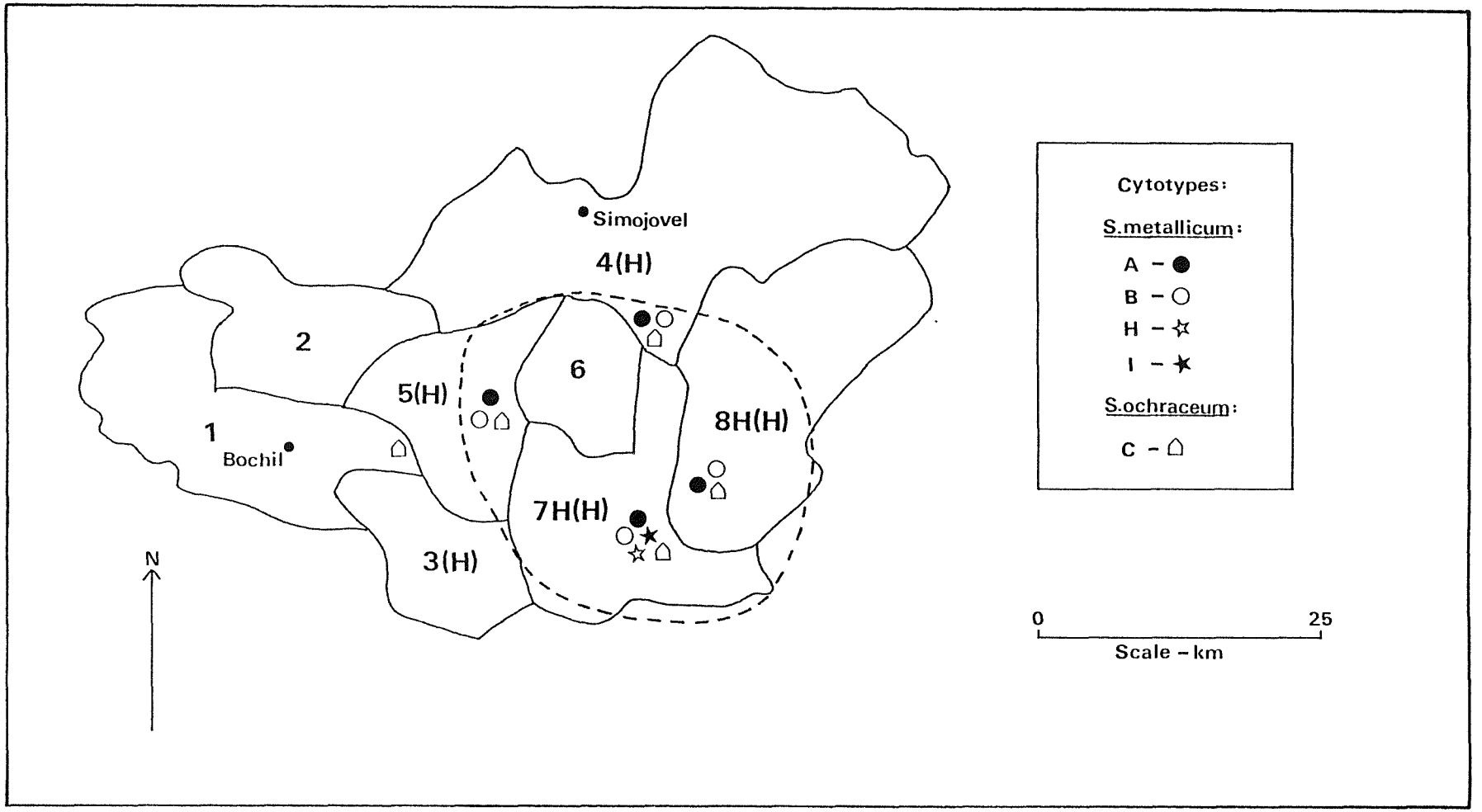
If the surveys are correct, the number of people with the disease in this focus has fallen from 4,000 in 1963 (Chávez Núñez, 1963a) to 199 in 1978 (Martínez Reynoso, 1979a), much more than in the other foci (see section 1.4.2), suggesting that the amount of transmission that these

Table 7.2 Prevalence of onchocerciasis in the municipalities of the Chamula focus.

Municipality :	Disease Prevalence in 1978* :
1. Bochil	0%
2. Jititol	0%
3. Larrainzar	0%
4. Simojovel	0.64%
5. El Bosque	0.20%
6. Chalchihuitan	0%
7. Chenalho	0.95%
8. Pantelho	3.45%

* Martínez Reynoso (1979a)

Figure 7.2. Distribution of the cytotypes of S.ochraceum and S.metallicum in relation to onchocerciasis in the Chamula focus.
(Numerals refer to municipalities in Table 7.2.)
(H) = municipality with high prevalence of disease in 1959 (Morales Cisernos, 1963)
H = municipality with high prevalence of disease in 1978 (Martínez Reynoso, 1979a)



people are subjected to has fallen. As the disease in Chamula is now most prevalent in the regions where there are still migrant coffee workers, this suggests that the disease is contracted in Soconusco. People from the other regions in the Chamula focus have taken to working in the local towns and therefore would not come into contact with the disease in Soconusco.

However, the migrant workers are most likely to harvest coffee on the highly-developed fincas, which also have the lowest levels of disease, so the chances of actually contracting the disease in Soconusco are lessened. Also, it is likely that these migrant workers predominately work on the land, both in and out of the coffee harvesting season, and therefore there is a high potential for contact with the vectors in both the Chamula and Soconusco foci. People who are now working in the towns in the Chamula focus are far less likely to contract the disease in any case, as they are not working on the land where transmission occurs.

In this study S.ochraceum larvae were collected in the municipalities of Bochil, El Bosque, Chenalho, Pantelho and Simojovel (see Figure 7.2). With the exception of Bochil, these are all within the limits of the focus. No specimens were collected in Larrainzar, but this would be expected as the only site sampled there was at high altitude (1820m). All specimens that were examined were found to be cytotype C (see Chapter 2), and it is therefore assumed that all S.ochraceum larvae collected in this focus were of this cytotype. S.ochraceum from this focus had not been examined cytologically before and cytotype C had not previously been recorded in Mexico. This cytotype was however described from Guatemala by Hirai et al (in preparation). There it was reported to be found in a non-endemic savanna-like area which differed from the onchocerciasis areas and it was said to have low anthropophily. The Chamula focus is much less steep and rugged and much more highly cultivated than the other two foci in Mexico. When collecting larvae for

the present study biting S.ochraceum were not seen. However, it is possible that adults were absent due to the time of year and the high altitude of some of the sites. S.ochraceum do bite in numbers in this focus in areas which are at lower altitude and inaccessible by road (Campaign against onchocerciasis, pers. comm.).

Transmission of onchocerciasis is actively occurring in the Soconusco and Oaxaca foci and it is interesting that the S.ochraceum cytotype is different in each of the disease areas (see Chapter 2). Cytotype C is the most distinct type from the other two as it has both fixed autosomal and sex chromosome differences. Cytotype B only differs from cytotype A in its sex chromosomes. If transmission is not occurring in the Chamula focus it could partly be explained if cytotype C was found to be a less efficient vector than the other two.

Larvae of S.metallicum were also collected in El Bosque, Chenalho, Pantelho and Simojovel. Cytotypes A, B, H and I were positively identified by cytotaxonomy from this focus (see Chapter 2). All the larvae collected were then divided into cytotypes morphologically and their distribution by municipality is shown in Figure 7.2. Cytotype A was found at all the localities where S.metallicum occurred and was by far the most abundant. Cytotype B was found in all the municipalities where S.metallicum was found, but its numbers were very low. Two larvae of cytotype H and three of cytotype I were each only found at one locality in Chenalho.

7.2.4 The Oaxaca Focus.

In 1962, the highest prevalences of onchocerciasis in Oaxaca were in the municipalities of Santiago Comaltepec, Ixtlan, Villa Alta, San Juan Yaee, San Pedro Yolox and in Valle Nacional (see Figure 7.3). Disease levels in some

populations of these communities were as high as 80% to 90% (García Sánchez and Chávez Núñez, 1962). In 1978, the highest prevalences of onchocerciasis in Oaxaca were in the municipalities of Santiago Comaltepec, San Juan Yaee, San Pedro Yolox, Santiago La Lopa and Santiago Camotlan (see Figure 7.3). The levels of disease in all the municipalities can be seen in Table 7.3 (Martínez Reynoso, 1979a).

Larval collections were made in the municipalities of Santiago Comaltepec, San Juan Yaee, San Pedro Yolox, Santiago La Lopa, Valle Nacional, San Juan Quiotepec, San Pablo Macuiltianguis, Tanetze de Zaragoza, Juquila Vijanos and Talea de Castro (see Figure 7.3). S.ochraceum larvae were examined cytologically from Comaltepec, Yaee, La Lopa, Tanetze, Juquila Vijanos and Talea, and all were found to be cytotype B (Chapter 2). All S.ochraceum larvae collected were therefore assumed to be cytotype B.

S.ochraceum B was not found in San Pedro Yolox, Macuiltianguis or Quiotepec, but this would be expected as these samples were all at high altitude, 1850m and above, where S.ochraceum is not generally found. Onchocerciasis is contracted in these municipalities in areas at much lower altitude which are inaccessible by road (S.Torres, pers. comm.). S.ochraceum was also absent in the two collections in Valle Nacional.

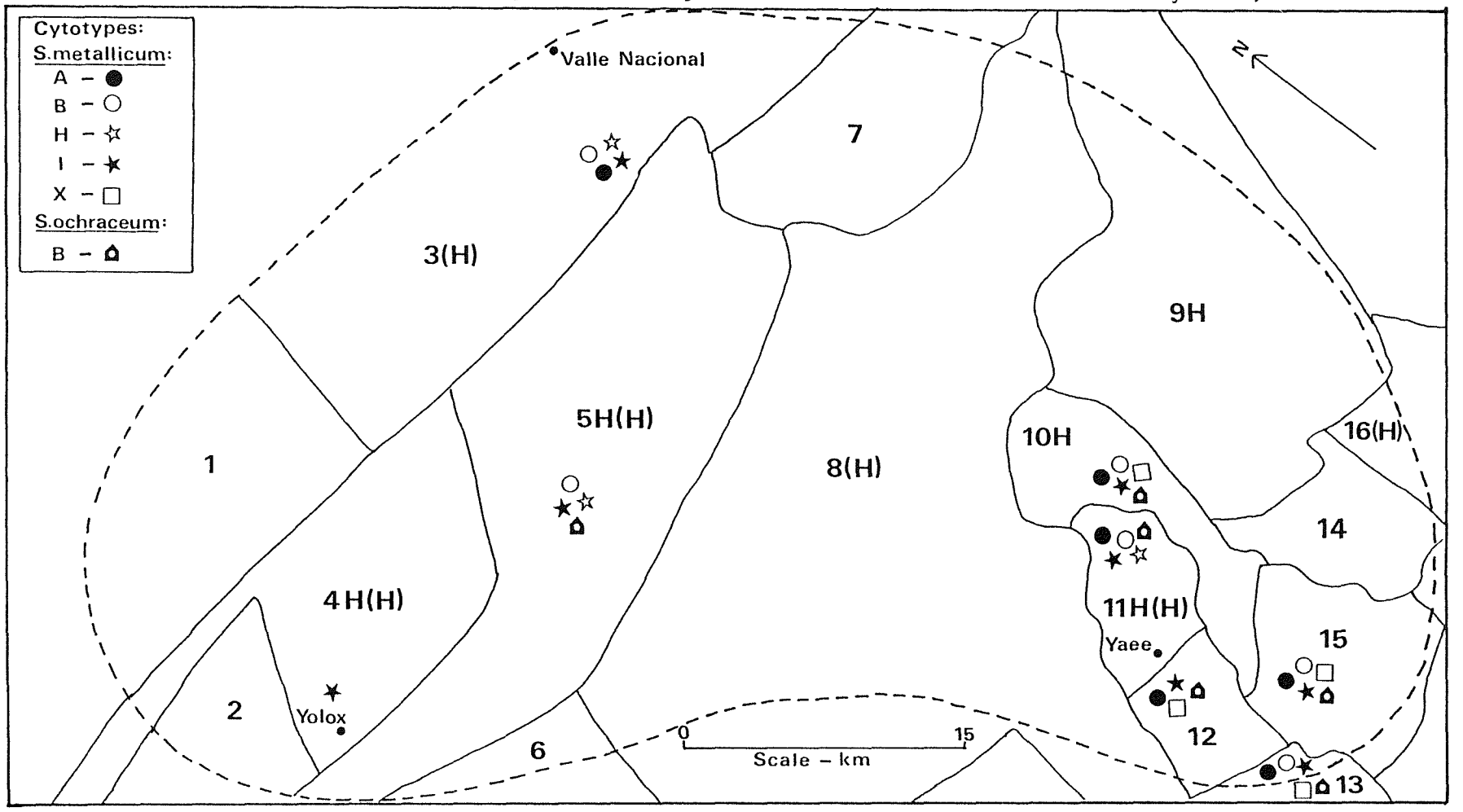
S.metallicum larvae were examined cytologically and cytotypes A, B, H, I and X were present in this focus (Chapter 2). All the larvae were divided into cytotypes by morphology and their distribution by municipality is shown in Figure 7.3. In this focus cytotype I was by far the most abundant and it occurred in all the municipalities except Macuiltianguis and Quiotepec, in both of which samples were collected at very high altitude and did not contain S.metallicum or S.ochraceum larvae. Cytotype A was less common and it occurred in all municipalities except

Table 7.3 Prevalence of onchocerciasis in the municipalities of the Oaxaca focus.

	Municipality :	Disease Prevalence in 1978* :
1.	San Felipe Usila	0.18%
2.	San Juan Quiotepec	9.88%
3.	Valle Nacional	9.75%
4.	San Pedro Yolox	30.89%
5.	Santiago Comaltepec	27.24%
6.	San Pablo Macuiltianguis	-
7.	Ayozintepec	1.16%
8.	Ixtlan de Juarez	11.21%
9.	Santiago Camotlan	27.13%
10.	Santiago La Lopa	30.35%
11.	San Juan Yaee	32.55%
12.	Tanetze de Zaragoza	9.25%
13.	San Juan Juquila Vijanos	1.78%
14.	San Juan Yatzona	3.72%
15.	Talea de Castro	4.31%
16.	Villa Alta	3.50%

* Martínez Reynoso (1979a)

Figure 7.3. Distribution of the cytotypes of S.ochraceum and S.metallicum in relation to onchocerciasis in the Oaxaca focus.
(Numerals refer to municipalities in Table 7.3.)
(H) = municipality with high disease prevalence in 1962 (García S. and Chávez N.,1962)
H = municipality with high disease prevalence in 1978 (Martínez Reynoso, 1979a)



Comaltepec, Yolox, Macuiltianguis and Quiotepec. Cytotype B was rare but occurred in all municipalities except Yolox, Tanetze, Macuiltianguis and Quiotepec. Cytotype H was rare and only occurred in Comaltepec, Valle Nacional and Yaee. Cytotype X occurred in Tanetze, Juquila Vijanos, La Lopa and Talea.

At the time the localities were sampled the highest levels of disease occurred in Santiago La Lopa (29%), Santiago Yagallo (27%) and San Juan Yaee (25%) (S.Torres, pers. comm.). S.ochraceum cytotype B and S.metallicum cytotypes A and I were identified from all these localities and S.metallicum cytotypes B and X were also found. A variety of vectors therefore could also be responsible for transmitting the disease in the Oaxaca focus.

S.metallicum cytotypes A, I and X were also identified at the site 160km to the south of the Oaxaca focus (see Figure 2.6), which is not an endemic area.

7.3 Conclusions.

This study has shown that a different cytotype of S.ochraceum occurs in each of the onchocerciasis foci in Mexico. The stream conditions in which each of these cytotypes was most abundant also differed, although this may partly be because different types of sites were sampled in each focus. The level of the disease varies between foci. In the Chamula focus the level of the disease is very low, whereas in the Soconusco and Oaxaca foci it is much higher. Although there are many factors which may be responsible for the level of the disease in different areas, one possible factor is a variable ability of the different S.ochraceum cytotypes to transmit the disease. If one cytotype had a low vectorial capacity this would be very important if a programme of larviciding was planned,

particularly as only one cytotype has been found in each focus to date.

Although S.ochraceum is the principal vector of the disease, S.metallicum is known to be a secondary vector (see section 1.4.3). Until recently, this has been considered as a single species with a wide distribution, and not a species complex. In this study, it was found to consist of five cytotypes in Mexico which had varying abundances and stream preferences. Different members of the S.metallicum complex are known to have variable vectorial abilities. In Venezuela S.metallicum is a very efficient vector and the cytotypes differ from those occurring in Mexico and Guatemala (see section 1.5.4). S.metallicum in Guatemala was split into two morphospecies, S.metallicum s.s. and S.horacioi, by Okazawa and Onishi (1980). Subsequently, Ito et al (1980) found evidence that S.metallicum s.s. may be a better vector than S.horacioi. S.metallicum s.s. most closely resembles cytotype A and S.horacioi most closely resembles cytotype H (see Chapter 4).

The five cytotypes of S.metallicum found in Mexico may therefore have varying abilities to transmit the parasite. From the information gained in this thesis on the distribution and abundance of their larvae it was impossible to conclude whether some cytotypes might be better vectors than others. However, if a larviciding programme were to be instigated in Mexico it would be important to assess the vectorial capacities of all the cytotypes by experimental and/or natural infection. This might show that all S.metallicum cytotypes occurring in Mexico were poor vectors compared with S.ochraceum and therefore control could be concentrated solely in areas where this last species was prolific. Conversely, some cytotypes of S.metallicum may turn out to be much better vectors than others.

As S.metallicum has always been considered as a secondary vector in Mexico and Guatemala, a cytotype with a good vectorial capacity may prove to be one which has a limited distribution. In this present study the ecological conditions of the streams in which each cytotype was most prevalent varied. Therefore, if it were necessary to control one or more cytotypes of S.metallicum, the streams in which they preferentially bred could be selected. A larviciding programme in Mexico would be costly and technically difficult due to the small size of the streams and their inaccessibility. A programme could be run much more efficiently if only the streams containing the most important vectors were treated.

S.callidum, another secondary vector of onchocerciasis in Mexico, is also known to be a species complex. In this study the larvae of this species were found to be very widely distributed in the three foci. It is also possible that the vectorial capacities of the different members of this species complex vary and although generally zoophilic, different cytotypes could be of importance in localised areas. Similarly, the three potential vectors, S.veracruzanum, S.haematoptum and S.gonzalezi, may be transmitting the disease in isolated areas.

Summary.

1. Larvae of S.ochraceum and S.metallicum from the three onchocerciasis foci in Mexico were identified by cytotaxonomy.

S.ochraceum cytotype A was present in the Soconusco focus, cytotype B in the Oaxaca focus and cytotype C in the Chamula focus. Cytotype C was recorded in Mexico for the first time.

S.metallicum cytotypes A, B, H and I were recorded from all three onchocerciasis foci and a previously undescribed form, named X, was identified from Oaxaca. The most obvious difference between cytotype X and the described cytotypes was a nucleolar organizer shift to the end of chromosome arm IIIl. Cytotypes B, H, and I had not previously been recorded in Mexico.

2. The possibility of obtaining identifiable chromosomes from S.ochraceum adults was investigated. However, although polytene chromosomes were present in the Malpighian tubules they were of poor quality and the cytotypes of the specimens could not be determined.

3. The head patterns and body colouration of S.metallicum larvae were examined and in the majority of cases the cytotypes could be distinguished from each other using these morphological differences. However, cytotype B could not be separated from cytotype X.

4. Cuticular hydrocarbon analysis was employed to investigate whether adult members within the S.ochraceum and S.metallicum species complexes could be separated. Some separation was achieved between members of the S.ochraceum species complex. S.metallicum specimens could not be separated into groups, but many of them were of uncertain

cytotype and flies of known cytotype are required initially for this technique.

5. The distributions and abundances of the different cytotypes of S.ochraceum and S.metallicum, and S.callidum, S.downsi and S.paynei (scored together with S.rubicundulum) were examined in relation to selected environmental variables. Multivariate analysis showed that the distributions of the larvae were significantly correlated with altitude, pH, stream size and temperature. Each S.ochraceum and S.metallicum cytotype was found to be associated with characteristic stream conditions. The proportions of the different S.metallicum cytotypes varied between the three onchocerciasis foci.

6. The distributions of the S.ochraceum and S.metallicum cytotypes in relation to the known distribution of the disease are discussed.

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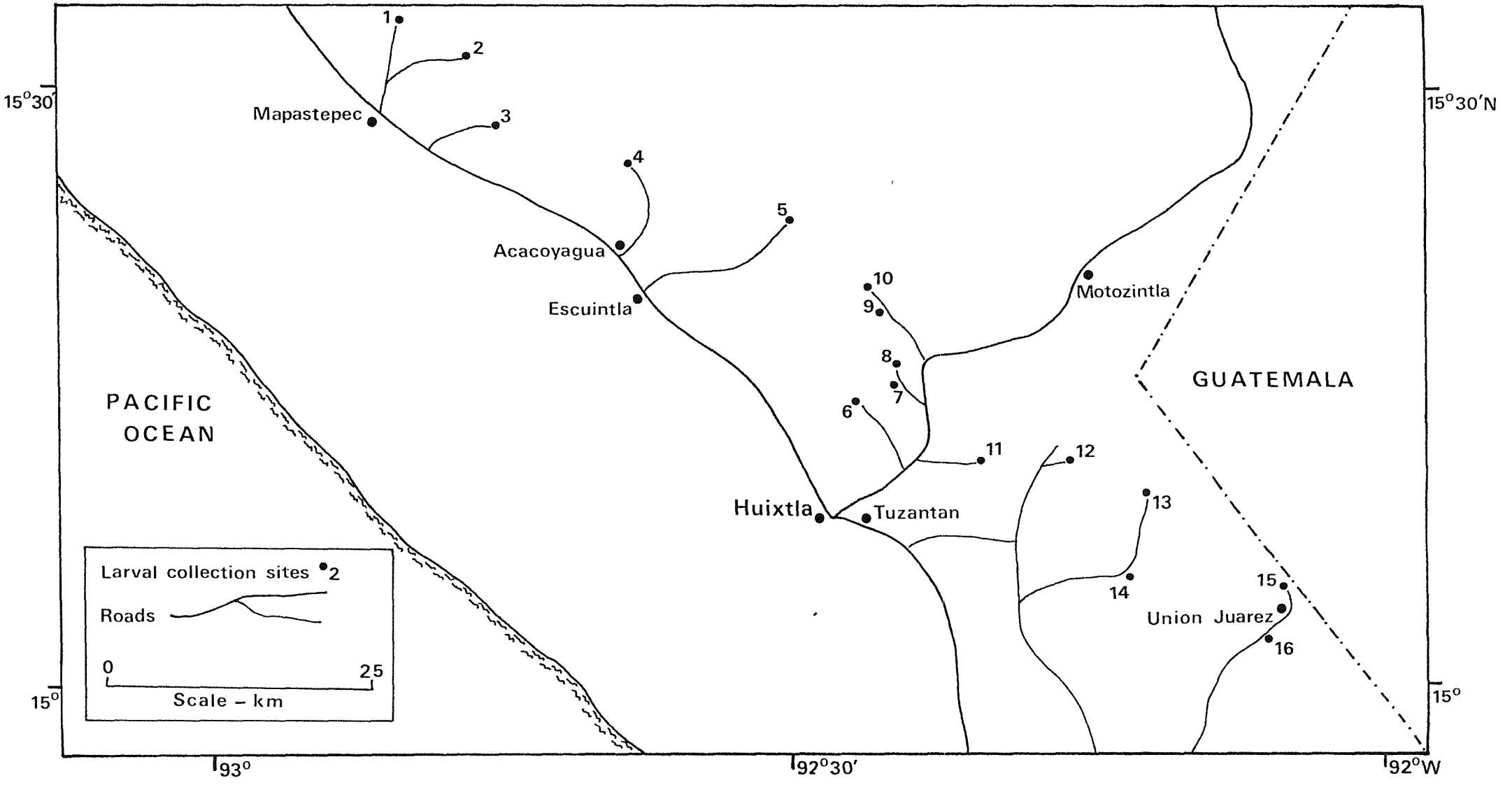
Appendices.

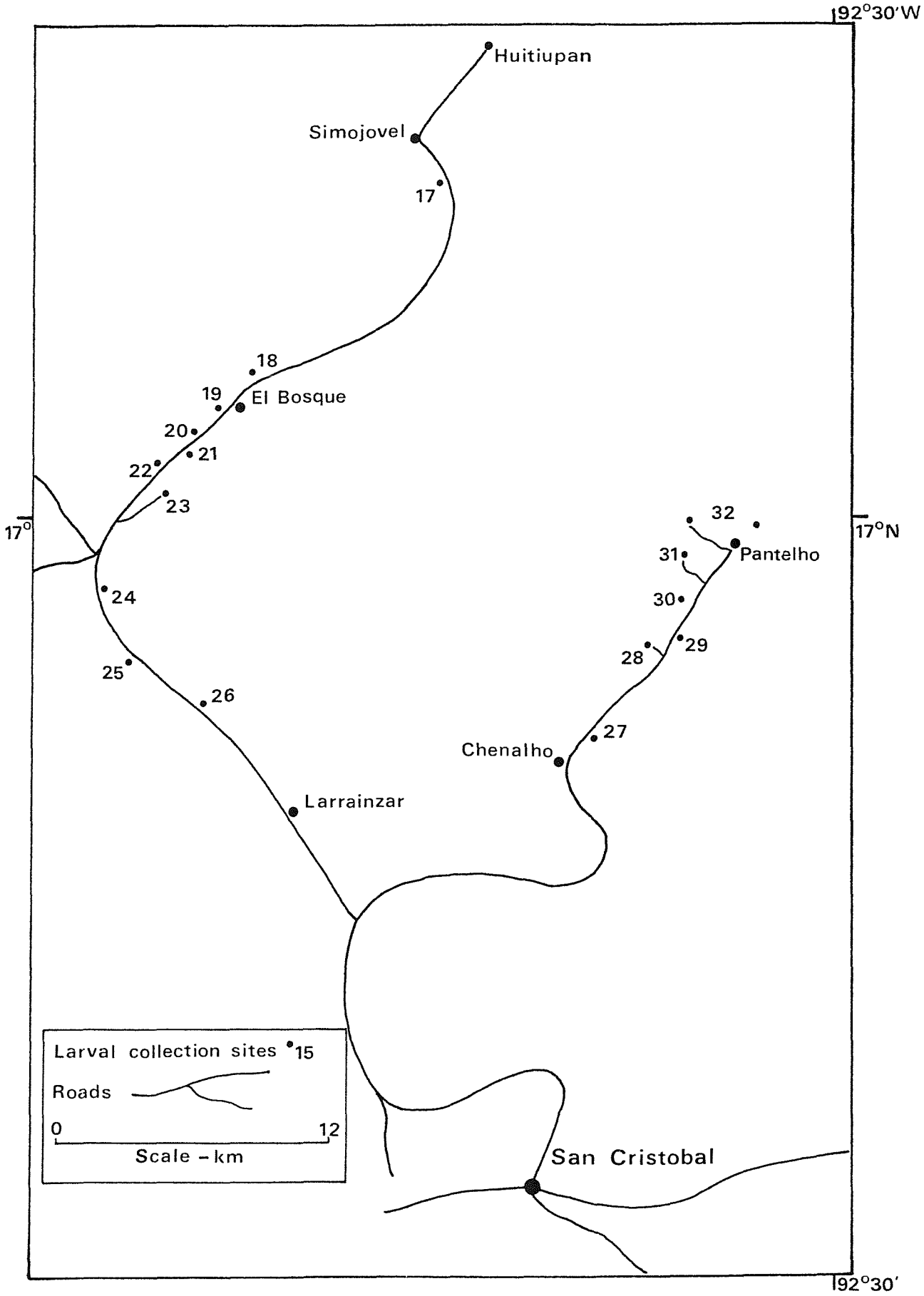
Appendix I (i) Collection sites in the Soconusco focus.

No.	Locality	Municipality
1	Tres de Mayo	Mapastepec
2	Panteleon Dominguez	Mapastepec
3	Nueva Costa Rica	Mapastepec
4	Golondrinas	Acacoyagua
5	Jalapa	Escuintla
6	Morelos	Huixtla
7	Varitas	Huixtla
8	Nueva America	Huixtla
9	Cuauhtehmoc	Motozintla
10	Francisco I. Madero	Motozintla
11	San Cristobal	Tuzantan
12	Finca Hamburgo	Tapachula
13	Chespal	Tapachula
14	Manacal	Tapachula
15	Talquian	Union Juarez
16	Once de Abril	Union Juarez


15°30'
15°30'N
15°
15°
93°
92°30'
92°W
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Mapastepec
Acacoyagua
Escuintla
Huixtla
Tuzantan
Motozintla
Union Juarez
PACIFIC
OCEAN
GUATEMALA
Larval collection sites
Roads
0
25
Scale – km

Appendix I (ii) Collection sites in the Chamula focus.

No.	Locality	Municipality
17	Simojovel	Simojovel
18	Poblado Berlin	El Bosque
19	El Bosque	El Bosque
20	San Pedro	El Bosque
21	San Miguel	El Bosque
22	Los Angeles	El Bosque
23	Los Platanos	El Bosque
24	Bochil (outskirts)	Bochil
25	San Caijetano	Larrainzar
26	Paraje Vayalemo	Larrainzar
27	Chenalho	Chenalho
28	Pechiquil	Chenalho
29	Vista Hermosa	Chenalho
30	Queshtic	Chenalho
31	Paraiso	Pantelho
32	Pantelho	Pantelho

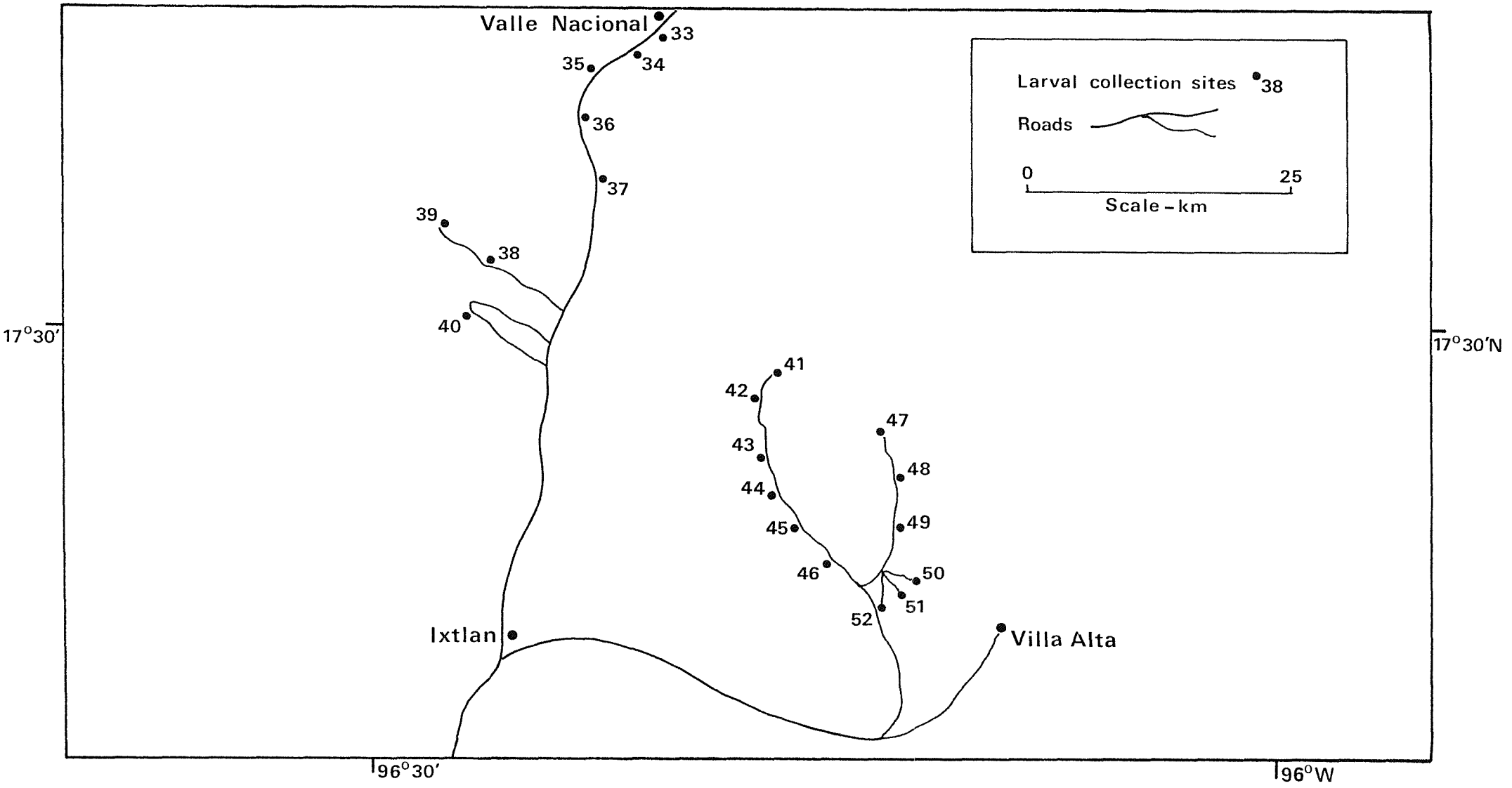

92°30'W
Huitiupan
Simojovel
17
18
19
El Bosque
20
21
22
23
17°
32
17°N
31
Pantelho
30
24
28
29
25
26
27
Chenalho
Larrainzar
Larval collection sites 15
Roads
0
12
Scale - km
San Cristobal
92°30'

Appendix I (iii) Collection sites in the Oaxaca focus.

No.	Locality	Municipality
33	San Mateo Yetla	Valle Nacional
34	Plan de Las Flores	Valle Nacional
35	Puerto Eligio	Santiago Comaltepec
36	Loma Metate	Santiago Comaltepec
37	La Esperanza	Santiago Comaltepec
38	San Pedro Yolox	San Pedro Yolox
39	San Juan Quiotepec	San Juan Quiotepec
40	San Pablo Macuiltianguis	San Pablo Macuiltianguis
41	San Juan Yaee	San Juan Yaee
42	Santa Maria La Chichina	San Juan Yaee
43	Santiago Yagallo	San Juan Yaee
44	Tanetze de Zaragoza	Tanetze de Zaragoza
45	San Isidro Reforma	Juquila Vijanos
46	Juquila Vijanos	Juquila Vijanos
47	Santiago La Lopa	Santiago La Lopa
48	Otatitlan de Morelos	Talea de Castro
49	San Bartoleme Yatoni	Talea de Castro
50	La Hacienda	Talea de Castro
51	El Porvenir	Talea de Castro
52	Las Delicias	Talea de Castro

Collection sites in south Oaxaca (outside focus)

53	Rio Molino)	not shown on map
54	Pluma y Hidalgo)	


Valle Nacional
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
Ixtlan
Villa Alta
Larval collection sites
38
Roads
0
25
Scale - km
17°30'
17°30'N
96°30'
96°W

Appendix II (i). Cytotaxonomic results of S.ochraceum larvae from the Soconusco focus, showing the sex chromosome categories.

Site No.	Name	Collection Date	Altitude (m)	Cytotype - A XoXo	XoY0	XoY2	XoY4
4	Golondrinas :						
	a	10.8.85	720	6	2	1	8
	d	22.1.87	950	11	-	1	8
	f	23.1.87	790	11	1	1	8
	g	23.1.87	750	14	1	1	17
5	Jalapa :						
	a	21.7.85	860	1	-	-	-
	a	9.8.85	860	1	-	-	-
	b	9.8.85	860	1	-	-	2
6	Morelos :						
	a	16.10.85	1100	8	-	-	5
	b	24.9.85	1100	4	-	-	3
	b	24.1.86	1100	-	-	-	2
	SE3	24.1.86	1000	1	-	-	5
	c	26.1.87	1130	5	1	1	3
	f	17.10.85	1100	4	-	-	5
	f	24.1.86	1100	6	1	-	2
	f	26.1.87	1100	3	-	1	3
	g	26.9.85	1140	5	2	1	7
	g	17.10.85	1140	5	2	3	4
	h	2.8.85	1160	5	1	1	5
	j	25.9.85	1280	8	-	-	6
	m	16.10.85	1280	2	2	-	5
	n	25.9.85	1280	4	-	2	3

Appendix II (ii). Cytotaxonomic results of S.ochraceum larvae from the Soconusco focus, showing the sex chromosome categories.

Site No.	Name	Collection Date	Altitude (m)	Cytotype - A XoXo	XoY0	XoY2	XoY4
9	Cuauhtehmoc :						
	a	5.2.87	1170	2	2	-	5
	b	5.2.87	1170	1	1	-	-
10	Madero :						
	c	6.2.87	1330	7	-	-	-
	d	6.2.87	1220	5	-	1	5
11	S.Cristobal :						
	b	7.8.85	1010	4	-	-	4
	NTE1	22.9.85	1000	1	-	-	-
	E2	22.9.85	1000	-	-	-	2
12	Hamburgo :						
	e	24.10.85	1000	-	-	-	1
	g	24.10.85	1000	5	1	-	2
	j	20.9.85	1200	-	-	-	3
	j	24.10.85	1200	6	-	-	7
13	Chespal :						
	a	8.8.85	800	12	-	-	10
	c	8.8.85	800	8	-	2	11
	d	8.8.85	800	17	2	1	12
14	Manacal :						
	a	10.2.87	820	8	-	1	6
	b	10.2.87	850	3	-	-	2
15	Talquian :						
	a	11.2.87	1510	1	-	-	1
Total for each category of cytotype A :				185	19	18	172

Total number of S.ochraceum cytotype A analysed = 394

Appendix II (iii). Cytotaxonomic results of S.ochraceum larvae from the Chamula focus, showing the sex chromosome categories.

Site No.	Name	Collection Date	Altitude (m)	Cytoype - C X0X0	X0Y1	X0Y2
21	S. Miguel :	27.2.87	1100	7	1	2
22	Los Angeles :	6.11.87	1260	6	8	2
23	Los Platanos : a	6.11.87	1280	3	-	1
24	(Bochil) :	7.11.87	1240	-	1	2
27	Chenalho : a	8.11.87	1350	1	-	-
28	Pechiquil : a	9.11.87	1320	12	16	2
	b	9.11.87	1320	-	3	1
29	Vista Hermosa a :	25.2.87	1480	3	6	-
	b	8.11.87	1510	-	1	-
30	Queshtic : a	25.2.87	1280	-	-	1
32	Pantelho : a	9.11.87	1100	3	-	-
Total for each category of cytotype C :				35	36	11

Total number of S.ochraceum cytotype C analysed = 82

Appendix II (iv). Cytotaxonomic results of S.ochraceum cytotype C larvae from the Chamula focus - frequency of polymorphic inversion IIs-8.

Site No.	Name	Inversion IIs-8 ss	si	ii
21	San Miguel	4	4	2
22	Los Angeles	10	6	-
23a	Los Platanos	2	2	-
24	(Bochil)	2	1	-
27a	Chenalho	-	-	1
28a	Pechiquil	16	12	2
28b	Pechiquil	3	1	-
29a	Vista Hermosa	2	6	1
29b	Vista Hermosa	1	-	-
30a	Queshtic	-	1	-
32a	Pantelho	1	1	1
	Total :	41	34	7

number of standard sequence = (41 x 2) + 34 = 116

number of inverted sequence = 34 + (7 x 2) = 48

frequency of inverted sequence IIs-8 = 29.3%

Appendix II (v). Cytotaxonomic results of S.ochraceum larvae from the Oaxaca focus, showing the sex chromosome categories.

Site No.	Name	Collection Date	Alt. (m)	Cytoype - B X1X1	X1X2	X1Y0	X1Y1	X2Y0
35	Puerto Eligio :	28.10.87	650	-	-	1	-	-
41	Yaee :	24.10.87	1300	2	-	3	2	-
42	La Chichina :	24.10.87	1330	5	2	7	-	-
43	Yagallo : c	24.10.87	1300	2	-	2	-	-
	d	24.10.87	1450	2	-	1	-	-
44	Tanetze : a	24.10.87	1260	2	-	1	-	1
46	Juquila Vijanos :	25.10.87	1580	-	-	1	-	-
47	La Lopa : a	27.10.87	1060	1	-	1	-	-
	b	27.10.87	1140	1	-	4	-	-
52	Las Delicias : b	26.10.87	1500	8	-	4	-	-
Total for each category of type B:				23	2	25	2	1

Total number of S.ochraceum cytotype B analysed = 53

Total number of all S.ochraceum cytotypes analysed = 529

Appendix III (i). Cytotaxonomic results of S.metallicum larvae from the Soconusco focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I
1	3 de Mayo :						
	a	18.10.87	300	5	-	1	-
	b	18.10.87	290	9	1	-	-
	c	18.10.87	300	9	7	-	-
2	Panteleon Dominguez :	19.10.87	270	2	1	-	-
3	Nueva Costa Rica a :	17.10.87	270	8	-	-	-
	b	17.10.87	280	1	-	-	-
	d	17.10.87	350	-	4	-	-
	e	17.10.87	410	-	4	-	-
	f	18.10.87	240	2	-	-	-
4	Golondrinas :						
	a	10.8.85	720	3	13	-	-
	b	19.7.85	850	3	-	-	-
	d	22.1.87	950	4	5	1	4
	f	23.1.87	790	12	-	5	-
	j	23.1.87	640	4	11	-	-
5	Jalapa :						
	b	21.7.85	860	7	-	-	2
	b	8.8.85	860	3	-	-	8
	d	24.1.87	660	6	-	-	-
	f	24.1.87	830	12	2	-	-

Appendix III (ii). Cytotaxonomic results of S.metallicum larvae from the Soconusco focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I
6	Morelos :						
	b	24.6.85	1100	4	-	-	-
	b	4.8.85	1100	9	-	-	-
	b	24.9.85	1100	16	-	-	-
	b	16.10.85	1100	4	-	4	5
	c	26.1.87	1130	-	-	-	1
	h	2.8.85	1160	-	-	-	11
	h	26.1.87	1160	-	-	-	3
	i	26.6.85	1280	-	-	1	9
	j	25.9.85	1280	9	-	8	9
	k	25.6.85	1280	1	-	1	2
	k	5.8.85	1280	8	-	9	3
	l	25.9.85	1280	1	-	6	1
	m	26.6.85	1280	-	-	-	7
7	Varitas :	8.2.87	700	16	-	-	-
8	Nueva America a :	8.2.87	860	17	-	-	-
9	Cuauhtehmoc :						
	a	5.2.87	1170	-	-	-	2
	b	5.2.87	1170	3	-	-	1
10	Madero :						
	d	6.2.87	1220	8	-	-	3
11	S.Cristobal :						
	c	8.2.87	990	2	-	-	1
	d	8.2.87	1010	16	-	-	-

Appendix III (iii). Cytotaxonomic results of S.metallicum larvae from the Soconusco focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I
12	Hamburgo :						
	a	19.9.85	800	12	-	-	-
	j	20.9.85	1200	1	1	-	-
13	Chespal :						
	b	8.8.85	800	10	3	1	-
	e	9.2.87	930	8	-	-	-
	f	9.8.85	930	5	-	-	-
14	Manacal :						
	b	10.2.87	850	3	-	-	-
15	Talquian :						
	a	11.2.87	1510	20	-	-	-
	b	11.2.87	1600	-	-	-	2
	c	11.2.87	1620	-	-	-	4
Cytotype totals for the Soconusco focus :				263	52	37	78

Total number of S.metallicum larvae analysed from the Soconusco focus = 430

Appendix III (iv). Cytotaxonomic results of S.metallicum larvae from the Chamula focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I
17	Simojovel :						
	a	28.2.87	830	9	-	-	-
	b	5.11.87	990	13	1	-	-
	c	6.11.87	950	3	-	-	-
18	Poblado Berlin :	6.11.87	980	12	-	-	-
19	El Bosque :	6.11.87	1080	1	-	-	-
20	S. Pedro :	6.11.87	1210	1	-	-	-
21	S.Miguel :	27.2.87	1100	13	-	-	-
23	Los Platanos :						
	a	6.11.87	1280	3	-	-	-
	b	6.11.87	1280	4	-	-	-
27	Chenalho :						
	a	8.11.87	1350	4	-	-	-
28	Pechiquil :						
	a	9.11.87	1320	4	1	-	-
	b	9.11.87	1320	6	-	1	-
29	Vista Hermosa a :	25.2.87	1480	-	-	-	2
	b	8.11.87	1510	10	3	-	1
30	Queshtic :						
	a	25.2.87	1280	17	-	-	-
32	Pantelho :						
	a	9.11.87	1100	19	1	-	-
	c	10.11.87	1100	1	1	-	-
Cytotype totals for the Chamula focus :				120	7	1	3

Total number of S.metallicum larvae analysed from the Chamula focus = 131

Appendix III (v). Cytotaxonomic results of S.metallicum larvae from the Oaxaca focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I	X
34	Plan de las Flores :	29.10.87	530	1	2	-	-	-
36	Loma Metate :	28.10.87	780	-	-	5	-	-
37	Esperanza : a	29.10.87	1580	-	-	-	12	-
	b	29.10.87	1390	-	-	-	12	-
	c	29.10.87	1390	-	-	-	4	-
38	Yolox : b	30.10.87	1860	-	-	-	1	-
41	Yaee :	24.10.87	1300	1	-	-	1	-
42	Chichina :	24.10.87	1330	1	-	-	3	-
43	Yagallo : a	24.10.87	1210	10	-	-	-	-
	b	24.10.87	1210	-	2	-	-	-
	c	24.10.87	1300	-	-	-	3	-
	d	24.10.87	1450	-	-	-	21	-
44	Tanetze : a	24.10.87	1260	3	-	-	4	5
	b	24.10.87	910	-	-	-	-	2
45	Reforma :	25.10.87	1510	2	-	-	10	9
46	Juquila Vijanos :	25.10.87	1580	-	-	-	9	-
47	La Lopa : a	27.10.87	1060	-	1	-	2	2
48	Otatitlan :	27.10.87	1560	-	-	-	18	-

Appendix III (vi). Cytotaxonomic results of S.metallicum larvae from the Oaxaca focus.

Site No.	Name	Collection Date	Altitude (m)	Cytotype A	B	H	I	X
49	Yatoni :	27.10.87	1590	-	-	-	6	-
50	La Hacienda : a	26.10.87	1030	1	-	-	-	5
51	El Porvenir : a	26.10.87	1200	5	-	-	1	-
	b	26.10.87	1170	1	-	-	3	-
	c	26.10.87	1170	-	-	-	4	4
	d	26.10.87	1190	-	-	-	-	2
52	Delicias : a	26.10.87	1400	10	3	-	19	1
	b	26.10.87	1500	-	-	-	-	1
54	Pluma y Hidalgo a :	31.10.87	1080	-	-	-	2	-
	b	31.10.87	1080	6	-	-	7	1
Cytotype totals for the Oaxaca focus :				41	8	5	142	32

Total number of S.metallicum larvae analysed from the Oaxaca focus = 228

Total number of S.metallicum larvae analysed from all three foci = 789

Appendix IV (i). Collection sites - descriptions of streams.

Site (see App I)	Date	Alt. (m)	Size (see Ch.6)	pH	Temp (°C)	Shade	Description
1(a)	18.10.87	300	1/2	5.9	23.0	Total	Stony and muddy bed Clear water Vegetation covering
1(b)	18.10.87	290	2	5.9	25.0	Part	Stony bed. Clear water Vegetation at edges
1(c)	18.10.87	300	2	5.6	24.0	Part	Stony bed. Clear water Vegetation at edges
2	19.10.87	270	2	5.3	24.0	Part	Stony and muddy bed Clear water Vegetation at edges
3(a)	17.10.87	270	2	5.9	24.0	Part	Rocky,stony,muddy bed. Clear water Vegetation at edges
3(b)	17.10.87	280	2/3	6.2	25.0	Part	Rocky and muddy bed Clear water Vegetation at edges
3(c)	17.10.87	350	1/2	5.6	26.0	Total	Stony and muddy bed Clear water Vegetation at edges
3(d)	17.10.87	350	3	5.9	26.0	Part	Rocky and stony bed Clear water Vegetation at edges
3(e)	17.10.87	410	3	6.2	26.0	Part	Rocky,stony,muddy bed. Clear water Scarce vegetation
3(f)	18.10.87	240	4	6.2	24.0	Part	Rocky,stony,muddy bed. Clear water Vegetation at edges
3(g)	18.10.87	150	3/4	5.9	25.0	Part	Muddy bed Clear water Scarce vegetation
4(a)	10.8.85	720	2/3	-	21.0	Part	Stony and silty bed Cloudy water Vegetation in water

Appendix IV (ii). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
4(b)	10.8.85	850	2	-	24.5	Part	Stony and muddy bed Muddy water Vegetation in water
4(c)	22.1.87	940	2	6.5	21.0	Part	Rocky and muddy bed Clear water Vegetation at edges
4(d)	22.1.87	950	2	6.2	18.0	Part	Silty bed Clear water Vegetation at edges
4(e)	22.1.87	830	2	6.5	20.0	Part	Rocky and silty bed Clear water Vegetation overhang
4(f)	23.1.87	790	3	6.5	18.0	Part	Rocky and silty bed Clear water Vegetation at edges
4(g)	23.1.87	750	3	6.2	19.0	Part	Rocky and muddy bed Clear water Vegetation at edges
4(h)	23.1.87	780	3/4	6.2	17.5	Part	Silty and rocky bed Clear water Vegetation at edges
4(i)	23.1.87	780	3	6.2	18.5	Part	Rocky and stony bed Clear water Vegetation at edges
4(j)	23.1.87	640	3/4	6.5	20.0	Part	Rocky bed Clear water Vegetation at edges
4(k)	23.1.87	640	3	6.2	18.0	Part	Silty bed Clear water Vegetation at edges
5(a)	8.8.85	860	2	-	22.0	Total	Stony and muddy bed Clear water Vegetation in water
5(b)	8.8.85	860	2	-	21.0	Total	Stony bed Clear water Vegetation in water

Appendix IV (iii). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
5(c)	21.7.85	860	2	-	22.0	Total	Rocky and silty bed Cloudy water Vegetation covering
5(d)	24.1.87	660	3	6.2	21.5	Part	Stony and silty bed Clear water Vegetation at edges
5(e)	24.1.87	830	3	6.5	20.5	Part	Rocky and muddy bed Clear water Vegetation at edges
5(f)	24.1.87	830	3	6.2	20.5	Part	Stony and silty bed Clear water Vegetation at edges
6(a)	4.8.85	1100	2	-	20.0	Part	Rocky and muddy bed Clear water Vegetation at edges
6(b)	24.6.85	1100	1/2	-	20.0	None	Stony and muddy bed Clear water Vegetation at edges
6(c)	26.1.87	1130	2	6.2	17.5	Total	Rocky and silty bed Clear water Scarce vegetation
6(d)	26.1.87	1100	2	-	16.0	Part	Stony and silty bed Clear water Vegetation in water
6(f)	2.8.85	1100	3	6.2	18.5	Part	Rocky and muddy bed Turbulent water Scarce vegetation
6(g)	2.8.85	1140	3	6.2	18.5	Part	Rocky bed Clear water Scarce vegetation
6(h)	2.8.85	1160	2	-	18.5	Part	Rocky and silty bed Clear water Vegetation at edges

Appendix IV (iv). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
6(i)	25.6.85	1280	1	-	19.0	Total	Stony and muddy bed Clear water Vegetation in water
6(j)	5.8.85	1280	1	-	18.5	Total	Stony and muddy bed Clear water Vegetation in water
6(k)	5.8.85	1280	1	-	18.5	Total	Muddy bed Clear water Emergent plants
6(l)	6.8.85	1280	1	-	18.5	Total	Stony and muddy bed Clear water Vegetation in water
6(m)	6.8.85	1280	1	-	18.0	Total	Stony and muddy bed Clear water Vegetation-covered
6(n)	6.8.85	1280	1/2	-	18.0	Part	Stony bed.Waterfall Clear water Vegetation in water
7	8.2.87	700	4	6.2	-	None	Rocky and muddy bed Clear water Vegetation at edges
8	8.2.87	860	2	6.2	-	Part	Rocky bed Clear water Vegetation at edges
9(a)	5.2.87	1170	1	6.2	-	Part	Muddy bed Cloudy water Emergent vegetation
9(b)	5.2.87	1150	2	-	-	Part	Stony bed Clear water Scarce vegetation
10(a)	6.2.87	1330	2	6.2	-	Part	Rocky and muddy bed Clear water Vegetation at edges

Appendix IV (v). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
10(b)	6.2.87	1350	2/3	6.2	-	Total	Muddy bed Cloudy water Scarce vegetation
10(c)	6.2.87	1330	2/3	6.2	-	Part	Rocky and muddy bed Clear water Vegetation at edges
10(d)	6.2.87	1220	3	6.2	-	Part	Muddy bed Clear water Vegetation at edges
10(e)	6.2.87	1200	2	6.2	-	Total	Muddy bed Clear water Vegetation at edges
11(a)	7.8.85	1010	3/4	6.2	22.0	Scant	Stony and muddy bed Clear water Vegetation at edges
11(b)	7.8.85	1020	3	6.2	22.5	Part	Stony and muddy bed Slightly cloudy Vegetation in water
11(c)	7.8.85	990	2	5.9	21.5	Total	Stony and muddy bed Clear water Vegetation in water
11(d)	8.2.87	1010	2	5.9	-	Scant	Stony and muddy bed Clear water Vegetation at edges
11(e)	9.2.87	1160	2	5.9	-	Part	Rocky and muddy bed Clear water Vegetation at edges
13(a)	8.8.85	800	2	-	22.0	Part	Rocky and silty bed Slightly cloudy Vegetation in water
13(b)	8.8.85	800	2	-	21.0	Part	Rocky and silty bed Clear water Vegetation in water
13(c)	8.8.85	800	2	-	19.5	Part	Rocky and silty bed Clear water Vegetation in water

Appendix IV (vi). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
13(d)	8.8.85	800	2/3	-	21.0	Part	Rocky and muddy bed Clear water Vegetation in water
13(e)	9.2.87	930	2	6.2	-	None	Rocky and muddy bed Dirty water Vegetation in water
13(f)	9.2.87	930	2	6.2	-	Part	Muddy bed Dirty water Scarce vegetation
14(a)	10.2.87	820	3	6.2	-	Scant	Rocky and muddy bed Slightly cloudy Vegetation at edges
14(b)	10.2.87	850	3	5.9	-	Part	Stony and muddy bed Clear water Vegetation at edges
15(a)	11.2.87	1510	3	5.9	-	Part	Stony bed Clear water Vegetation at edges
15(b)	11.2.87	1600	2	5.6	-	None	Stony and muddy bed Clear water Vegetation at edges
15(c)	11.2.87	1620	4	5.9	-	Part	Rocky and stony bed Clear water Some vegetation
16	10.2.87	760	5	6.2	-	None	Rocky and stony bed Clear water Vegetation at edges
17(a)	28.2.87	830	4	6.2	-	Part	Rocky bed.Waterfall Clear water Vegetation at edges
17(b)	5.11.87	990	1/2	5.3	18.0	Part	Rocky and muddy bed Clear water Vegetation in water
17(c)	6.11.87	950	2	6.2	19.0	Part	Rocky bed.Waterfall Clear water Scarce vegetation

Appendix IV (vii). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
18	6.11.87	980	2/3	6.5	18.0	Total	Rocky and muddy bed Clear water Scarce vegetation
19	6.11.87	1080	2	6.5	19.0	None	Rocky and muddy bed Clear water Scarce vegetation
20	6.11.87	1210	2	5.6	18.0	None	Rocky and muddy bed Clear water Vegetation at edges
21	27.2.87	1100	2	6.2	19.0	Total	Stony and muddy bed Clear water Scarce vegetation
22	6.11.87	1260	2	5.9	16.0	Part	Stony and muddy bed Clear water Vegetation at edges
23(a)	6.11.87	1280	2	5.3	18.0	Part	Stony and muddy bed Clear water Vegetation at edges
23(b)	6.11.87	1280	1/2	5.0	19.0	Part	Rocky and muddy bed Clear water Scarce vegetation
24	7.11.87	1240	3	6.2	15.0	Part	Rocky and stony bed Clear water Vegetation at edges
25	7.11.87	1720	2	5.0	13.0	Total	Stony and muddy bed Clear water Vegetation at edges
*26	7.11.87	1820	2/3	5.3	13.0	None	Stony and silty bed Clear water Vegetation at edges
27(a)	8.11.87	1350	3	6.2	15.0	Part	Stony and muddy bed Slightly cloudy Vegetation at edges
27(b)	8.11.87	1470	2	6.2	16.0	Total	Stony and muddy bed Clear water Vegetation-covered

* stream not used in analysis

Appendix IV (viii). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
28(a)	9.11.87	1320	3	6.2	18.0	Scant	Stony and muddy bed Clear water Scarce vegetation
28(b)	9.11.87	1320	1	6.2	18.0	Part	Stony and muddy bed Clear water Scarce vegetation
29(a)	25.2.87	1480	2	6.2	17.0	Part	Muddy bed Slightly cloudy Vegetation at edges
29(b)	8.11.87	1510	1	6.2	18.0	Part	Stony and muddy bed Clear water Vegetation at edges
30(a)	25.2.87	1280	3	5.9	19.0	Part	Rocky and muddy bed Clear water Vegetation at edges
30(b)	25.2.87	1290	3	5.9	18.0	Part	Rocky and muddy bed Clear water Vegetation at edges
31	23.2.87	1350	2	5.9	17.0	Part	Stony bed.Drying up Slightly cloudy Scarce vegetation
32(a)	9.11.87	1100	3	6.5	19.0	Scant	Rocky and muddy bed Clear water Vegetation at edges
32(b)	9.11.87	1080	3	6.5	20.0	None	Rocky and muddy bed Clear water Scarce vegetation
32(c)	10.11.87	1100	3	6.2	20.0	Part	Rocky and silty bed Clear water Scarce vegetation
33	29.10.87	440	2	5.0	19.0	Part	Rocky bed Clear water Scarce vegetation
34	29.10.87	530	2	5.0	19.0	Part	Rocky and silty bed Clear water Vegetation at edges

Appendix IV (ix). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
35	28.10.87	650	2	5.3	22.0	Total	Rocky and silty bed Clear water Scarce vegetation
36	28.10.87	780	2	5.3	20.0	Total	Rocky and muddy bed Clear water Vegetation in water
37(a)	29.10.87	1580	1	5.0	15.0	None	Stony bed Clear water Vegetation at edges
37(b)	29.10.87	1390	1/2	5.6	16.0	Total	Stony and silty bed Clear water Vegetation at edges
37(c)	29.10.87	1390	2/3	5.6	16.0	Part	Rocky and silty bed Clear water Scarce vegetation
38(a)	30.10.87	1850	3	5.0	12.0	Total	Stony bed Clear water Scarce vegetation
38(b)	30.10.87	1860	2	5.3	13.0	Total	Stony bed Clear water Vegetation-covered
39(a)	30.10.87	1930	2	5.0	11.0	Total	Rocky and silty bed Clear water Scarce vegetation
*39(b)	30.10.87	1930	2	5.3	12.0	Total	Rocky and silty bed Clear water Scarce vegetation
*40(a)	30.10.87	2320	2	5.3	12.0	Part	Stony bed Clear water Vegetation at edges
*40(b)	30.10.87	2040	2	5.3	14.0	None	Stony bed Clear water Scarce vegetation
41	24.10.87	1300	2	5.6	19.0	Total	Stony bed Slightly cloudy Vegetation in water

* stream not used in analysis

Appendix IV (x). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (^{o}C)	Shade	Description
42	24.10.87	1330	2	5.0	17.0	Part	Rocky bed Clear water Vegetation in water
43(a)	24.10.87	1210	2	5.9	21.0	None	Rocky bed Clear water Vegetation at edges
43(b)	24.10.87	1210	2	5.6	20.0	None	Rocky bed Clear water Vegetation at edges
43(c)	24.10.87	1300	2	5.3	17.0	Part	Rocky and stony bed Clear water Vegetation at edges
43(d)	24.10.87	1450	2	5.3	17.0	Total	Rocky and stony bed Clear water Vegetation-covered
44(a)	24.10.87	1260	1/2	5.0	18.0	Part	Stony bed Clear water Vegetation in water
44(b)	24.10.87	910	2	5.3	20.0	None	Rocky and stony bed Slightly cloudy No vegetation
44(c)	24.10.87	1020	2/3	5.3	20.0	Part	Stony bed Clear water Vegetation at edges
45	25.10.87	1510	1	5.0	17.0	Total	Stony bed Clear water Vegetation-covered
46	25.10.87	1580	1	5.3	16.0	Part	Stony bed Clear water Vegetation in water
47(a)	27.10.87	1060	2	5.9	19.0	None	Rocky bed Clear water Vegetation at edges
47(b)	27.10.87	1140	2	5.9	19.0	Total	Rocky bed Clear water Vegetation at edges

Appendix IV (xi). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
48	27.10.87	1560	2	5.0	16.0	Part	Rocky and sandy bed Clear water Vegetation at edges
49	27.10.87	1590	2	5.0	16.0	Total	Stony and sandy bed Clear water Vegetation at edges
50(a)	26.10.87	1030	2	5.6	20.0	Total	Rocky bed Clear water Vegetation-covered
50(b)	26.10.87	1020	4	5.3	19.0	None	Rocky and muddy bed Slightly cloudy Vegetation at edges
50(c)	26.10.87	1080	2/3	5.9	19.0	Total	Rocky and muddy bed Clear water Vegetation at edges
51(a)	26.10.87	1200	2	5.0	18.0	Total	Muddy bed Slightly cloudy Vegetation-covered
51(b)	26.10.87	1170	4	5.3	15.0	None	Stony and muddy bed Clear water No vegetation
51(c)	26.10.87	1170	2	5.3	18.0	Part	Stony bed Clear water Vegetation in water
51(d)	26.10.87	1190	3	5.6	18.0	Part	Stony and muddy bed Clear water Scarce vegetation
52(a)	26.10.87	1400	2	5.3	20.0	None	Stony bed Clear water Vegetation in water
52(b)	26.10.87	1500	2	5.3	20.0	Part	Stony bed Clear water Vegetation at edges
*53	31.10.87	2130	2/3	5.6	13.0	Scant	Muddy bed Clear water Scarce vegetation

* stream not used in analysis

Appendix IV (xii). Collection sites - descriptions of streams.

Site	Date	Alt. (m)	Size	pH	Temp (°C)	Shade	Description
54(a)	31.10.87	1080	2	5.0	19.0	Part	Rocky and silty bed Clear water Scarce vegetation
54(b)	31.10.87	1080	2	5.9	19.0	None	Rocky and muddy bed Clear water Scarce vegetation

Appendix V (i) Collection sites - *Simulium* larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : *S.ochraceum* cytotype		*S.metallicum* cytotypes		Other *Simulium*	
1a	18.10.87	-		A	43	*S.callidum*	11
				*B	3		
				H	2		
1b	18.10.87	-		A	50	*S.callidum*	11
				B	2		
1c	18.10.87	-		A	55	*S.callidum*	6
				B	59	*S.downsi*	3
2	19.10.87	-		A	99	*S.callidum*	8
				B	4	*S.downsi*	2
3a	17.10.87	-		A	78	*S.callidum*	1
				*B	1		
3b	17.10.87	-		A	5	*S.callidum*	1
3c	17.10.87	-		*A	3	*S.callidum*	1
3d	17.10.87	-		B	5	*S.callidum*	13
3e	17.10.87	-		*A	1	*S.callidum*	38
				B	10		
3f	18.10.87	-		A	2	*S.callidum*	18
						?*S.paynei*	8
						S.downsi	1
3g	18.10.87	-		*A	1	?*S.rubicundulum*	65
4a	19.7.85	*A	24	?	39	*S.callidum*	6
4a	10.8.85	A	67	A	23	*S.callidum*	28
				B	80		
				*H	6		
4b	19.7.85	-		A	40	-	
4b	10.8.85	-		*A	317	-	
				B	?		
4c	22.1.87	*A	4	A	6	*S.callidum*	6

Appendix V (ii) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
4d	22.1.87	A	52	A	13	S.callidum	11
				B	13		
				H	3		
				I	15		
4e	22.1.87	-		*A	34	S.callidum	12
				*B	2	?S.paynei	64
						S.sp	1
4f	23.1.87	A	58	A	138	S.callidum	93
				B	?		
				H	6		
				*I	3		
4g	23.1.87	A	135	*A	1	S.callidum	109
				*B	2		
4h	23.1.87	*A	253	*A	12	?S.paynei	4
				*B	4		
4i	23.1.87	*A	9	*A	13	S.callidum	9
				*B	19		
4j	23.1.87	*A	4	A	6	S.callidum	67
				B	20	?S.paynei	3
4k	23.1.87	*A	6	*B	2	S.callidum	14
				*I	1		
5a	21.7.85	A	2	*A	109	S.callidum	24
5a	8.8.85	A	6	*A	352	S.callidum	159
				*H	1	?S.paynei	4
5b	21.7.85	*A	2	A	132	-	
				I	5		
5b	8.8.85	A	7	A	32	S.callidum	2
				I	10		
5c	21.7.85	*A	3	*A	23	S.callidum	7
5c	8.8.85	-		*A	18	S.callidum	171

Appendix V (iii) Collection sites - *Simulium* larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : *S.ochraceum* cytotype		*S.metallicum* cytotypes		Other *Simulium*	
5d	24.1.87	-		A	98	*S.callidum*	193
						S.downsi	1
5e	24.1.87	-		*A	573	*S.callidum*	15
						S.paynei	1
5f	24.1.87	-		A	122	*S.callidum*	50
				B	6	*S.downsi*	1
				*I	2		
6a	20.6.85	*A	7	*A	6	-	
				*H	2		
				*I	2		
6a	22.6.85	*A	5	*A	2	*S.callidum*	1
				*H	2		
				*I	10		
6a	24.6.85	*A	32	*A	30	*Cnephia sp.*	1
				*H	9		
6a	4.8.85	*A	29	*A	28	*S.callidum*	2
				*H	3		
6a	24.9.85	*A	3	*A	45	*S.callidum*	1
				*H	7		
				*I	5		
6a	16.10.85	A	64	*A	19	*S.callidum*	11
				*B	3		
				*I	10		
6b	22.6.85	-		*A	113	*Cnephia sp.*	1
				*I	1		
6b	24.6.85	-		A	83	-	
				*H	3		
				*I	9		
6b	29.6.85	-		*A	52	?*S.paynei*	14
6b	4.8.85	*A	3	A	238	-	
				*H	1		

Appendix V (iv) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
6b	24.9.85	A	43	A	66	-	
				*H	8		
				*I	16		
6b	16.10.85	*A	6	A	90	-	
				H	7		
				I	28		
6b	24.1.86	A	2	*A	9	-	
				*B	1		
				*I	10		
6c	24.9.85	*A	5	*A	1	-	
				*H	9		
6c	24.1.86	-		*A	200+	-	
6c	26.1.87	A	22	*A	2	-	
				I	3		
6d	20.6.85	*A	6	*A	31	S.callidum	2
				*I	2	?S.paynei	13
6d	24.9.85	*A	50	*A	30	S.callidum	2
				*I	5	?S.paynei	3
6d	18.10.85	*A	9	*A	1	S.callidum	28
				*I	4	?S.paynei	25
6d	26.1.87	-		*A	89	S.paynei	1
SE3	24.1.86	A	96	*A	11	S.callidum	1
				*I	34		
SE4	24.1.86	*A	2	*A	14	?S.paynei	7
				*I	6		
6e	26.9.85	*A	6	*A	6	S.callidum	31
				*H	28	?S.paynei	2
6e	17.10.85	*A	9	*A	11	S.callidum	3
				*H	16	?S.paynei	2
				*I	15		

Appendix V (v) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
6f	21.6.85	*A	2	*H	4	S.callidum	16
				*I	5	Cnephia sp.	2
6f	2.8.85	*A	3	*A	36	S.callidum	42
				*H	20		
				*I	39		
6f	26.9.85	*A	36	*A	43	S.callidum	8
				*H	9	?S.paynei	4
				*I	34		
6f	17.10.85	A	75	*A	12	S.callidum	54
				*H	3		
				*I	79		
6f	24.1.86	A	23	*A	5	-	
				*I	5		
6f	26.1.87	A	39	*A	2	-	
6g	18.6.85	*A	1	*H	5	S.callidum	8
				*I	14	Cnephia sp.	1
6g	23.6.85	*A	20	*H	18	S.callidum	11
				*I	71	Cnephia sp.	2
6g	2.8.85	*A	27	*H	33	S.callidum	7
6g	26.9.85	A	71	*H	1	S.callidum	58
				*I	10		
6g	17.10.85	A	70	*I	12	S.callidum	77
6g	26.1.87	*A	3	*A	2	-	
				*I	2		
6h	18.6.85	*A	9	*I	25	-	
6h	23.6.85	*A	4	*H	2	S.callidum	2
				*I	42	Cnephia sp.	2

Appendix V (vi) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
6h	2.8.85	A	68	*A I	1 68	S.callidum	47
6h	26.1.87	*A	9	I	7	-	
6i	19.6.85	-		*A *H *I	1 7 14	S.callidum	1
6i	25.6.85	-		H I	9 83	-	
6i	5.8.85	-		*A *H *I	2 2 1	-	
6i	25.9.85	*A	13	*A *H *I	26 10 15	Cnephia sp.	4
6i	16.10.85	*A	2	*I	24	Cnephia sp.	5
6j	19.6.85	-		-		Cnephia sp.	59
6j	25.6.85	-		*A *H *I	3 4 18	Cnephia sp.	116
6j	5.8.85	*A	3	*A *H *I	84 19 3	Cnephia sp.	10
6j	25.9.85	A	36	A H I	37 36 54	S.callidum	15
6j	16.10.85	*A	18	*I	47	S.callidum	6
6k	19.6.85	-		*H *I	1 1	Cnephia sp.	58
6k	25.6.85	-		A H I	3 2 3	Cnephia sp.	125

Appendix V (vii) Collection sites - *Simulium* larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : *S.ochraceum* cytotype		*S.metallicum* cytotypes		Other *Simulium*	
6k	5.8.85	-		A H I	90 23 5	*Cnephia sp.*	1
6k	25.9.85	*A	27	*A *H *I	9 9 57	*Cnephia sp.*	1
6k	16.10.85	-		*H *I	2 6	-	
6l	19.6.85	-		-		*Cnephia sp.*	45
6l	26.6.85	-		-		*Cnephia sp.*	74
6l	6.8.85	-		*A *H *I	15 21 9	-	
6l	25.9.85	*A	12	A H I	8 38 6	-	
6l	16.10.85	-		*H *I	2 6	-	
6m	19.6.85	-		*H *I	13 68	*Cnephia sp.*	2
6m	26.6.85	*A	1	*H I	1 35	*Cnephia sp.*	2
6m	6.8.85	*A	6	*A *H *I	1 5 21	-	
6m	25.9.85	*A	12	*H *I	5 23	-	
6m	16.10.85	A	30	*A *H *I	4 7 82	-	

Appendix V (viii) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
6n	19.6.85	-		*I	4	Cnephia sp.	8
6n	26.6.85	-		*I	32	Cnephia sp.	74
6n	6.8.85	*A	16	*H *I	13 72	S.callidum	1
6n	25.9.85	A	50	*H *I	3 96	S.callidum	43
6n	16.10.85	*A	15	*I	59	S.callidum	8
6o	17.10.85	*A	2	*I	3	S.callidum	7
7	8.2.87	-		A	164	S.downsi	2
8	8.2.87	-		A	62	-	
9a	5.2.87	A	18	I	13	-	
9b	5.2.87	A	3	A I	4 1	-	
10a	6.2.87	-		*A	84	S.paynei	35
10b	6.2.87	-		*A	12	S.paynei S.nigricornis	48 50
10c	6.2.87	A	16	*A	9	-	
10d	6.2.87	A	20	A I	27 4	?S.paynei S.nigricornis	1 1
10e	6.2.87	-		*A	253	-	
11a	22.7.85	*A	6	*A	142	S.callidum S.downsi ?S.paynei	145 81 7
11a	7.8.85	-		*A	26	S.callidum S.downsi ?S.paynei	185 110 51

Appendix V (ix) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
11a	8.2.87	-		*A	84	S.downsi	1
11b	22.7.85	*A	3	*A	44	S.callidum	16
						?S.paynei	1
11b	7.8.85	A	55	*A	118	S.callidum	75
						S.downsi	23
						?S.paynei	6
11b	8.2.87	-		*A	3	-	
				*I	1		
11c	22.7.85	-		*A	320	?S.paynei	6
				*I	1		
11c	7.8.85	-		*A	39	?S.paynei	20
11c	8.2.87	-		A	9	-	
				I	2		
11d	8.2.87	-		A	103	S.callidum	2
						S.downsi	2
11e	9.2.87	-		*A	269	-	
12a	19.9.85	-		A	12	-	
12a	24.10.85	-		*A	10	-	
12b	24.10.85	-		*A	2	S.callidum	18
						S.downsi	1
12b	22.1.86	*A	1	*A	40	S.callidum	11
12c	19.9.85	*A	1	-		S.callidum	35
						S.downsi	179
						S.paynei	5
12c	22.1.86	-		*A	136	S.callidum	1
						S.downsi	5

Appendix V (x) Collection sites - *Simulium* larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : *S.ochraceum* cytotype		*S.metallicum* cytotypes		Other *Simulium*	
12d	19.9.85	*A	2	*A	131	*S.callidum*	15
						?*S.paynei*	8
12d	24.10.85	*A	1	*A	85	*S.callidum*	2
12d	22.1.86	-		*A	10	-	
12e	18.9.85	*A	2	*A	68	*S.callidum*	41
				*H	1	*S.paynei*	11
12e	24.10.85	A	6	*A	29	*S.callidum*	85
12f	18.9.85	-		*A	19	*S.callidum*	47
						S.paynei	5
12f	24.10.85	*A	3	*A	27	*S.callidum*	15
						S.downsi	1
12g	18.9.85	-		*A	163	*S.callidum*	5
						?*S.paynei*	1
12g	24.10.85	A	16	*A	28	*S.callidum*	49
12h	18.9.85	-		*A	68	*S.callidum*	2
						?*S.paynei*	4
12h	24.10.85	-		*A	4	-	
12i	20.9.85	*A	8	*A	42	*S.callidum*	97
				*B	3	*S.downsi*	2
						S.paynei	18
12i	24.10.85	*A	23	*A	65	*S.callidum*	18
				*B	4		
12i	22.1.86	-		*A	28	-	
				*I	1		
12j	20.9.85	*A	38	A	57	*S.callidum*	45
				B	6	*S.downsi*	2
						?*S.paynei*	2

Appendix V (xi) Collection sites - *Simulium* larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : *S.ochraceum* cytotype		*S.metallicum* cytotypes		Other *Simulium*	
12j	24.10.85	A	44	*A	5	*S.callidum*	1
12j	22.1.86	-		*A	16	*S.callidum*	47
						?*S.paynei*	4
12k	20.9.85	*A	22	*A	34	*S.callidum*	59
				*B	2	*S.downsi*	1
				*I	1		
12k	22.1.86	-		*A	6	*S.callidum*	1
				*H	2		
12l	20.9.85	A	12	*A	39	*S.callidum*	5
				*I	3		
12l	24.10.85	*A	28	*A	4	*S.callidum*	15
				*I	10		
12l	22.1.86	-		*A	1	-	
				*H	1		
13a	18.7.85	*A	36	*A	294	*S.callidum*	13
13a	8.8.85	A	70	*A	184	*S.callidum*	33
				*B	5	?*S.rubicundulum*	4
13b	18.7.85	*A	14	*A	115	*S.callidum*	7
13b	8.8.85	*A	44	A	124	*S.callidum*	64
				B	3		
				H	1		
13c	18.7.85	-		*A	141	*S.callidum*	37
						S.downsi	7
						?*S.rubicundulum*	53
						S.sp.	1
13c	8.8.85	A	63	*A	37	*S.callidum*	172
				B	?	*S.downsi*	3
						?*S.rubicundulum*	182
						?*S.paynei*	2

Appendix V (xii) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
13d	18.7.85	*A	22	*A	55	S.callidum	58
						S.downsi	1
						?S.rubicundulum	4
						Cnephia sp.	1
13d	8.8.85	A	83	*A	66	S.callidum	88
						?S.rubicundulum	2
13e	9.2.87	-		A	54	?S.paynei	9
13f	9.2.87	-		A	21	-	
14a	10.2.87	A	31	*A	69	S.callidum	9
14b	10.2.87	A	18	A	22	-	
				*I	2		
15a	11.2.87	A	2	A	96	?S.paynei	1
15b	11.2.87	-		I	2	-	
15c	11.2.87	-		I	10	-	
16	10.2.87	-		-		S.callidum	7
						S.sp.	55
17a	28.2.87	-		A	28	-	
17b	5.11.87	*C	1	A	151	S.callidum	13
				B	3		
17c	6.11.87	-		A	5	S.paynei	13
18	6.11.87	-		A	52	-	
				B	?		
19	6.11.87	-		A	13	-	
				B	?		
20	6.11.87	*C	6	A	3	-	
21	27.2.87	C	26	A	20	-	

Appendix V (xiii) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
22	6.11.87	C	59	*A *B	7 4	S.callidum	7
23a	6.11.87	C	12	A	10	?S.paynei	3
23b	6.11.87	*C	4	A B	27 ?	S.paynei	7
24	7.11.87	C	3	-		S.callidum	1
25	7.11.87	*C	2	-		S.tricornis S.veracruzanum S.sp.	2 2 7
26	7.11.87	-		-		S.veracruzanum	27
27a	8.11.87	C	1	A	38	S.callidum S.downsi	9 41
27b	8.11.87	-		*A	11	S.callidum	7
28a	9.11.87	C	69	A B	13 1	S.callidum S.paynei	8 10
28b	9.11.87	C	12	A H	32 2	?S.paynei	3
29a	25.2.87	C	15	I	2	-	
29b	8.11.87	C	2	A B I	41 3 1	S.callidum ?S.paynei	1 3
30a	25.2.87	C	1	A	75	S.paynei	39
30b	25.2.87	-		-		S.paynei	41
31	23.2.87	-		*A	2	-	
32a	9.11.87	C	3	A B	179 3	S.callidum	27
32b	9.11.87	-		*A	3	S.yepocapense	1

Appendix V (xiv) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
32c	10.11.87	-		A	1	S.callidum	1
				B	5	S.downsi	89
33	29.10.87	-		*A	7	S.callidum	7
				*H	1		
				*I	3		
34	29.10.87	-		A	5	S.callidum	43
				B	3		
35	28.10.87	B	1	-		-	
36	28.10.87	-		H	52	-	
37a	29.10.87	-		*B	3	S.sp	7
				I	206		
37b	29.10.87	-		I	54	S.callidum	6
37c	29.10.87	-		I	9	S.callidum	57
38a	30.10.87	-		-		S.callidum	10
38b	30.10.87	-		I	1	S.callidum	51
39a	30.10.87	-		-		S.callidum	10
						?S.paynei	6
39b	30.10.87	-		-		S.deleoni	6
						S.parrai	43
						S.sp	25
40a	30.10.87	-		-		S.ruizi	32
						?S.anduzei	26
						?S.d'andretta	7
40b	30.10.87	-		-		?S.d'andretta	14
						S.sp	33
41	24.10.87	B	14	A	1	S.callidum	70
				I	5		

Appendix V (xv) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
42	24.10.87	B	25	A	1	S.callidum	36
				*H	1		
				I	11		
43a	24.10.87	-		A	22	S.callidum	3
43b	24.10.87	-		B	4	S.callidum	19
43c	24.10.87	B	7	I	23	S.callidum	98
						?S.menchacai	1
43d	24.10.87	B	12	*B	1	S.callidum	38
				I	109		
44a	24.10.87	B	7	A	12	S.callidum	25
				I	23	?S.paynei	10
				X	48	?S.rubicundulum	3
44b	24.10.87	-		*A	5	S.callidum	21
				X	4	?S.paynei	98
						?S.rubicundulum	11
44c	24.10.87	-		*I	6	S.callidum	17
				*X	1	?S.paynei	2
45	25.10.87	-		A	26	-	
				I	43		
				X	49		
46	25.10.87	B	2	*B	20	-	
				I	55		
47a	27.10.87	B	2	*A	2	S.callidum	7
				B	3	S.paynei	4
				I	11		
				X	3		
47b	27.10.87	B	29	*A	1	S.callidum	17
				*I	1		
48	27.10.87	*B	3	I	83	S.callidum	3

Appendix V (xvi) Collection sites - Simulium larvae present.
(* most probable cytotype based on focus/morphology)

Site	Date	Species Present : S.ochraceum cytotype		S.metallicum cytotypes		Other Simulium	
49	27.10.87	-		I	17	S.callidum	8
						?S.paynei	1
						S.deleoni	40
50a	26.10.87	-		A	3	S.callidum	43
				X	17	?S.paynei	126
						?S.rubicundulum	12
50b	26.10.87	-		*B	?	S.callidum	4
				*X	?7		
50c	26.10.87	-		-		S.paynei	6
51a	26.10.87	-		A	25	-	
				I	5		
51b	26.10.87	-		A	1	S.callidum	21
				I	3	S.sp	23
51c	26.10.87	-		*A	3	S.callidum	28
				I	17		
				X	4		
51d	26.10.87	-		*A	3	S.callidum	7
				X	2		
52a	26.10.87	*B	1	A	50	?S.paynei	8
				B	21	S.deleoni	2
				I	89		
				X	18		
52b	26.10.87	B	36	*I	14	-	
				X	6		
53	31.10.87	-		-		S.deleoni	31
54a	31.10.87	-		I	7	?S.paynei	3
54b	31.10.87	-		A	37	S.callidum	7
				I	30		
				X	2		

Appendix VI (i) The six environmental factors used in the canonical correspondence analysis showing the classes used.

Altitude : Class	Range (m)
1	0 - 304
2	305 - 609
3	610 - 914
4	915 - 1219
5	1220 - 1524
6	1525 - 1829
7	1830 - 2134

Temperature : Class	Range (°C)
1	<10
2	10 - 11
3	12 - 13
4	14 - 15
5	16 - 17
6	18 - 19
7	20 - 21
8	22 - 23
9	>24

Size : Class	Type
1	1
2	1/2
3	2
4	2/3
5	3
6	3/4
7	4
8	4/5
9	5

pH : Class	Range (pH)
1	4.6 - 5.0
2	5.1 - 5.5
3	5.6 - 6.0
4	6.1 - 6.5

Shade : Class	Type
1	None
2	Scant
3	Part
4	Total

Water Clarity : Class	Type
1	Clear
2	Slightly cloudy
3	Cloudy

Appendix VI (ii) Output of CANOCO.

Weighted correlation matrix (weight = sample total)

SP AX1*	1.0000						
SP AX2	.0907	1.0000					
SP AX3	-.0131	.0844	1.0000				
SP AX4	.0743	.0455	-.0657	1.0000			
EN AX1*	.7499	.0000	.0000	.0000	1.0000		
EN AX2	.0000	.4800	.0000	.0000	.0000	1.0000	
EN AX3	.0000	.0000	.4447	.0000	.0000	.0000	1.0000
EN AX4	.0000	.0000	.0000	.3836	.0000	.0000	.0000
ALTITUDE	.5859	.0308	-.2452	-.0008	.7814	.0641	-.5514
SIZE	-.4767	-.0268	-.2327	.1599	-.6358	-.0558	-.5232
pH	-.6005	.1174	-.1369	-.1269	-.8008	.2445	-.3139
TEMP	-.3990	-.3365	.1512	-.1196	-.5321	-.7010	.3400
CLARITY	-.1496	-.0154	.0704	.0044	-.1996	-.0322	.1583
SHADE	-.1112	-.0150	.1189	.1291	-.1483	-.0313	.2674

	SP AX1	SP AX2	SP AX3	SP AX4	EN AX1	EN AX2	EN AX3

EN AX4	1.0000						
ALTITUDE	-.0020	1.0000					
SIZE	.4169	-.3155	1.0000				
pH	-.3309	-.3545	.4069	1.0000			
TEMP	-.3117	-.6335	.0642	.2559	1.0000		
CLARITY	.0116	-.0984	-.0354	.1703	.2869	1.0000	
SHADE	.3366	-.0612	-.2270	.1707	.0693	.0179	1.0000

	EN AX4	ALT	SIZE	pH	TEMP	CLAR	SHADE

* where SP AX1 = sample scores on the first ordination axis which are derived from the species scores by weighted averaging.

EN AX1 = sample scores on the first ordination axis which are linear combinations of the environmental variables.

Appendix VI (iii) Output of CANOCO.

Variance Inflation Factors (VIF)

Variable	VIF
Altitude	1.9867
Size	1.4850
pH	1.4456
Temperature	1.8911
Clarity	1.1338
Shade	1.1662

Species scores

Name	Axis 1	Axis 2	Axis 3	Axis 4	Weight
S.metallicum :					
cytotype A	-0.42	-0.33	0.31	-0.88	5284
cytotype B	-0.58	-0.22	2.32	0.05	314
cytotype H	0.63	0.53	0.60	-0.19	217
cytotype I	3.24	1.01	-0.47	-0.71	1077
cytotype X	3.96	-2.41	2.44	3.09	154
S.ochraceum :					
cytotype A	-1.15	2.51	-0.36	0.94	1198
cytotype B	2.70	-1.98	0.12	3.73	139
cytotype C	1.31	1.09	-3.33	-0.92	216
S.callidum	-0.37	-0.28	-0.09	1.24	2511
S.downsi	-1.00	-2.89	-5.13	0.20	278
S.paynei/ S.rubicundulum	0.10	-0.51	0.49	0.34	926

Appendix VI (iv) Output of CANOCO.

Sample scores

Name	Axis 1	Axis 2	Axis 3	Axis 4	Weight
1a	-0.38	-0.29	0.34	-0.41	59
1b	-0.42	-0.32	0.30	-0.48	63
1c	-0.51	-0.34	1.12	-0.30	123
2	-0.43	-0.37	0.25	-0.67	113
3a	-0.42	-0.33	0.33	-0.84	80
3b	-0.41	-0.32	0.24	-0.52	6
3c	-0.41	-0.32	0.21	-0.35	4
3d	-0.43	-0.26	0.58	0.91	18
3e	-0.41	-0.27	0.41	0.95	49
3f	-0.26	-0.44	-0.08	0.81	29
3g	0.10	-0.50	0.48	0.33	66
4a	-0.69	0.68	0.83	0.39	204
4b	-0.42	-0.33	0.31	-0.88	317
4c	-0.58	0.40	-0.01	0.37	16
4d	-0.25	1.28	0.08	0.38	107
4e	-0.12	-0.42	0.40	0.06	112
4f	-0.49	0.27	0.05	0.15	298
4g	-0.80	1.25	-0.22	1.06	247
4h	-1.09	2.30	-0.28	0.84	273
4i	-0.60	0.23	0.88	0.18	50
4j	-0.43	-0.17	0.42	0.84	100
4k	-0.44	0.51	0.03	0.97	23
5a	-0.41	-0.28	0.18	-0.20	522
5b	0.20	0.32	0.05	-0.51	51
5c	-0.37	-0.28	-0.06	1.04	189
5d	-0.39	-0.31	0.02	0.53	292
5e	-0.42	-0.33	0.30	-0.82	589
5f	-0.37	-0.31	0.22	-0.25	181
6a	-0.71	1.04	-0.01	0.08	62
6b	-0.04	-0.18	0.24	-0.84	95
6c	-0.61	2.13	-0.33	0.62	27
6d	-0.41	-0.33	0.31	-0.86	90
6f	0.75	0.24	0.00	-0.06	140
6g	-0.19	1.24	0.14	0.42	67
6h	0.67	1.23	-0.33	0.40	184
6i	0.73	0.28	0.27	-0.57	5
6j	-0.16	-0.07	0.32	-0.70	109
6k	-0.06	-0.11	0.33	-0.73	118
6l	0.80	0.34	0.29	-0.52	45
6m	1.93	1.17	-0.26	-0.33	33
6n	2.18	1.17	-0.31	-0 36	102
7	-0.43	-0.36	0.24	-0.86	166
8	-0.42	-0.33	0.31	-0.88	62
9a	0.69	1.88	-0.41	0.25	31
9b	-0.24	0.90	-0.04	-0.17	8
10a	-0.27	-0.38	0.36	-0.52	119
10b	0.00	-0.47	0.45	0.10	60
10c	-0.89	1.49	-0.12	0.29	25
10d	-0.41	0.86	-0.01	-0.14	52
10e	-0.42	-0.33	0.31	-0.88	253

Appendix VI (v) Output of CANOCO.

Sample scores (continued)

Name	Axis 1	Axis 2	Axis 3	Axis 4	Weight
11a	-0.50	-1.09	-1.47	0.66	372
11b	-0.59	0.03	-0.38	0.17	277
11c	-0.24	-0.39	0.37	-0.46	59
11d	-0.43	-0.38	0.20	-0.82	107
11e	-0.42	-0.33	0.31	-0.88	269
13a	-0.58	0.35	0.14	-0.18	296
13b	-0.54	0.22	0.10	0.05	236
13c	-0.29	-0.01	0.11	0.66	465
13d	-0.65	0.67	-0.07	0.54	239
13e	-0.34	-0.36	0.33	-0.70	63
13f	-0.42	-0.33	0.31	-0.88	21
14a	-0.62	0.48	0.08	-0.18	109
14b	-0.56	0.95	-0.02	-0.09	42
15a	-0.43	-0.28	0.29	-0.83	99
15b	3.24	1.01	-0.47	-0.71	2
15c	3.24	1.01	-0.47	-0.71	10
16	-0.37	-0.28	-0.09	1.24	7
17a	-0.42	-0.33	0.31	-0.88	28
17b	-0.41	-0.32	0.29	-0.70	168
17c	-0.04	-0.46	0.44	0.01	18
18	-0.42	-0.33	0.31	-0.88	52
19	-0.42	-0.33	0.31	-0.88	13
20	0.74	0.62	-2.12	-0.90	9
21	0.56	0.47	-1.75	-0.90	46
22	0.90	0.77	-2.41	-0.67	77
23a	0.48	0.33	-1.42	-0.75	25
23b	-0.14	-0.21	-0.04	-0.66	38
24	0.89	0.75	-2.52	-0.38	4
25	1.31	1.09	-3.33	-0.92	2
27a	-0.66	-1.49	-2.88	-0.17	89
27b	-0.40	-0.31	0.15	-0.05	18
28a	0.82	0.63	-2.17	-0.61	101
28b	0.08	0.04	-0.56	-0.78	49
29a	1.54	1.08	-2.99	-0.89	17
29b	-0.26	-0.25	0.27	-0.71	51
30a	-0.23	-0.38	0.34	-0.46	115
30b	0.10	-0.51	0.49	0.34	41
31	-0.42	-0.33	0.31	-0.88	2
32a	-0.39	-0.30	0.23	-0.59	212
32b	-0.42	-0.33	0.31	-0.88	3
32c	-0.97	-2.70	-4.63	0.19	96
33	0.27	-0.04	0.04	0.01	18
34	-0.39	-0.28	0.09	0.96	51
35	2.70	-1.98	0.12	3.73	1
36	0.63	0.53	0.60	-0.19	52
37a	3.18	0.99	-0.43	-0.69	209
37b	2.88	0.88	-0.43	-0.51	60
37c	0.12	-0.10	-0.14	0.98	66
38a	-0.37	-0.28	-0.09	1.24	10
38b	-0.30	-0.25	-0.10	1.20	52

Appendix VI (vi) Output of CANOCO.

Sample scores (continued)

Name	Axis 1	Axis 2	Axis 3	Axis 4	Weight
39a	-0.19	-0.36	0.12	0.90	16
41	0.31	-0.47	-0.08	1.50	90
42	1.22	-0.65	-0.06	1.74	74
43a	-0.41	-0.33	0.26	-0.62	25
43b	-0.41	-0.27	0.33	1.03	23
43c	0.45	-0.14	-0.15	1.03	128
43d	2.32	0.47	-0.32	0.09	160
44a	2.11	-0.97	0.90	1.43	128
44b	0.12	-0.52	0.45	0.51	139
44c	0.67	-0.08	-0.04	0.79	26
45	2.73	-0.71	0.91	0.83	118
46	2.23	0.61	0.27	-0.39	77
47a	1.50	-0.17	0.35	0.54	32
47b	1.56	-1.28	0.03	2.66	48
48	2.95	0.72	-0.40	-0.27	44
49	2.05	0.57	-0.32	-0.09	27
50a	0.32	-0.62	0.53	0.75	201
50b	-0.37	-0.28	-0.09	1.24	4
50c	0.10	-0.51	0.49	0.34	6
51a	0.19	-0.11	0.18	-0.85	30
51b	0.06	-0.13	-0.12	0.92	25
51c	1.14	-0.03	0.00	0.62	52
51d	0.34	-0.65	0.43	1.02	12
52a	1.76	0.10	0.38	-0.23	187
52b	2.97	-1.28	0.22	2.55	56
54a	2.30	0.55	-0.18	-0.39	10
54b	1.14	0.15	0.02	-0.51	76

Biplot scores of environmental variables

Name	Axis 1	Axis 2	Axis 3	Axis 4
Altitude	0.368	0.020	-0.156	-0.001
Size	-0.299	-0.017	-0.148	0.106
pH	-0.377	0.075	-0.089	-0.084
Temp	-0.251	-0.215	0.096	-0.079
Clarity	-0.094	-0.010	0.045	0.003
Shade	-0.070	-0.010	0.076	0.085

Appendix VI (vii) Output of CANOCO.

Canonical coefficients.

Name	Axis 1	Axis 2	Axis 3	Axis 4
Altitude	0.317	-0.240	-0.310	-0.058
Size	-0.233	-0.148	-0.191	0.238
pH	-0.321	0.171	-0.130	-0.223
Temperature	-0.059	-0.440	-0.063	-0.114
Clarity	-0.045	0.058	0.051	0.073
Shade	-0.078	-0.059	0.047	0.187

*t-values of canonical coefficients

Name	Axis 1	Axis 2	Axis 3	Axis 4
Altitude	3.96	-2.98	-3.85	-0.68
Size	-3.37	-2.12	-2.74	3.27
pH	-4.71	2.49	-1.90	-3.10
Temp	-0.76	-5.59	-0.80	-1.38
Clarity	-0.74	0.95	0.84	1.15
Shade	-1.28	-0.95	0.75	2.89

* strictly speaking canonical coefficients have too large a variance for t-values to be applicable.

Appendix VI (viii) Output of CANOCO.

Results of the Monte Carlo significance test for the first eigenvalue.

Data - first eigenvalue = 0.332

99 random sets - first eigenvalues :

0.133	0.055	0.102	0.149	0.152	0.136	0.095	0.172
0.081	0.170	0.112	0.068	0.171	0.109	0.148	0.173
0.126	0.095	0.111	0.111	0.128	0.153	0.103	0.141
0.100	0.100	0.086	0.087	0.135	0.138	0.111	0.127
0.132	0.102	0.130	0.125	0.099	0.121	0.195	0.167
0.103	0.119	0.117	0.129	0.088	0.110	0.085	0.087
0.157	0.089	0.194	0.121	0.102	0.141	0.082	0.133
0.084	0.103	0.110	0.100	0.117	0.128	0.115	0.140
0.126	0.097	0.102	0.131	0.152	0.108	0.092	0.187
0.087	0.138	0.194	0.148	0.108	0.091	0.077	0.170
0.125	0.167	0.078	0.138	0.095	0.196	0.114	0.104
0.129	0.102	0.130	0.113	0.113	0.087	0.102	0.148
0.107	0.119	0.082					

P-value < 0.01

Results of Monte Carlo significance test for the complete trace.

Sum of eigenvalues for the trace = 0.624

99 random sets - complete trace :

0.430	0.411	0.387	0.494	0.484	0.584	0.431	0.564
0.440	0.400	0.355	0.308	0.667	0.526	0.524	0.613
0.581	0.488	0.535	0.420	0.391	0.397	0.461	0.495
0.500	0.389	0.463	0.418	0.450	0.534	0.482	0.443
0.512	0.317	0.441	0.422	0.536	0.473	0.430	0.709
0.646	0.424	0.447	0.432	0.376	0.426	0.363	0.404
0.485	0.444	0.561	0.553	0.467	0.642	0.348	0.546
0.346	0.423	0.436	0.340	0.423	0.573	0.471	0.476
0.447	0.551	0.484	0.613	0.545	0.474	0.381	0.567
0.474	0.415	0.602	0.594	0.497	0.500	0.320	0.481
0.457	0.573	0.392	0.644	0.495	0.650	0.552	0.714
0.469	0.532	0.526	0.432	0.487	0.442	0.452	0.495
0.549	0.522	0.394					

P-value = 0.08