STUDIES ON *IN VITRO* COLONIZATION, KARYOMORPHOLOGY, AND TEMEPHOS SUSCEPTIBILITY OF *SIMULIUM DAMNOSUM* THEOBALD COMPLEX.

BY

MIKE YAW OSEI-ATWENEBOANA

A THESIS SUBMITTED TO THE UNIVERSITY OF GHANA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN ZOOLOGY

DEPARTMENT OF ZOOLOGY
UNIVERSITY OF GHANA
LEGON, GHANA

DECLARATION

I certify that this work has not been submitted to any other university for any degree. The experimental work was carried out by me. I have given the due acknowledgment to all from whom guidance and directions were received.

Mike Yaw Osei-Atweneboana (Student)

Prof.W.Z . Coker (Supervisor)

Dr M.D. Wilson (Supervisor)

Dr. D.N.D Wilson (Supervisor)

DEDICATION

To my Dad Frank Osei-Atweneboana, my uncle Mac James Granaham, Dr. M.D. Wilson and Miss Veronica Konadu.

ACKNOWLEDGMENTS

I wish to acknowledge with sincere thanks, my supervisors at the Noguchi Memorial Institute for Medical Research, (NMIMR), Drs. M.D.Wilson and D.A. Boakye for their guidance, advice, constructive suggestions and criticisms throughout the thesis research. I am also grateful to my supervisors at Zoology Department, Professor W.Z Coker and Dr. D.N Wilson for their contributions, guidance, and advice throughout the project.

My heartfelt thanks go to Dr. Philip McCall for sharing some of his brilliant ideas with me, and for providing the pheromone extract from ovaries of female *Simulium damnosum* s.l. that was used for part of this study. I am also thankful to Professor R.J. Post of Wageningen Agricultural University, The Netherlands, for giving me more insight into cytotaxonomy, and to Miss M.A. Cobblah, lecturer at the Zoology Department for her advice and encouragement. I also appreciate the support, encouragement and company of Edward Crabbe, Anita Ofosu-Okyere and Bridgette-Marian Ogoe (Mrs.), my laboratory mates at NMIMR. I am thankful to Mr. Alfred Duodu, of the Electron Microscopy Unit at NMIMR for all photographs used in this work.

I am also grateful to all those in Zoology Department, University of Ghana, who in one way or the other contributed towards this project. My utmost gratitude goes to Veronica Konadu, Boakye Mensah, Baffour Gyawu Jnr. and Oheneba Osei Akoto for their love, care and bearing with me, during the period of this project.

I thank Mr. Maxwell Appawu, Head of Parasitology Unit, NMIMR for his advice and support and Mrs Aba Wilmot for her encouragement and advice. Finally, I wish to express my sincere thanks to Professor Francis Nkrumah, Director of NMIMR for allowing me to work at the Institute.

I thank the Almighty God for seeing me through this programme.

The present study was supported by Research Grants awarded to Drs. M.D. Wilson and D.A. Boakye.

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ABSTRACT

The strategy of using insecticides against *Simulium damnosum* s.l. vectors, adopted by Control Programmes for human onchocerciasis in West Africa has led to increased vector biology studies and has at the same time also revealed the paucity of our knowledge in this area. To effectively control the vectors means that laboratory colonies need to be established to enable experiments to be carried out. It also means there is a need to intensify research on aspects of the biology that determine the epidemiological importance of the various species.

For the first part of the present study, a simple but effective technique was developed and used in various ways, including simulated group oviposition, to study *in vitro* oviposition of female flies. It was found that using a dripping effect of water was an improvement over the water immersion method that was used by most researchers.

For the second part of the study, cytotaxonomic studies for species distribution and for the revision of *S. sanctipauli* s.l. populations from the major river systems in South West Ghana, were carried out. Two different populations of *S. sanctipauli* s.s were recognized with a newly identified inversion, that occurred in the R. Tano populations which is named inversion IS-25.

For the final part of the study, tests were carried out on *S. sanctipauli* s.s. populations from two sites on the Rivers Pra and Tano, with the aim of relating chromosomal inversions to Temephos (an organophosphate insecticide) susceptibility levels. It was found that *S. sanctipauli* s.s. populations from Sutri rapids (R.Tano) were resistant to Temephos, whilst those from Sekyere-Heman (R. Pra) were susceptible at the recommended diagnostic dose.

A chromosomal analysis however, did not show the presence of any unique inversion or differences in the chromosomal inversion frequencies between the general populations and

the resistant population. Therefore it was concluded that no chromosomal inversions were responsible for the resistance that was detected on the R. Tano.

CHAPTER ONE

GENERAL INTRODUCTION

Human onchocerciasis, a severely debilitating blinding disease is caused by infection with the parasitic filarial nematode *Onchocerca volvulus* (Leuckart, 1893). It is one of the most common causes of infectious blindness world-wide, and the second most devastating infectious disease following poliomyelitis in terms of its economic consequences to an afflicted community (WHO, 1987). The disease is usually widespread in rural areas, and causes a lot of distress and disfigurement, but it is the blindness that accounts for its main socio-economic impact. Thus, it can cause a reduction in an individual's ability to carry out everyday tasks, especially in those people who exist by subsistence farming (Mackenzie *et al.*, 1985). In the worst affected villages of savanna areas of West Africa for example, up to about 15% of a whole population (including children) and more than 40% of the males of working age may be blind (Duke, 1990). In these villages, the mortality rate among the blind persons over 30 years of age is three to four times higher than for sighted people of the same age group (Prost and Vaugelade, 1981).

The prevalence and the severity of onchocerciasis as well as the magnitude of the associated social and economic effects vary widely in different geographical areas where the disease occurs. The disease is confined to the Tropics because its transmission is limited by climate and habits of the vector. The prevalence figures given by WHO (1987) indicate that, of the global total of persons infected, and of the numbers that are blind as a result of the disease, more than 99% live in the Tropical belt of Africa. There is also a small extension into the Arabian peninsula in Yemen

and adjacent areas of Saudi Arabia, and relatively small foci in Latin American countries of Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela (Duke, 1990).

Onchocerciasis is still endemic in 34 countries, 26 in the African Region, six in the Region of the Americas and two in the Eastern Mediterranean Region. Current estimations suggest that about 17.7 million of the world's population are infected with the disease, of whom some 270,000 are blind; in addition, a further 500,000 are severely visually disabled (WHO, 1995). It must however, be noted that these figures are certainly an underestimate and do not fully reflect the importance of the disease and its implication; more accurate data are needed from a number of countries. Furthermore it is difficult to estimate the visual disabilities caused by onchocerciasis, since the constriction of the visual field as a result of optic nerve involvement is not usually assessed in routine survey procedures (WHO, 1995).

Duke *et al.* (1966) were the first to report on the existence of forest and savanna strains of *O. volvulus*. These two postulated strains showed associations with epidemiological differences observed in the forest and savanna forms of the disease. They also showed that parasites from savanna developed very poorly in forest flies but well in savanna flies. Also the forest strain develops well in the forest flies but very poorly in savanna flies. This relationship of the taxonomy of both parasites and vectors to the pattern of the disease has been reported. Waddy (1962) reported that high rates of blindness occur in savanna countries, and that the blindness rate can reach as high as 15% in the savanna areas, with 100% infectivity. However, the infectivity rate can be similarly high in the forest bioclimate zones, but the ocular

manifestation of the disease may be very much less severe with overall blindness rates typically around 0.5% (Duke,1990).

In Ghana, according to the survey carried out by the Onchocerciasis Control Programme, 123,000 people have been infected by the disease, out of this 7,400 are blind. (WHO, 1995). The areas in Ghana seriously affected by the disease include the Northern Region, Brong-Ahafo Region, Ashanti, Volta and a small part of Eastern Region. Infections still occur along the river Pra in the Central Region, probably also along the Otchi river and the Tano and Ankobra rivers in the Western Region.

In West Africa the disease is transmitted exclusively by members of the *Simulium damnosum* Theobald complex. Up till 1966, *S. damnosum* was considered taxonomically uniform because of its rather constant morphology (Freeman and de Meillon, 1953). However, subsequent cytotaxonomic investigations showed that *S. damnosum* is not a single species but a complex of sibling species (Dunbar, 1969; Vajime and Dunbar, 1975), some of which are restricted to either the savanna or the forest. These biotypes are known to show profound differences in their epidemiological importance (Post and Boakye, 1992).

The *S. damnosum* species complex in West Africa has been described on the basis of the interspecific inversion differences in the banding sequences of the larval silk gland polytene chromosomes (Vajime and Dunbar, 1975). Six most common cytospecies were first described by Vajime and Dunbar (1975), but subsequently cytotaxonomic studies have increased this species complex to 15 cytotypes (Post and Crampton, 1988). However, not all these cytospecies are epidemiologically important, hence vector control strategies seek to discriminate against the more important vectors (Post and Boakye, 1992). Currently, there are nine sibling species within the

S. damnosum complex found in the Onchocerciasis Control Programme areas in West Africa. These are grouped under three subcomplexes: The *S. damnosum* subcomplex, comprising *S. damnosum* s.s., *S. sirbanum* and *S. dieguerense*; The *S. squamosum* subcomplex is made up of *S. yahense* and *S. squamosum*; and the *S. sanctipauli* subcomplex consists of *S. sanctipauli* s.s., *S. soubrense, S. leonense* and *S. konkourense.* (Boakye *et al.*, 1993; Boakye, 1993; Wilson and Post,1994).

It is generally the sibling species associated with the savanna zone i.e the *S. damnosum* subcomplex which are effective vectors of the blinding form of the disease. This observation forms the basis of the strategy of control adopted by the WHO Onchocerciasis Control Programme for West Africa (OCP), i.e., to control the more severe form of the disease that is associated with the savanna areas. To achieve this, it uses insecticides against the larval stages of the savanna vector species wherever they occur (Kurtak, 1990).

The OCP was set up in 1974 with two objectives, to combat the disease that was widespread and severe, and to remove a major obstacle to economic development in the area. The original area of the Programme consisted of 654,000 km^2 of Guinea and Sudan savanna in seven countries of West Africa, namely Mali, Côte d'Ivoire, Burkina Faso, Ghana, Togo, Benin and Niger. A first southward extension of its treated area was made to cover extra area in Côte d'Ivoire in 1978-1979 and brought the total area to 764,000 km^2. The area of operation however, was later extended in the early 1980's to include Guinea, Sierra Leone and Senegal and extensions further south in Benin, Togo and Ghana, such that the OCP now covers some 1,300,000 km^2 ranging from Sahel to rainforest (Philippon *et al.*,

1990). The activities of OCP spanning 20 years has generated a lot of interest and stimulated research studies in onchocerciasis. The major areas of the disease most researched include among others, the biology of the vectors, insecticides and evolution of resistance and disease transmission.

However, one of the major obstacles to onchocerciasis research still remains the inability to establish laboratory colonies of the vectors. The availability of such colonies would greatly increase our knowledge of *S. damnosum* biology, especially in the areas of genetic inheritance, systematics of the vector as applied to cytotaxonomic identifications and population studies, evolution of insecticide resistance, and testing of new control agents. In order to achieve such colonization, it is necessary to rear adults from the egg stage, and then induce the subsequent adults to mate, blood-feed, and oviposit. Each stage of the cycle is critical, but all of these present great difficulties that make permanent laboratory colonisation still impossible to achieve.

The OCP's mandate in the subregion expires in the year 2002 and with it will also end the opportunities that enabled the explosion of field research that accounted for the better knowledge of the disease. Of paramount importance to the OCP and the scientific community therefore is the need to establish self-perpetuating colonies so that the vectors could be investigated away from their natural breeding sites and at times of the year when little or no material is naturally available. Interest in the establishment of *S. damnosum* s.l. colonies has been rekindled because of the development of a very simple set-up for rearing blackflies by Boakye and colleagues at OCP (OCP unpublished data). It occupies very little

space and consists of a small plastic bucket and a water pump of the type used for indoor aquaria, and has been used successfully to rear eggs to adults.

For this study some of the methods used to carry out some of the very critical stages in rearing of *S. damnosum* s.l. were revisited and experiments carried out with the aim of improving upon them. The stages chosen were, laboratory survival of adult flies, mating and oviposition inducement. Specifically, experiments were carried out to obtain;

a) long term survival of *S. damnosum* s.l. at ambient temperatures that ensure that maximum numbers of blood-fed females live long enough till oviposition and beyond,

b) the best method that will result in maximum numbers of females mating in the laboratory,

c) a method that will combine effective egg inducement with high fertility rate,

d) to test the rearing system developed by Boakye, D. A.(unpublished data) and if necessary to modify it to improve upon it's efficiency.

As mentioned above, the existence of OCP stimulated onchocerciasis research and one of the major areas of vector biology that was extensively studied was cytotaxonomy. This is evident from the number of publications on the subject from the OCP area. The cytotaxonomic studies were mainly for identification and studies on insecticide resistance in members of the *S. damnosum* species complex. Of the members, species belonging to the *S. sanctipauli* subcomplex were the most studied. Furthermore, this species subcomplex is also the most reviewed

(Meredith *et al.*,1983; Post, 1984; 1986; Surtees *et al,* 1988; Boakye *et al.*, 1993; Boakye, 1993)

Two reasons necessitated the second part of the study involving cytotaxonomy, a standard reference for species identification and starting point for species distribution. Principally, as mentioned earlier, the strategy adopted for onchocerciasis control by OCP targeted only the savanna vectors. and as a result only the northern savanna half of Ghana was included in the OCP areas of the control programme. The south western half was however, not considered.

Hence there is virtually no comprehensive information of the status of onchocerciasis in southern Ghana and whatever exists is very patchy, being data collected by various researchers with differing research objectives. But the available evidence indicates that there are several areas of hyperendemicity. For example, a 1989 survey of 1722 workers of Twifo Oil Palm Plantation, found 1448 (84.1%) to have onchocerciasis. (Wilson, M.D. unpublished data)

Moreover, the picture of distinct forest and savanna distributions is not the case on the ground, because of deforestation and also of human migration. Deforestation activities are creating derived savanna enclaves within the forest that will result in the southward extension of the savanna vectors and parasite (Baker *et. al.*, 1990), thus extending the need for control. The reason is that the derived savanna will be suitable for the perennial breeding of savanna vector species. Human migration along the north-south axis of West Africa is also leading to the introduction of the savanna parasite strains into forested areas.

The rivers Tano, Pra-Ofin and tributaries form two of the four main river systems of south-western Ghana. They are characterised by large breeding sites

around which are focalised zones hyperendemic for onchocerciasis. The extent of blindness or visual impairment resulting from infections with onchocerciasis has not really been determined. A study conducted by the OCP in a small area of Brong Ahafo also revealed that a very high proportion of children are infected and most important of all with blinding onchocerciasis. Previous prospections (Wilson, M..D Pers. comms.) have all revealed that the savanna flies, especially *S. damnosum* sensu stricto occur in these river basins. There is also intense deforestation presently going on, mainly as a result of human activities such as logging, farming, surface mining etc.. The region is also characterised by intense human migration which is mostly in the north-south direction.

The second reason is that *S. sanctipauli* is the most common species found below latitude 6°N. This species is the most reviewed, has been extensively studied , and has the ability to develop resistance and new inversions (Post, 1984; 1986; Surtees *et al* 1988; Boakye *et al* 1993; Boakye 1993). Despite all these developments, the taxonomic status of *S. sanctipauli* in areas in Ghana outside the OCP is not known very well, as detailed studies have not been carried out. And it is likely that detailed studies might reveal new inversions and inversion frequencies unique to the populations in South Western Ghana.

For the reason given above the second part of the study therefore will seek:

1) To provide a comprehensive cytotaxonomic data for south-western Ghana, as a database for *S. sanctipauli* populations that are found in the rain forest belt of south-western Ghana;

2) To detect new inversions, inversion frequencies and sex-linked inversions if any for taxonomic purposes;

3) And also to determine if the inversion scored showed seasonal and clinal variation.

The last part of the study was undertaken because the problem of development and spread of insecticide resistance has necessitated the need for the frequent check of insecticide susceptibility levels of *S. damnosum* s.l., from different localities since the development of resistance in a particular species, can easily lead to the spread of resistance to other river systems (Kurtak, 1990).

Both the Tano and Pra-Ofin river basins are heavily farmed along the banks, especially for cocoa and a lot of pesticides, usually Dieldrin® are used. It has frequently been observed that the use of agricultural insecticides in areas around river systems where farming is carried out extensively, affects the susceptibility levels of *S. damnosum* population, by increasing the tolerance levels. The consequence is that resistance to Abate® rapidly develops, thus necessitating the use of more expensive insecticides for vector control.

Again, it has been postulated that resistant *S. sanctipauli* populations on the River Comoe are chromosomally identical to some of the populations found on the Tano (Boakye *et al.,* in press), If this is not noticed and reported earlier, it may subsequently produce resistant population and will result in the dependence on more expensive insecticides for vector control. In anticipation of possible vector control activities in both basins, it was found necessary to conduct insecticide susceptibility tests, to recognise and document the insecticide susceptibility levels for future and further studies.

The objectives of the third part of the study are two-fold, first, to determine first, the susceptibility levels of *S. sanctipauli* s.s. and secondly the relationship between the surviving larvae and chromosomal inversions.

The specific objectives are:

1) to determine the susceptibility levels of *S. sanctipauli* s.s. populations in the River Pra at Sekyere-Heman and River Tano at Sutri Rapids.

2) to find out if any difference found in susceptibility levels could be related to chromosomal inversions.

CHAPTER TWO

LITERATURE REVIEW

2.1 HUMAN ONCHOCERCIASIS : DISEASE AND SYMPTOMATOLOGY

Onchocerciasis, commonly called "river blindness" is caused by the filarial worm *Onchocerca volvulus* (Nematoda: Filaridae). The parasite, was first described in 1893 from three human subjects in the Gold Coast, now Ghana (Leuckart, 1893). The worm is a parasite in all its life cycle, and in man as the definitive host, it grows and becomes adult and reproduces. During the period between infection and appearance of microfilariae in the skin, there are usually no signs of the disease, but as soon as a heavy worm load develops various clinical and pathological manifestations ensue. The main clinical manifestations of onchocerciasis are skin lesions, onchocercal nodules, lymphadenopathy and ocular lesions (Duke, 1990).

Onchocercal nodules are subcutaneous tumours that harbour the adult *O. volvulus* and when visible or palpable on the surface are found mainly over bony prominences on the head, shoulder, girdle, rib, trochanter, knee and ankles. The nodules are rarely considered to have medical importance, causing the patient little or no discomfort, but when on the head, they may erode the bone (Muller, 1975; Manson-Bahr and Apted, 1982; Duke, 1990).

The early skin symptoms of infection include pruritic rash which is composed of numerous small circular raised discrete papules and in the hyperendemic areas of

Africa, the skin shows gross scarring. The skin also thickens owing to subcutaneous oedema and this leads to the characteristic 'peau d'orange' effect. This effect is often associated with lymph gland enlargement, especially in the groin and changes in pigmentation of the skin also occur, which may be increased or decreased. Depigmented spots are usually surrounded by areas of pigmentation, this symptom is commonly described as `leopard skin'. In Central America gross skin changes are less marked even when microfilariae are abundant. However, in the early stage there is pruritus and some people, especially children, may suffer a severe form of dermatitis known in Guatemala and Mexico as 'erisipela de la costa' and mal morado'. This is characterised by reddish lesions on the face and an adult may show a thickened smooth white face.

In the onchocerciasis foci of Yemen and Sudan, a severe itching papular dermatitis known as `sowda' is also common (WHO, 1987). In later stages and in chronic infections, reactions lead to heavier lichenoid changes and thickening of the skin, which give the appearance of lizard skin (xeroderma) and finally there is atrophy with loss of elasticity and a premature aged appearance (presbyderma). Skin changes can be unpleasant but are rarely considered to have important medical significance. However, with severe itching there can be loss of sleep and skin damage caused by scratching. The use of burning paraffin to obtain relief has been recorded (WHO, 1987).

When the eye is involved, a few microfilariae may invade the cornea, and response to the local presence or local microfilariae death produces fluffy opacities in early and late infections. Individual opacities, however, clear spontaneously but are succeeded by others. The symptoms at this stage are irritation of the eye, lacrimation and photophobia. Heavier and chronic infections lead to sclerosing keratitis in the

cornea and this is largely responsible for the exceptionally high onchocercal blindness in the Sudan-Guinea savanna zones of West Africa (Anderson and Fuglsang, 1977; Duke, 1990). Inflammation of the iris and the ciliary body of the eye with secondary glaucoma is also a major cause of blindness. Iridocyclitis can occur, often with microfilariae, flare, keratic precipitate cells in the anterior chamber. Pigment is deposited on the anterior capsule of the lens and the iris become bound down by posterior synechiae. The iris may display patches of atrophy. Progressive loss of vision takes place because the pupil becomes occluded with attendant secondary glaucoma or cataract (Nelson, 1970; Muller, 1975; Anderson and Fuglsang, 1977; Duke,1990).

Disturbance of the retinal pigment epithelium leads to marked sclerosis of the choroid and the retinal vessels. As the retina and choroid degenerate, choroidal and optic nerve atrophy result. Optic nerve atrophy may also result when it is invaded directly, leading to gross reduction of peripheral visual fields, key-hole vision or total blindness (WHO, 1987; Duke, 1990). There are, however, differences in the situation of the lesions in African and Central American onchocerciasis foci. In general, in the savanna area of West Africa, blindness is mainly due to sclerosing keratitis, whereas in the rain forest area blindness is often due to posterior segment lesions. In the Central American foci in Guatemala and Mexico blindness usually results from anterior uveitis (WHO, 1987).

In heavy microfilariae infection of the skin, the lymphatic system becomes involved when some of the parasites get to the lymph nodes where they produce lymphadenitis with subsequent fibrosis. Enlarged inguino-femoral nodes result, which hang in pockets of skin known as hanging groin, and predisposes the patient to hernia.

Lymphatic enlargement of the scrotum and hydrocele and enlarged testes also results in scrotal elephantiasis (Nelson, 1970; Duke, 1990).

The involvement of the central nervous system has also been noted (Mazzotti, 1959; Rodger, 1960). Raper and Ladkin (1950) claim that *O. volvulus* is the causative agent of the "Nakalanga syndrome" (the pygmy dwarfing resulting from damage to the pituitary gland) in Uganda.

2.2 THE EPIDEMIOLOGY AND SOCIOECONOMIC IMPACT OF ONCHOCERCIASIS

Onchocerciasis caused by *O. volvulus*, has human beings as the only vertebrate host. Infection with *O. volvulus* like other filaria infections are characterized by coincidence between the degree of human infection and the intensity of exposure to infected vectors. The epidemiology of the disease is not uniform throughout its distribution because different disease patterns are associated with different variants or strains of the parasite, difference in vector competence and feeding characteristics of local blackfly populations, and with difference in the human host responses to the parasite (WHO,1995).

The most recent prevalence figures indicate that, of all the global total of persons infected, and of the numbers that are blind as a result of the disease, more than 99% live in the tropical belt of Africa (WHO,1995). Onchocerciasis is still endemic in 34 countries, 26 in the African Region, six in the Region of the America and two in the Eastern Mediterranean Region. Current estimations, suggest that about 17.7 million of

the world's population are infected with the disease, of whom some 270,000 are blind; in addition, a further 500.000 are severely visually disabled (WHO, 1995).

In West Africa it is customary to make an implicit association between the clinical and parasitological patterns of onchocerciasis and bioclimatic zones by referring to savanna and forest onchocerciasis. Foci of "savanna type" (with heavy microfilarial loads and associated high rates of serious ocular lesions and blindness due to onchocerciasis), are only very exceptionally found south of latitude 7-8°N. The savanna area north of 8°N is the domain of *S. sirbanum* and *S. damnosum* s.s. and locally of small foci of *S. squamosum*; and with the exception of few unusual concentrations, especially in the rainy season, the *S. sanctipauli* subcomplex does not appear to play a significant role as a vector. South of latitude 7°N, the *S. sanctipauli* subcomplex and *S. yahense* predominate as vectors (WHO, 1987).

In West Africa and across the Sudan, no foci of "savanna type" onchocerciasis are known that are not associated with the presence of *S. sirbanum* and *S. damnosum* s.s. On the other hand foci of "forest type" onchocerciasis are always associated with transmission by the *S. sanctipauli* subcomplex and/ or *S. yahense* in West Africa, or with *S squamosum* and *S. mengense* in Cameroon (WHO, 1987)

Nevertheless, where the forest merges gradually into the savanna (6-8°N), there is a wide zone in which foci of blinding hyperendemic onchocerciasis do occur. Although the blindness rates are not usually as high as in the true savanna, the relationship between microfilarial loads and the frequency of serious ocular lesion is less clear.

The socio-economic consequences of the disease are most marked in the hyperendemic belt that extends across sub-Saharan Africa, excluding the West

African countries in the original OCP areas, where the burden of onchocercal blindness has been greatly reduced as a result of control measures (WHO, 1995). Within Africa, blindness rates in hyperendemic communities not under control may rise to 15% and up to 40% of adults may show severe ocular impairment.

The prevalence and the severity of onchocerciasis as well as the magnitude of the associated social and economic effect vary widely in different geographical areas where the disease occurs. As a results of the devastating nature of the disease, the intolerable misery due to its effects on the eye and skin, and fear of becoming blind, leads to the disintegration of social structure and abandonment of homes and farm lands.

2.3 THE DISTRIBUTION AND LIFE CYCLE OF *SIMULIUM*

The vectors of human onchocerciasis belong to the genus *Simulium* (Diptera: Simuliidae). The Simuliidae, is a family of small, usually stout blood-sucking insects with a humped thorax; they are commonly known as "blackflies". Simuliidae are of medical importance because of the various ways in which they affect man and animals. They are vectors of both human and animal onchocerciasis in Tropical Africa, Mexico, Central and South America and the Yemen (WHO, 1995; Duke, 1990). So far about 1,300 species names have been proposed for blackflies (Stone, 1965). These include 240 species from Neotropical Region (Vulcano, 1967), 140 species from Ethiopian Region (Crosskey, 1969), 400 species from Paleartic Region (Rubtzov, 1969), 120 from the Neartic Region (Stone, 1965), 190 from the Oriental Region (Crosskey, 1973a), and 50 species from the Australian Region (Colless and McAlpine, 1970).

Their bites are injurious, often causing oedema, pruritus and secondary infections due to scratching, and may also be intolerable nuisance because of their abundance and habit of landing and crawling about on the head, face, legs and body. As blood suckers, simuliids also transmit other parasites of both domestic and wild animals and birds.

The main vectors of human onchocerciasis worldwide are, *S. damnosum*, *S. neavei*, *S. ochraceum*, *S. metallicum* and *S. exiguum*, and are all complexes of sibling species. In Africa and the Southern Arabian peninsula, where about 99% of the disease occurs, two species complexes, *S. damnosum* s.l. and *S. neavei* s.l. are the main vectors of onchocerciasis (WHO, 1995).

The *S. damnosum* complex is the most important vector in West Africa, the Sudan, parts of Congo basin, Tanzania, Uganda, Ethiopia and probably Malawi and the Yemen. The members of this complex in West Africa include *S. damnosum* s.s., *S. sirbanum*, *S. sudanense*, *S. dieguerense*, *S. sanctipauli* s.s., *S. soubrense*. *S. leonense*, *S. konkourense*, *S. squamosum*, *S. yahense* and *S. mengense*.

Most members of *S. damnosum* s.l. include both anthropophilic and non-anthropophilic forms, for example in West Africa, all the members of the *S. damnosum* complex bite man, and may also bite other animals such as cattle etc. In East and Central Africa, species of the *S. neavei* complex also bite both man and other animals (WHO, 1995). This complex is made up of *S. neavei* s.s., *S. woodi* and *S. ethiopians*, of which *S. neavei* s.s. is the most important vector. The broad distribution of the species of *S. damnosum* s.l. in the OCP area within West Africa is shown in Fig. 1.1.

The requirement for fast flowing water restricts the distribution of the West African vectors, *S. damnosum* s.l. This usually coincides with areas where the precambrian basement rock forms the land surface and is exposed in the river-beds in the shape of the resistant rapids that break the flow and provide the classic breeding site (Crosskey, 1981). In the more northern regions of the savanna, the breeding sites are localised to perennial rivers with precambrian rapids and the periodic wet season extensions breeding ranges to rivers that cease in the dry season. In the south the distribution is limited by lagoons, coastal plains and sea. There are no east-west limitations on the distribution of the breeding sites of the vectors.

The cytospecies distribution of *S. damnosum* s.l. is closely linked with particular climatic/vegetation zones arranged in latitudinal bands of increasing dryness from south to north (Vajime and Quillévéré, 1978). The *S. damnosum* s.s. and *S. sirbanum* occupy

to north (Vajime and Quillévéré, 1978). The *S. damnosum* s.s. and *S. sirbanum* occupy the savanna zone as far as 14^0N. This is also the northern limit of the endemic area for onchocerciasis, while the southern limit extends well beyond the savanna zone. These species find their way into the forest along the major watercourses. The *S. soubrense* and *S. sanctipauli* are forest-dwelling, but to the north of the great dense forest it is also found in the zone of light wet forest and in the places even in the savanna up to 10^0N. *S. yahense* is limited to small forest watercourses. *S. squamosum*, probably covers several different entities and is widespread in both forest and savanna areas (WHO, 1987).

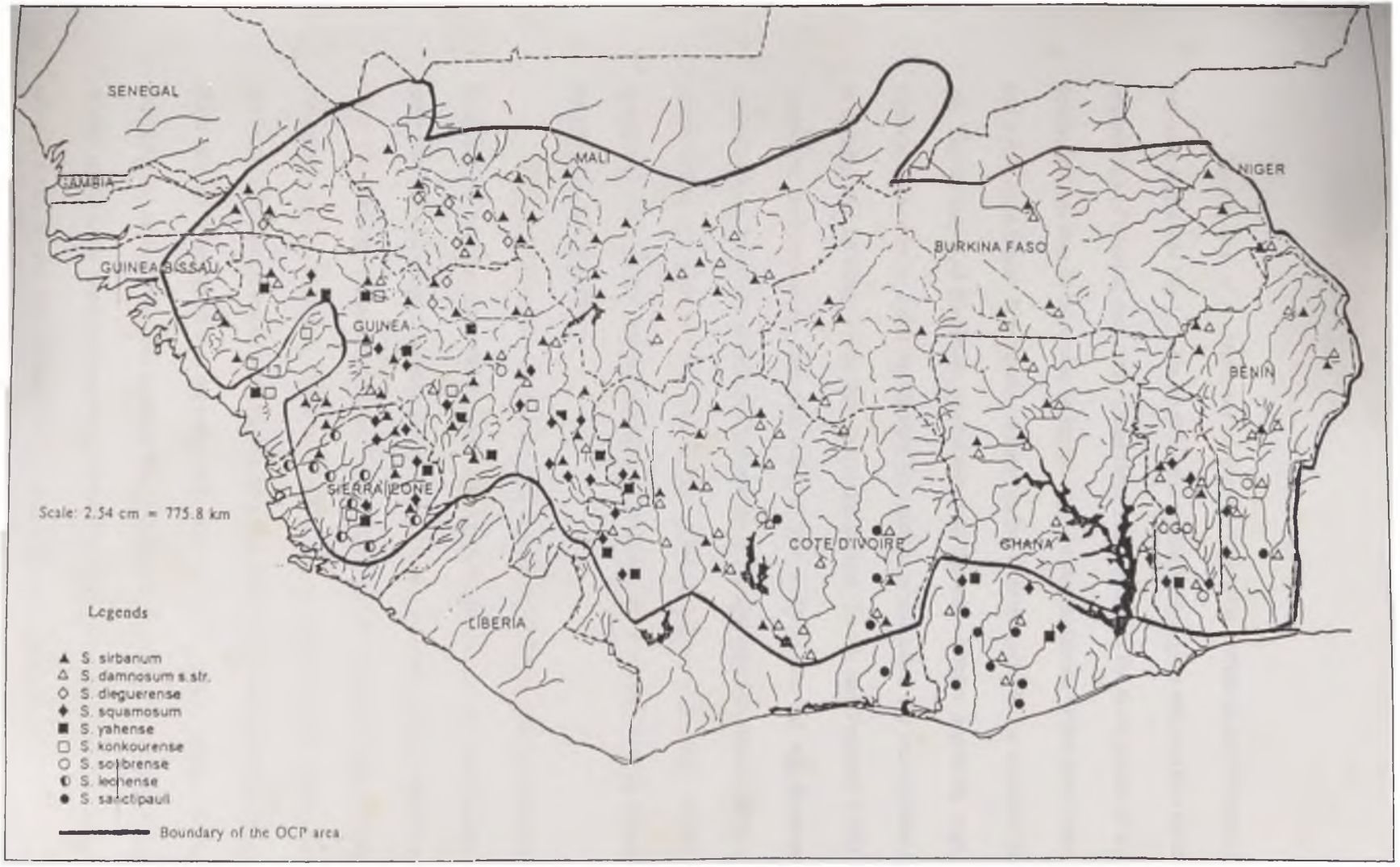


Figure 1.1
The distribution of cytospecies of the *Similium damnosum* complex in the Onchocerciasis Control Programme area of West Africa. (Source: unpublished OCP data).

The life cycle of *S. damnosum* s.l. involves both adult, terrestrial and the aquatic pre-adult stages (see Fig. 1.2) The adult females are haematophagous and may take up to one millilitre of blood at each meal. These blood meals condition the development of the ovaries and less than 24 hours after laying the eggs, the female takes a further blood meal and a new cycle begins. It completes as many as five or more cycles in the course of its life. The life-cycles of Simuliidae generally involve the adult females depositing eggs, which are in masses or chains on substrate (in zones at or just below the water surface) or actually into the water, where they sink to the bottom (Davies and Peterson, 1956). Embryonic development can take only a day or two in the tropics, but egg diapause occurs in many temperate species and in these life in the egg may last several months; overwintering can occur in this way in the Holarctic regions (Crosskey, 1973a). Depending on the species and the prevailing temperature, the period of hatching ranges from few hours to months.

The young larvae then move to the part of the river or stream that has adequate food material, oxygen, and other requirements. Most blackfly larvae are filter-feeders, using paired cephalic fans to remove sestonic particles that commonly range in size from 10 to 100 microns in diameter (Chance, 1970a). Larvae have from six to eight instars during their growth (Crosskey, 1973). In *S. damnosum* s.l. there are seven instars, the last instar spins cocoon around itself, then pupates after five days to several weeks. Pupal stage may last from about two days to a little over a week in most species before emerging into adults. It has been reported that, *S. damnosum* complex and other African species appear to be capable of regulating the time of pupation so that adult emergence can occur during the day (Disney, 1969). The adults mate immediately

mate immediately after emergence. The females generally live up to three weeks while the males may live for a few days only after mating.

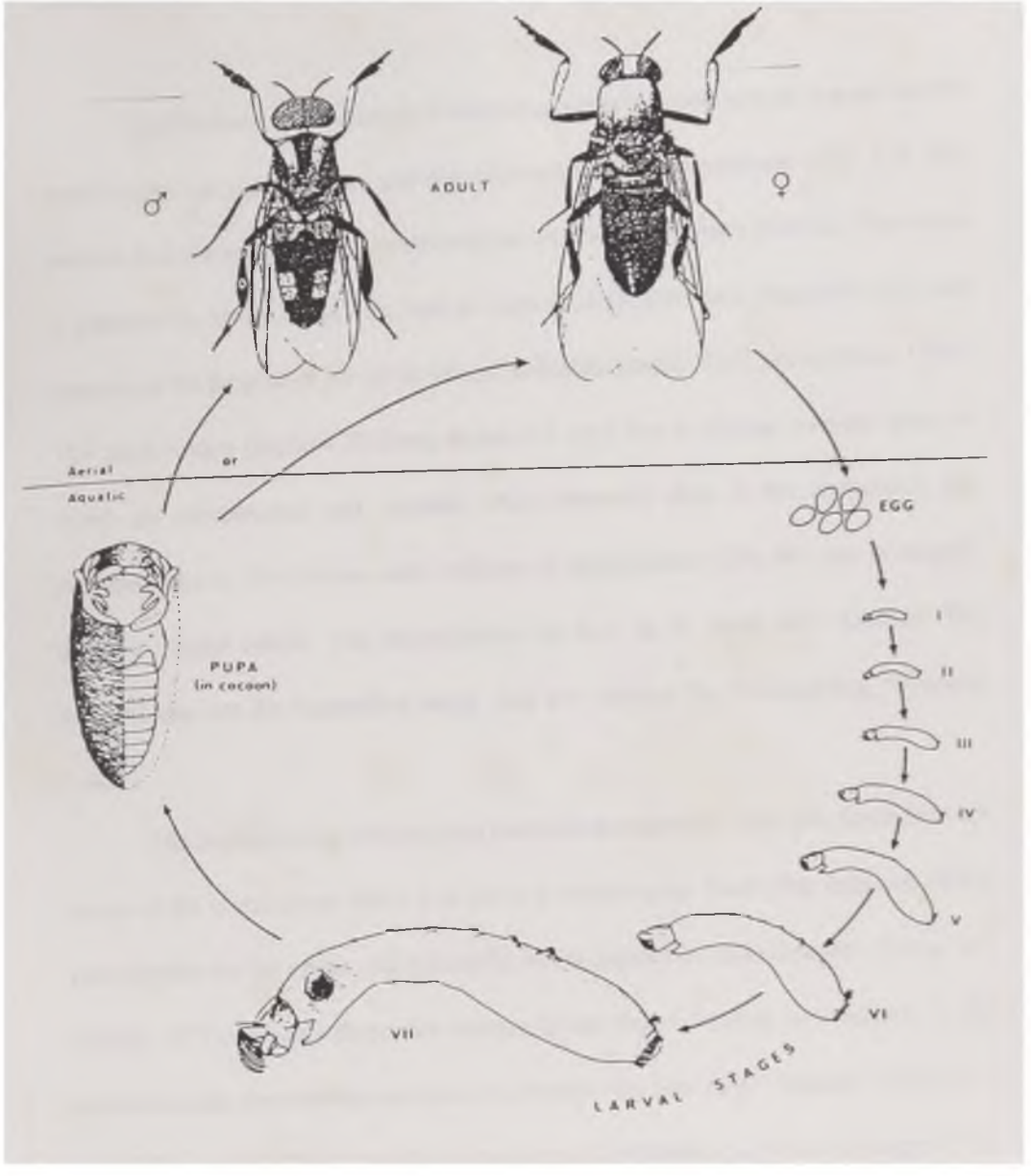


Figure 1.2
The life cycle of *Simulium damnosum* (Theobald, 1903) s.l.. From Crosskey (1990).

2.4 LIFE CYCLE AND TRANSMISSION OF *ONCHOCERCA VOLVULUS*

The life cycle of the parasite, *Onchocerca volvulus* involves both human and the insect vector, as the definitive and the intermediate host respectively (Fig. 1.3) The parasite is acquired in man by exposure to the bites of the infective blackfly. The worm is parasitic in all its entire life, and in man as definitive host, becomes adult and reproduces for the greater part of its life, estimated at around 12-15 years (Duke, 1990). The adult worms (females 30-80cm, males 3-5 cm.) live in fibrous nodules, some of which are subcutaneous and palpable while others lie deep in the connective and muscular tissues. The female emits millions of microfilariae (250-300 μm in length), into the dermal tissues. The microfilariae can live up to about 6-24 months. The microfilariae are the transmitted stage, and are ingested by bloodfeeding *Simulium* vectors.

The ingested miocrofilariae then penetrate the stomach walls into the abdominal cavity of the vector, from where they move to other organs. Soon after ingestion of the microfilariae by the vector, the successful worm appears in various organs (Omar and Garms, 1977), but only those that migrate to the thorax develop successfully. In the thorax muscles they shorten and fatten to become the first stage "sausage" larvae (L_1) (190-350 microns). The L_1 moults to the second larval stage (L_2) which is longer-(350-550 microns) and the subsequent moult forms the infective third larval stage, (L_3). The L_3's are motile and migrate to the head of the fly where they remain infective till they are released with the next bloodmeal (WHO, 1995) .

The infective larvae escape by the proboscis into the bite wound and then migrate into the human host. In the human host they moult twice without multiplying,

to reach the adult stage, as the mature worm under the skin. Their presence will cause a fibrotic reaction of the dermal layers of skin generating the characteristic nodules. About nine to twelve months after infection, the female parasite matures and starts to shed a new generation of microfilariae. The first microfilariae produced by adult females may appear in the skin some 10-15 months after infestation (Crosskey, 1990).

Although the route of the immature worm inside the human host is unknown, they appear to be attracted to existing nodules and may settle on their surface to form satellite or composite nodules (WHO, 1995).

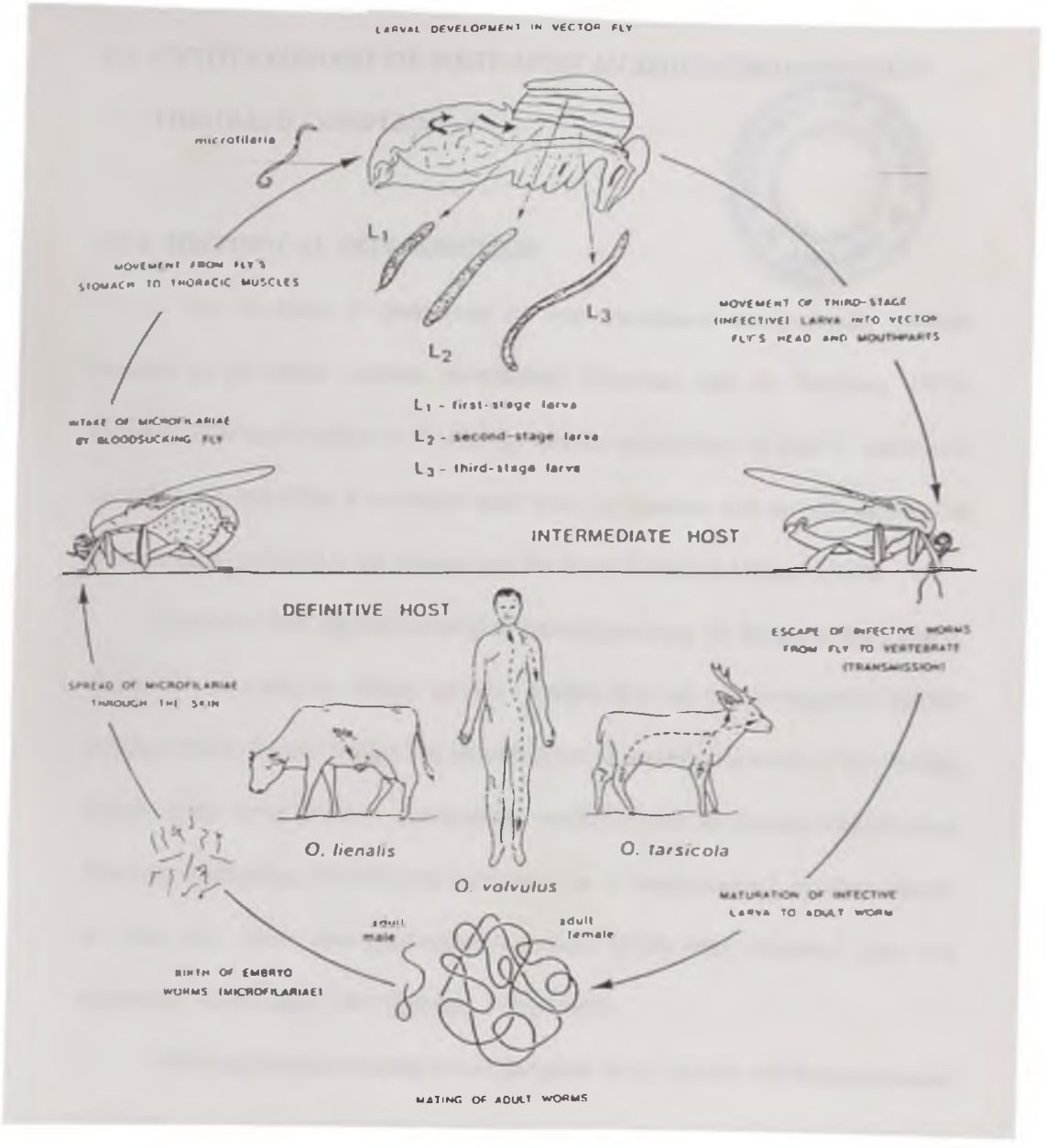


Figure 1.3
Schematic illustration of the life cycle and transmission of *Onchocerca volvulus* (Leuckart, 1893). From Crosskey (1990)

2.5 CYTOTAXONOMY OF WEST AFRICAN *SIMULIUM DAMNOSUM* THEOBALD COMPLEX

2.5:1 HISTORICAL INTRODUCTION

Up till 1966, *S. damnosum* s.l. was considered taxonomically uniform because of its rather constant morphology (Freeman and de Meillion, 1953). However, continued studies on the biology and the morphology of adult *S. damnosum* s.l. led to the belief that it contained more than one species with notable differences between the flies found in the savanna and the forest (Lewis and Duke, 1966).

Dunbar (1966) applied cytological techniques using the larvae to show that *S. damnosum* s.l. could be sibling species complex that are morphologically similar. Dunbar (1969) showed further that identification of specific segments of the banding pattern of the larval polytene chromosome could be used for species identification. The early application of cytological techniques to *S. damnosum* s.l. resulted initially in four, and later nine cytological segregates which were classified into two subgroups "Sanje" and "Nile" (Dunbar, 1966; 1969).

McCrae (1968) correlating those segregates from Uganda with known areas of onchocerciasis foci and with morphological features stated that Dunbar's cytotaxonomic studies illuminated long recognized but confusing problem of anthropophilism and non-anthropophilism. A further seven cytological segregates were recognized, six of which were reported as three pairs of closely related species or subspecies (Dunbar and Vajime, 1972). Also Vajime (1973) recognized eight segregates, which were later elevated to the status of species by Vajime and Dunbar (1975). The authors proposed the following names for the eight West African

segregates; *S.* (*Edwardsellum.*) *squamosum* Enderlein, *S.* (*Edwardsellum.*) *yahense*, *S.* (*Edwardsellum.*) *soubrense*, *S.* (*Edwardsellum.*) *sanctipauli*, *S.* (*Edwardsellum.*) *damnosum* Theobald, *S.* (*Edwardsellum.*) *sirbanum*, *S.* (*Edwardsellum.*) *sudanense*, and *S.* (*Edwardsellum.*) *dieguerense*. The first six species are known to be the most widespread cytospecies in West Africa.

Nine cytospecies of *S. damnosum* s.l. have so far been described in the Onchocerciasis Control Programme area of West Africa (Vajime and Dunbar, 1975; Boakye, 1993). About 40 species of the *S. damnosum* s.l. have been described so far in Africa (Crosskey, 1990).

2.5.2 CYTOTAXONOMIC PRINCIPLES

The method for identifying sibling species in the *S. damnosum* complex involves micromorphological studies of the giant chromosomes of the larval silk glands in stained preparations. The *S. damnosum* s.l. has a chromosomal set (karyotype) of three pairs, but these appear as three elements in the polytene karyotype, because of tight pairing between homologous chromosomes. Each chromosome is subdivided into units depending on its relative proportion of the total complement length (TCL), thus chromosome I which forms 42% has 42 units numbered 1-42. Chromosomes II and III form 30% and 28% respectively, and are numbered from 43-72 and-73-100 (Rothfels and Dunbar, 1953; Rothfels, 1956).

The main structural rearrangements of the chromosomes used in cytotaxonomy of *S. damnosum* s.l. are fixed diagnostic inversions and most commonly floating inversions within each particular species. Sex chromosomes (X and Y) differences may be also used; The inversions associated with the sex chromosomes may be in homozygous or heterozygous condition. Each inversion is either given a letter or a figure, with fixed inversions being species diagnostic.

2.5.3 CURRENT CYTOTAXONOMY OF *SIMULIUM DAMNOSUM* SPECIES COMPLEX

Nine cytospecies of *S .damnosum* s.l. have so far been described in the Onchocerciasis Control Programme area of West Africa. These fall into three subcomplexes; *S. squamosum* subcomplex, *S. sanctipauli* subcomplex, *S. damnosum* subcomplex. The *S. squamosum* subcomplex, includes *S. squamosum, S. yahense*. The *S. sanctipauli* subcomplex includes *S. sanctipauli* s.s., *S. soubrense, S. leonense,* and *S. konkourense*. The *S. damnosum* subcomplex is made of *S. damnosum* s.s., *S. sirbanum*, *S. sudanense* and *S. dieguerense*. (Vajime and Dunbar, 1975; Boakye, 1993; Boakye *et al.*, 1993).

Simulium squamosum is considered the standard cytotype of the *S. damnosum* s.l., because the sequence of banding pattern of the polytene salivary gland chromosomes is the closest to the East African species from which it is differentiated by the inversions IS-1 and IL-3 (Vajime and Dunbar, 1975). The banding pattern of the polytene salivary gland chromosomes of *S. squamosum* is then used as the standard cytotype to which those of the other members of *S. damnosum* s.l. are compared. *S. squamosum* in most areas of West Africa has ectopic pairing of chromosomes I, II and III at their centromeres (see Fig. 2) Sex determination system associated with an alteration of the centromere region of the chromosome I has been reported (Vajime and Dunbar, 1975). Sex determination system has also been found to be associated with the other chromosomes in different populations (Meredith, 1987a ; Boakye, 1993).

Simulium squamosum is characterised by the fixed inversions, IS-I and-IL-3.

(see Fig 3), and the standard chromosome II, showing no fixed inversion. However, IL-3 has been found to be polymorphic in some populations of this species in Nigeria and Guinea (Boakye, unpublished WHO 1993 report). Various floating inversions on all of the three chromosomes have been reported, with their populations differing along its distributional range (Boakye, 1993). The centromeres of the second and third chromosomes are more prominent in this species and in *S. yahense* more so than in the other members of the complex in West Africa.(Boakye, 1993).

Simulium yahense is the closest of the West African *S. damnosum* species complex to *S. squamosum*. The two species show no fixed inversion differences (Quillévéré, 1975), except the inversion IIL-18 (see Fig 4) which is routinely used to distinguish *S. yahense*. However, this inversion is also found floating in *S. squamosum* population. As a result of this, the two species are best separated by enzyme electrophoresis (Meredith and Townson, 1981; Garms and Zillmann, 1984; Thomson *et al*., 1990), but can also be distinguished morphologically (Garms and Zillmann, 1984)

The *S. sanctipauli* subcomplex, includes *S. sanctipauli* s.s., *S. soubrense*, *S. konkourense* and *S. leonense* (Boakye *et al*., 1993). Members of the *S. sanctipauli* subcomplex were initially separated from *S. squamosum* by the inversions IL-6, IIL-4.6, and the triple inversion IIIL-2.17.4. (Vajime and Dunbar, 1975). But Post (1986) in his revision of *S. sanctipauli* subcomplex retained the inversions IIL-4.6. and IIIL-2.17.4. (Fig 5 and 6) and revised IL-6 to IL-P&Q. He also introduced two new inversions (IL-A and IIL-A), to distinguish the then three species of the subcomplex namely, *S. sanctipauli* s.s., *S. soubrense*, and *S. soubrense* 'B'. However, Boakye *et al.* (1993) reported that the break-points for IL-6 of Post (1986) were incorrect but maintained that a single inversion difference and not the double inversion IL-PQ normally occurred in

S. sanctipauli s.l. This inversion is apparently similar to IL-B of Post (1986) and it is therefore designated as such. However, the inversion IL-B as described by Post (1986) does not exist just as the inversions IIIL-C1.C2. Recently the inversions, IIL-6 and IIIL-2.17.4, have been reported by Boakye *et al.* (1993) as fixed in Ghana, but polymorphic in various population in West Africa. In view of these findings, the only fixed inversion diagnostic for the members of *S. sanctipauli* s.l. is IIL-4 (Boakye *et al.*, 1993). Each member in addition to this inversion has some other fixed inversions, which differentiate it from the other species of the sub-complex.

S. sanctipauli s.s. is characterised by inversion IIL-A (Post, 1986) as an intraspecific inversion, and two other fixed inversions IIL-6 and IIIL-2. A geographical variant of this species designated as *S. sanctipauli* Djodji form was found in Togo and Ghana. This form has no fixed inversion differences from other populations of *S. sanctipauli* except that sex determination is associated with the inversion IS-21. Males of the Djodji forms are heterozygous for the inversion, which was absent in the females. (Surtees *et al.*, 1988).

Post (1986) recognised all members of the *S. sanctipauli* subcomplex that lacked the inversions IL-A (Fig 7) and IIL-A (Fig 8) as *Simulium soubrense*. Recent observations have however shown that there are a lot of fixed inversion differences between various populations of *S. soubrense* which are sometimes found breeding in sympatric situations. Hence some have been raised to species status and the rest considered as geographic variants or forms (Boakye *et al.*, 1993).

The various forms described under *S. soubrense* are the Beffa form (Meredith *et al.*, 1983), the Farmington and St. Paul forms (Kashan and Garms, 1987), and the Chûtes Milo form (Boakye *et al.*, 1993). The Chûtes Milo form is recognised by the

presence of fixed inversion IIL-6 and IIIL-2, and the absence of inversion IL-A and IIL-A, these fixed inversions are homozygous in all populations. The 'Beffa' form shows a distinctive sex- linkage which is associated with the inversion IIS-6b (Fig. 9) (Meredith *et al.*, 1983), this is present in the male as a heterozygote condition but absent in females. It has IIIL-24 (Fig. 10) as fixed inversion and IIL-D (Fig. 11) as floating inversion, the inversion IIL-7.D (see Fig. 11) is however absent in the Chûtes Milo form. The 'Farmington' form differs by the absence of any sex-linked inversion and the inversion IIL-D. The St. Paul form shows sex-linkage with the inversion IIIL-5, but the inversions IIL-D.7 and IIIL-4.17 are present.

Simulium konkourense (Boakye *et al.*, 1993) differs from *S. soubrense* by having the inversion IIL-7.D fixed, it also lacks inversion IIIL-24 which is fixed in *S. soubrense*. *S. konkourense* includes two geographical variants, the Konkoure form (Quillévéré *et al.*,1982) and the Menankaya form (Boakye *et al.*, 1993), both are found mainly in Guinea (WHO, 1995). The two forms were previously considered as variants of *S. soubrense* because they lacked the inversions IL-A and IIL-A. These two forms however, show clinal variation in the proportion of floating inversions along their distributional range with two extremely different chromosomal forms at the ends (Boakye, 1993). They can however, be distinguished from each other by the absence of the inversion IIL-X (Fig. 12) in Menankaya but floating in the Konkoure forms.

Simulium leonense (Boakye et al., 1993) was originally thought to be a form of *S. soubrense*, known as *S. soubrense* 'B' (sensu Post, 1986). It has IL-A as the diagnostic inversion, IIS-7 (see Fig. 11) and IIIL-2 are also present as fixed inversions. Sex-linkage is found to be associated with IIIL-4.17 in most populations. This cytospecies occurs mainly in Sierra Leone and its border areas with Guinea.

S. damnosum subcomplex differs from *S. squamosum* by the inversions IL-1 and IIIL-2 (Fig. 13 and 14). *S. damnosum* s.s. is characterised by the presence of a complex rearrangement of four inversions termed IIL-C (Fig. 15), which is fixed (Post, 1986). It also has IL-1 and IIIL-2 as fixed inversions which are also present in *S. sirbanum*. The inversion IIL-C.8 (Fig. 16) is associated with sex determination in most populations of *S. damnosum* s.s. Females are homozygous for inversion IIL-C/C, while most males are heterozygous with the IIL-C/C.8.(Fig. 17).

Simulium sirbanum, and *S. sudanense*, were originally considered as two distinct species (Vajime and Dunbar, 1975), but both are now considered as one species, and classified as *S. sirbanum* (Vajime 1989; Fiasorgbor and Cheke, 1992). Individuals of this species are separated from *S. squamosum* by the fixed inversions IL-1, IIL-C.8 and IIIL-2. It also differs from *S. damnosum* s.s by a fixed inversion IIL-C.8. Sex-determination in this species is related to the inversion IS-3/3 (Fig. 18). Most males are found to be heterozygous for the inversion IS/3 (Fig. 19), while the females may be either homozygous for the inversion or the inversion may be lacking. Routinely this species is diagnosed by the sequence IIL-C.8/C.8 (see fig. 16), IIL-C/C.8.3 (Fig. 20) IIL-C.8.3/C.8.3 (Fig. 21) or (Boakye *et al.*, 1993). *S. sirbanum* is the most widely distributed and important vector of human onchocerciasis in the OCP area. It has been recorded from all over the river basin in the area, although it occurs predominantly in the savanna zone where two distinct forms occur (Fiasorgbor and Cheke, 1992).

Another savanna cytotype of *S. damnosum* subcomplex, *S. dieguerense* is rare and the adults are completely unknown. The type material was obtained from Dieguera (R. Bafing, Mali). It was first recognised as having four inversions IS-2, IL-

12, IIL-3.8 and IIIL-2 as being different from the standard *S. squamosum* (Vajime and Dunbar, 1975). However, Post (1982) revised and considered the diagnostic criteria, replacing IL-12, and by IL-35 (Fig. 22) and IIL-C respectively and IIIL-2. Later Boakye and Mosha (1988) reported sex-linkage to an altered segment of the centromere region of chromosome 1 (Fig. 23) and like other savanna cytotype, they all share IIIL-2 as fixed inversions, but it differs from the others by having a fixed and unique inversion IL-35. Floating inversions found in this species are IIL-68 and IIIL-28.

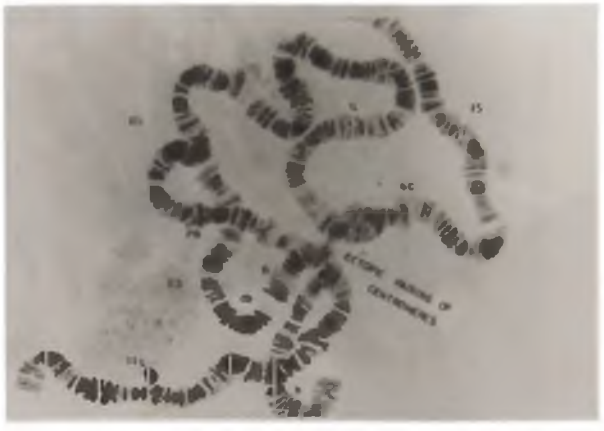


Fig 2 Full chromosome complement of *Simulium squamosum* showing the ectopic pairing of the centromeres. (B) ring of balbiani. (b) bliste(bd) double bubble; (PB) para-balbiani (C) centromere (NO) nucleolar organiser

Fig 3 Standard chromosomal sequence of *Simulium squamosum*, showing inversion IS-1/1 and IL-3/3.(C) centromere (NO) nucleolar organiser

Fig 4 Chromosome II of *Simulium yahense*, showing inversion IIL-18/18
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 5 Chromosome II of *Simulium sanctipauli* s.s. showing inversions IIL-4.6.A/4.6.A
(C) centromere (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

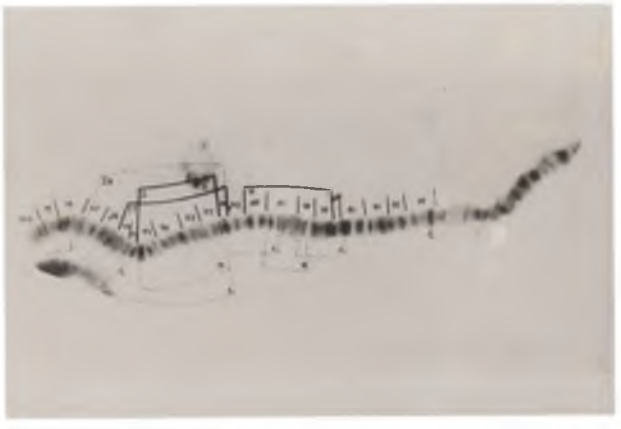


Fig 6 Chromosome III of *Simulium sanctipauli* s.s. showing inversion IIIL- 2.17.4/2.17.4
(C) centromere

Fig 7 Chromosome I of *Simulium sanctipauli* s.s. showing IS-A/A, IL-B/B
(C) centromere. (NO) nucleolar organiser

Fig 8 Chromosome II of *Simulium sanctipauli* s.s. showing inversion IIL-4.6.7/4.5.7 with particular reference to inversion IIL-A.
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

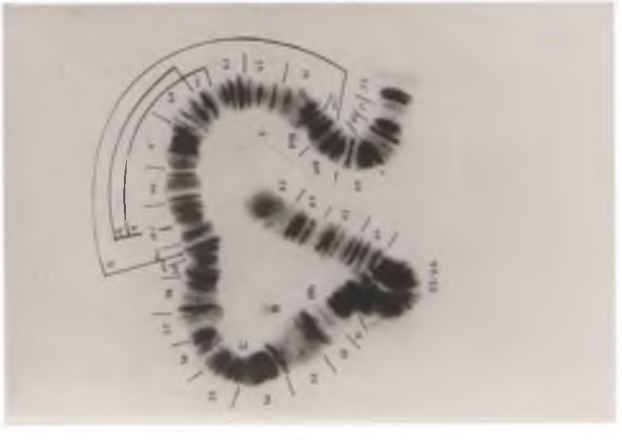


Fig 9 Chromosome II of *Simulium sanctipauli* s.l. showing inversion IIS/6b. This kind of inversion is peculiar to the "Beffa" form of *Simulium soubrense* which shows a distinctive sex-linkage associated with this inversion
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

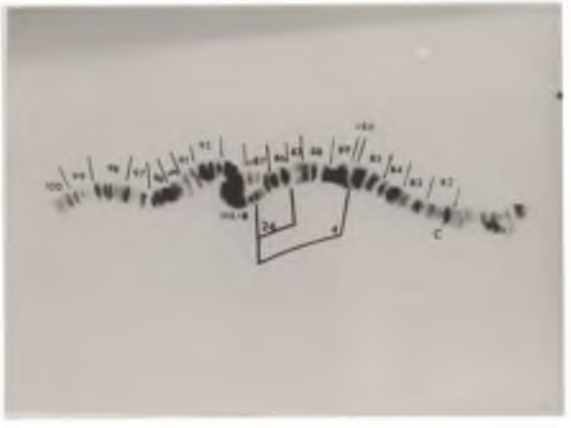


Fig 10 Chromosome III of *Simulium sanctipauli*.s.s. showing three inversions. including IIIL-24 and IIIL/B
(C) centromere

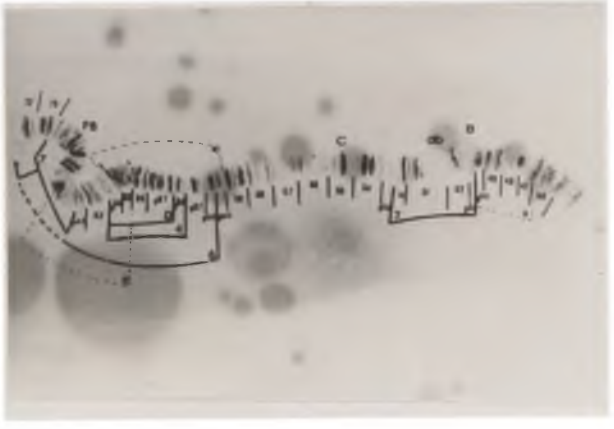


Fig 11 Chromosome II of *Simulium leonense*, showing inversion IIS-7/7 and IIL-4.6.D.7/4.6.D.7
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 12 Chromosome II of *Simulium konkourense*, konkoure form, showing inversion IIL-4.X/4.X
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 13 Chromosome I of *Simulium diegueren*se showing inversion IL-3.1.35/3.1.35
(C) centromere (NO) nucleolar organiser

Fig 14 Chromosome III of *Simulium damnosum* s.s. *Simulium sirbanum*, showing inversion IIIL-2/2
(C) centromere (b) blister

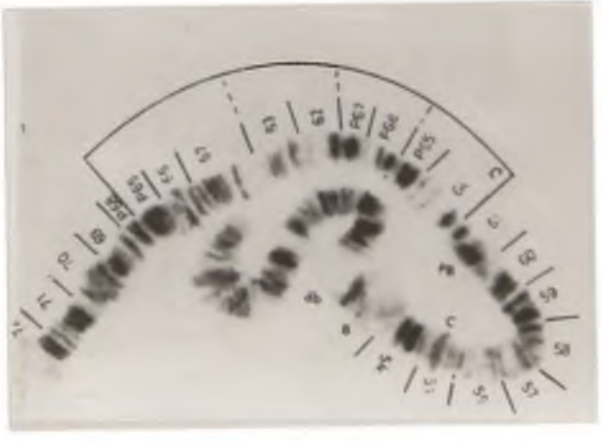


Fig 15 Chromosome II of *Simulium damnosum* s.s., showing inversion IIS-st/st and IIL-C/C
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 16 Chromosome II of *Simulium sirbanum/ Simulium dieguerense* showing IIS-st/st and IIL-C.8/C.8
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

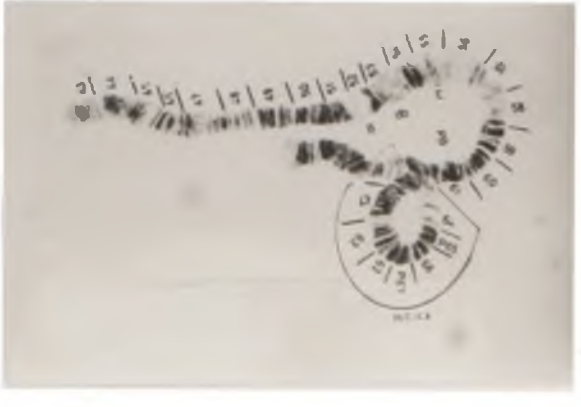


Fig 17 Chromosome II of *Simulium damnosum* s.s. with inversions IIS- st/st and IIL-C/C.8
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 18 Chromosome I of *Simulium damnosum* s.s./ *Simulium sirbanum*, showing inversion IS-2.3/2.3 and IL/2
(C) centromere (NO) nucleolar organiser

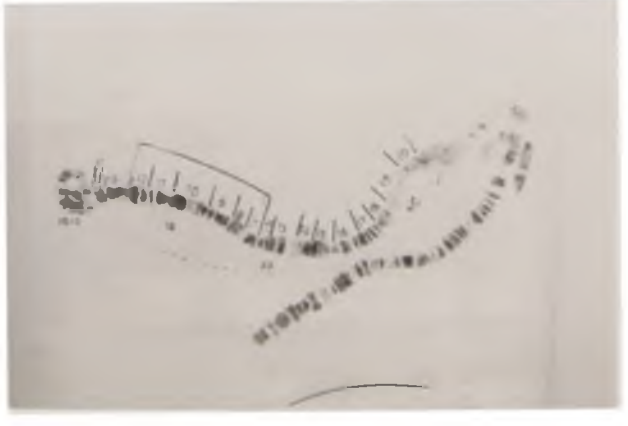


Fig 19 Chromosome I of *S. damnosum* s.s./ *Simulium sirbanum*, showing inversion IS-3 heterozygous; IS-2/2.3
(C) centromere (NO) nucleolar organiser

Fig 20 Chromosome II of *Simulium damnosum* s.s., showing inversion IIS-st/st and IIL-C/C.8.3
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

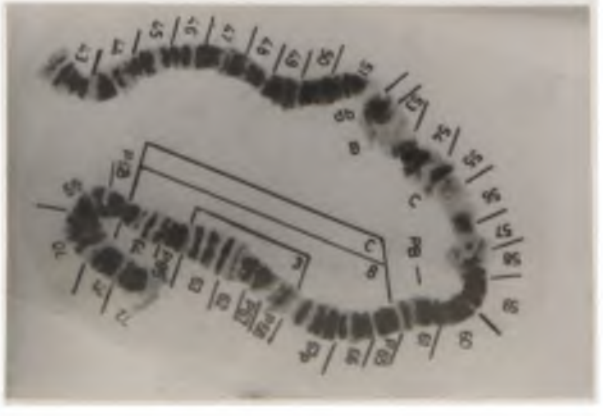


Fig 21 Chromosome II of *Simulium sirbanum* showing inversion IIS-st/st and IIL-C.8.3/C.8.3
(C) centromere; (B) ring of balbiani; (bd) double bubble; (PB) para-balbiani

Fig 22 Chromosome I of *Simulium dieguerense*, showing inversion IS-2/2 and IL-35/35
(C) centromere (NO) nucleolar organiser

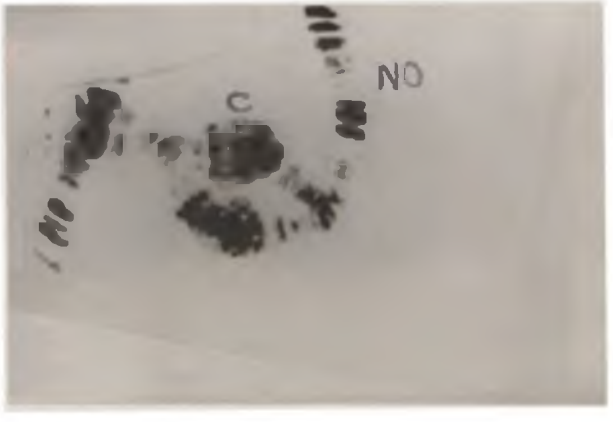


Fig 23 Shows altered centromere region of chromosome I, found in *Simulium dieguerense* males.
(C) centromere (NO) nucleolar organiser

2.6 CONTROL OF ONCHOCERCIASIS

Early attempts to control *Simulium* vectors of onchocerciasis were made in Chiapas state, Mexico in 1932. These involved the clearance of vegetation surrounding the breeding site on regular basis, but had little effect on fly densities. However the same control measure successfully led to the control of *S. neavei*. The latter control scheme was successful because the vector, *S. neavei* has restricted flight range and only occurred when there was dense undergrowth and tree cover (Buckley 1951). At Riana in the Nyanza province of Kenya, eradication was also achieved for the same reason by the complete removal of dense undergrowth with the elimination of a number of small trees (Buckley, 1951). However control by these means was not found to be feasible in West Africa, since the *S. damnosum* s.l. vector has extensive flight range (Walsh, 1990).

Larviciding was considered an attractive approach to control because the larvae were found to be sensitive to low concentrations of many poisons. In principle, there are two approaches to the control of onchocerciasis, either through the reduction of the vector numbers thereby reducing transmission or by killing the parasite. The control of vector populations of *S. damnosum* s.l. is achieved by using insecticides against the larvae and is the method employed in the Onchocerciasis Control Programme (OCP). The OCP was launched in 1974 with the aim of reducing transmission of onchocerciasis to the level at which it will not be a public health problem. (Philippon *et al.*, 1990). The Onchocerciasis Control Programme now covers over 1.2 million km^2 in 11 countries in West Africa (Curtis *et al.*, 1993) Vector control is primarily directed against the savanna vectors which are associated with the severe forms of the disease, specifically *S. sirbanum* and *S. damnosum* s.s.

(Philippon *et al.*, 1990; Hougard *et al.*, 1993). Control is achieved by weekly aerial application of especially designed formulation of insecticide upstream of their breeding sites. The insecticide of first choice was an emulsifiable formulation of the organophosphates insecticide, temephos (Abate$^{(R)}$), which was solely used because it carried well down stream and because it was relatively harmless to the remainder of the riverine ecosystem, including food fish.

Resistance to temephos appeared in 1980 in a population of *S. sanctipauli* s.s. in the Bandama river in Southern Côte d'Ivoire (Guillet *et al.*, 1980). Later similar resistant populations of the same species were identified in other river basins in Côte d'Ivoire. In 1981 chlorphoxim replaced temephos, but soon a similar pattern of resistance was detected (Kurtak *et al.*, 1981). A search was instituted for substitute insecticides which are effective against temephos resistant larvae as well as being environmentally acceptable. The first of these was the bacterial agent *Bacillus thuringiensis* H-14, which is toxic virtually only to *Simulium* and mosquito larvae and to which serious levels of resistance have not yet been detected. More recently, other synthetic insecticides, permethrine, carbosulfan, phoxim and pyraclofos have all been introduced and are effective against temephos-resistant larvae: Permethrin is actually more effective against temephos resistant than susceptible larvae (Kurtak *et al.*, 1987).

However, due to the resistance of some vector species to temephos and cross resistance with other organophosphate, the present strategy involves the rotational use of these six insecticides including *Bacillus thuringiensis* serotype H-14 (B.th.), phoxim (organophosphate), pyraclofos (organophosphate), permethin (synthetic pyrethroid) and carbosulfan (carbamate) (Kurtak, 1990; Hougard *et al.*,

1993). As a measure of success of the control operations, it is estimated that only 1.1% of children are expected to suffer from onchocerciasis in the central area of the OCP. Also, the entomological indices show that in 85-90% of the original area, the target species, *S. sirbanum* and *S. damnosum* s.s., have been reduced to densities which are incapable of maintaining transmission (WHO, 1988).

Chemotherapy can also be an effective method of controlling onchocerciasis if it results in the elimination of the adult parasite from the human body. Until recently the standard drugs used against *O. volvulus* infections were diethylcarbamazine (DEC) which kills the microfilariae, and Suramin which also kills the adult worm. However, neither of them can be employed on a large scale for the treatment of the disease, because of severe and dangerous reaction in the skin and eye. In addition, suramin is intrinsically toxic and therefore impracticable to be administered by repeated intravenous injections, and moreover the levels at which it is tolerated do not ensure effective killing of the adult worm.

In 1982, the onchocerciasis chemotherapy project (OCT) by the WHO was established with the primary aim of finding an effective non-toxic macrofilaricidal drug or as a secondary alternative microfilaricide. Two new drugs, a microfilaricide, ivermectin from Merck, Sharp and Dohme (MSD) and a macrofilaricide, CGP6140 from Ciba-Geigy Ltd, have emerged as a result of OCT's activities.

Ivermectin appears to be effective and non-toxic at single dose. It causes little or no Mazzotti reaction and reduces the concentration of microfilariae in the eye and that of more recent and acute anterior segment lesions (Awadzi *et al.*,

1985). Mass chemotherapy with Ivermectin is now the main drug of choice for onchocerciasis control.

2.7 PRINCIPLES OF INSECTICIDE SUSCEPTIBILITY TESTS

The term insecticide resistance has been defined by WHO (1956) as: the development of an ability in a strain of insects to tolerate doses of toxicants which will prove lethal to the majority of individuals in a normal population of the same species.

Tests for insecticides susceptibility of blackflies rely on bioassays with pesticides. Usually on larvae two main components of the bioassay that affect efficiency are, the duration of exposure to the insecticides and the amount of insecticides to which insects are exposed (Tabashnik *et al.*, 1993), if the duration of bioassay is too brief, it may not distinguish consistently susceptible and resistant individuals, and this is especially more so with slow-acting insecticides. Most bioassays for resistance involves the use of series of concentration of insecticides and the percentage mortality observed at each concentration in the series is analyzed with probit regression methods (Finney, 1971).

The ability to distinguish between susceptible and resistant individuals varies with insecticides concentration. Thus, bioassays that use one optimal or nearly optimal concentration (sometimes called discriminating or diagnostic concentration) are more effective for evaluating resistance than bioassays that use several concentrations, some of which are relatively poor at distinguishing between susceptible and resistant individuals (Roush and Miller, 1986).

The susceptibility of the larvae varies with their stage of development, young larvae are more susceptible than older ones. Pupae are far less susceptible than the larvae, and cannot be used for susceptibility test, since they do not feed (WHO, 1970). In mosquitoes, larval insecticide susceptibility test involves the exposure by immersion in solution or emulsion of insecticide, of 3rd-4th stage mosquito larvae and counting the mortality after a standard interval of time (WHO,1970), whilst in *S. damnosum* s.l., only the 5th and 6th instars are used for the larval insecticide susceptibility test (WHO, 1981).

To detect the appearance of insecticide resistant strains of blackfly larvae, a baseline for the species is initially established, either before the widespread use of insecticide or with specimens from untreated area. Then several tests are performed at various localities and at different seasons, to assess normal biological variation. Subsequent tests are conducted at regular intervals to determine any significant change in susceptibility (WHO, 1981).

2.8 INSECTICIDE RESISTANCE

To date, over 400 species of insects and mites are resistant to one or more pesticide (Georghiou and Mellon, 1983), and this includes the species of the *S, damnosum* complex. It was observed as early as 1914 in California, that, *Aspidiotus perniciosus*, a scale insect pest of citrus could no longer be controlled by doses of lime sulphur even when applied in doses that were ten times greater than had been successfully used in previous years (Melander,1914). A few years later,

the resistance of the red scale, *Aonidiella aurantii* to hydrogen cyanide was reported (Quayle, 1916). Insecticide resistance is widespread and involves many insects of agricultural and medical importance to different insecticides, e.g. the development of resistance in housefly, *Musca domestica,* to DDT, (Sacca, 1947). Studies conducted in Nigeria (Walsh, 1970b) reported the indication of reduced susceptibility to DDT in *S. damnosum*. Guillet *et al.* (1977) tested *S. damnosum.* s.l. larvae from areas within and adjacent to the O.C.P in 1977, and found evidence of precontrol resistance to DDT in five localities which they attributed to a probable run-off into the rivers from agricultural spraying.

Resistance to temephos first appeared in 1980 in a population of *S. sanctipauli* s.l. on the Bandama river in southern Côte d' Ivoire (Guillet *et al.*, 1980). Later this spread to other populations of the same species in other river basins in Côte d' Ivoire. In 1981 Chlorphoxim was introduced to replace temephos, resistance was soon detected (Kurtak *et al.,* 1981). Careful monitoring of resistance to temephos have shown it to have spread to all river basins in all different species at one time or another within the OCP area (Kurtak,1990). Meredith *et al.* (1986) detected full resistance at a site on river Tano in south-western Ghana where larviciding has never been undertaken, probably due to migration of resistant population from rivers in southern Côte d' Ivoire. Also, Traore-Lamizana *et al.* (1985) reported resistance to temephos in members of the *S. damnosum* complex in Cameroon.

CHAPTER THREE

EXPERIMENTS TOWARDS LABORATORY COLONIZATION OF *SIMULIUM DAMNOSUM* SPECIES COMPLEX

3.1 GENERAL INTRODUCTION

The laboratory colonization of organisms such as *Drosophila melanogaster*, *Anopheles gambiae* etc. has proved to be an important tool for scientific research. It has the added advantages of enabling experiments to be conducted under controlled conditions and also making materials available especially where distance from the laboratory to the field is far. However, in the case of *Simulium damnosum* s.l. all attempts at establishing permanent colonies in the laboratory have so far not been successful.

Despite this, the laboratory maintenance of wild bloodfed adult females of *S. damnosum* sibling species, for long enough time to stimulate oviposition, and the rearing of eggs to late instar larvae for chromosomal identification and full karyotyping is achievable. This technique continues to be an important research tool used by the Onchocerciasis Control Programme (Raybould *et al.*, 1979; Boakye, pers. comm.). This however, is limited in scope because further studies will be enhanced if permanent colonies of onchocerciasis vectors can be established. Thus, experiments can be carried out away from their natural breeding sites, and also at times of the year when little or no live material is available. For many experimental studies with adult female Simuliids involving laboratory rearing of *Simulium* species, survival of the female flies till oviposition is important. Also laboratory infection with *Onchocerca* parasites, will require the availability of flies at all times.

For vector control strategy of onchocerciasis, the identity of the biting female is very important, since not all the cytospecies of *S. damnosum* species complex are

epidemiologically significant. It is generally the sibling species associated with the savanna zone which are effective vectors of the blinding form of the disease whilst those associated with the forest zone transmit a less serious form (Philippon *et al.*, 1990).

As a result of these differences in the vectorial capacities and the profound differences in the epidemiological importance of *S. damnosum* species complex, a vivid identification of the female blackflies attacking man is of utmost importance. Until now, despite much progress in morphological taxonomy (Quillévéré *et al.*, 1977; Garms 1978; Garm and Cheke, 1985; Dang and Peterson, 1980; Wilson *et al.*, 1993), chromosomal differences in the larvae remain the most reliable characters for species determination within the complex.

Therefore, the need to obtain larvae in the laboratory from the man biting female blackflies for cytotaxonomic analysis becomes very necessary. For this reason the laboratory maintenance and rearing of species of the *S. damnosum* species complex, starting with wild-caught man biting adult females and rearing in the laboratory to late-instar larvae for cytotaxonomic determination, has proved to be an important research tool. Also if larvae, pupae and adults of *S. damnosum* s.l. are reared from a single egg batch, and one of the reared larvae is identified cytotaxonomically, the female parent and her progeny can be used in the search for morphological differences between the species. Considerable progress in morphological taxonomy has already resulted from studies of material provided in this way (Wilson *et al.*, 1993). In addition, reared larvae can be used for testing their susceptibility to insecticides, and the adults obtained this way can be used in experimental transmission studies to determine their vectorial capacities.

The laboratory colonization of *S. damnosum* s.l., involves maintenance of bloodfed flies to time of oviposition, oviposition inducement of gravid female flies, rearing of eggs to adults, mating of male and female flies and bloodfeeding mated females in the laboratory. The completion of all these stages of their life-cycle is associated with peculiar problems which make the realization of permanent

colonization more difficult. Much work, however, has been done by various workers to help overcome some of the problems. For instance Lewis *et al.* (1961) made a breakthrough by inducing gravid females *S. damnosum* s.l. to oviposit either by placing them in water or by decapitation. Marr (1962) was also able to induce oviposition by CO_2 anaesthesia. Permanent colonisation is yet to be achieved for any of the vectors of *Onchocerca volvulus*.

This study, reports some of the techniques that have been developed either by modification or simplification of some of the existing ones to enhance the progress towards permanent laboratory colonization of the *S. damnosum* species complex.

3.2 SPECIFIC OBJECTIVES

The main objectives set for the present study were, the following.

(a) To determine the appropriate tube size that will ensure long term survival of blackflies in the laboratory.

(b) To develop an oviposition system that will facilitate effective egg oviposition inducement and high fertility rate.

(c) To assess and modify if necessary, a very simple, cheap and easily transportable rearing system, developed by Boakye, D.A (unpublished data)

(d) To determine the mating rate of blackflies in the laboratory using laboratory reared adults.

3.3 THE LABORATORY MAINTENANCE OF THE ADULT FEMALE FLIES *SIMULIUM DAMNOSUM* S.L.

3.3.1 INTRODUCTION

Simuliids have usually been kept in the laboratory, either, individually in small tubes (Lewis, 1960; Duke, 1962) or together in large tubes, beakers, cages or paper cups (Davies, 1953; Wenk, 1965; McMahon, 1968). Containers of glass and certain kinds of plastics suffer from the disadvantage that the flies are sometimes trapped in condensation on the side of the vessels (Raybould and Mhiddin, 1974). However, the provision of relatively constant temperature and high humidity can overcome this problem and enhance their survival in the laboratory (Duke ,1962).

In the Onchocerciasis Control Programme, adult female flies are usually kept singly in plastic tubes of 4.5 x 1.5 cm (Raybould *et al.*, 1982) and maintained at low temperatures, but mortality rates were rather high (Boakye, personal communication). One of the reasons suggested for the high mortality experienced was tube size, but this has never been investigated. In this study two tubes of different sizes were used to determine their effect on the survival rate of adult female flies. The small sized tubes are those used in the OCP and the larger tube, roughly two times longer, but of the same diameter.

3.3.2 MATERIALS AND METHODS

Flies were collected at the villages of Sekyere-Heman (5° 11'N, 1° 36'W) on the River Pra and at Sutri rapids on River Tano (2° 38'30"W, 5° 23'12"N) using humans as bait. The flies were placed individually in a 9.8 x 1.5 cm plastic tube with polythene push-in-caps and kept cool in an ice-chest during transportation. In the laboratory, each fly was transferred into a fresh clean tube, but the cap was cut out in the centre, and a piece of fine silk-netting placed over the mouth of the tube before capping. A strip of filter-paper was placed in the tube to serve as a walkway and resting site for the flies, and also to absorb the excreta. The flies were fed on saturated sucrose solution from impregnated cotton-wool that was placed on the silk-netting. The soaked cotton-wool was changed regularly to prevent fungal infection. The tubes were then placed in an ice-chest and maintained at temperatures ranging between 23-25° C, with care taken to prevent attack by ants providing a water barrier.

Laboratory survivorship was compared for tubes of two different sizes, the standard OCP tube (4.5 x 1.5 cm), and a much longer tube (9.8 x 1.5 cm). Fifty female flies were used for each set of tubes. Each set of flies were put together in groups of ten using rubber bands, and placed in the same container at a relatively constant temperature. The flies were examined each morning, and mortalities and numbers surviving were recorded over the period of time until the last fly died.

In all the three experiments that were carried out, a total of 300 flies were used. The students t-test was used to determine the significance of the difference in the mean number of days and the ANOVA was used to test for significance of different mortality rates obtained. Both statistics were performed using the SPSS^X Statistical software package on a desk top computer.

3.3.3 RESULTS

The results obtained are shown in Table 1 and Figure 24. The flies kept in the two different sized tubes showed different mortality rates. Mortality of flies in the small tube began after the first day, and the longest time the last fly survived was 19 days. The mean number of days calculated for the small tube was 8.8 days.

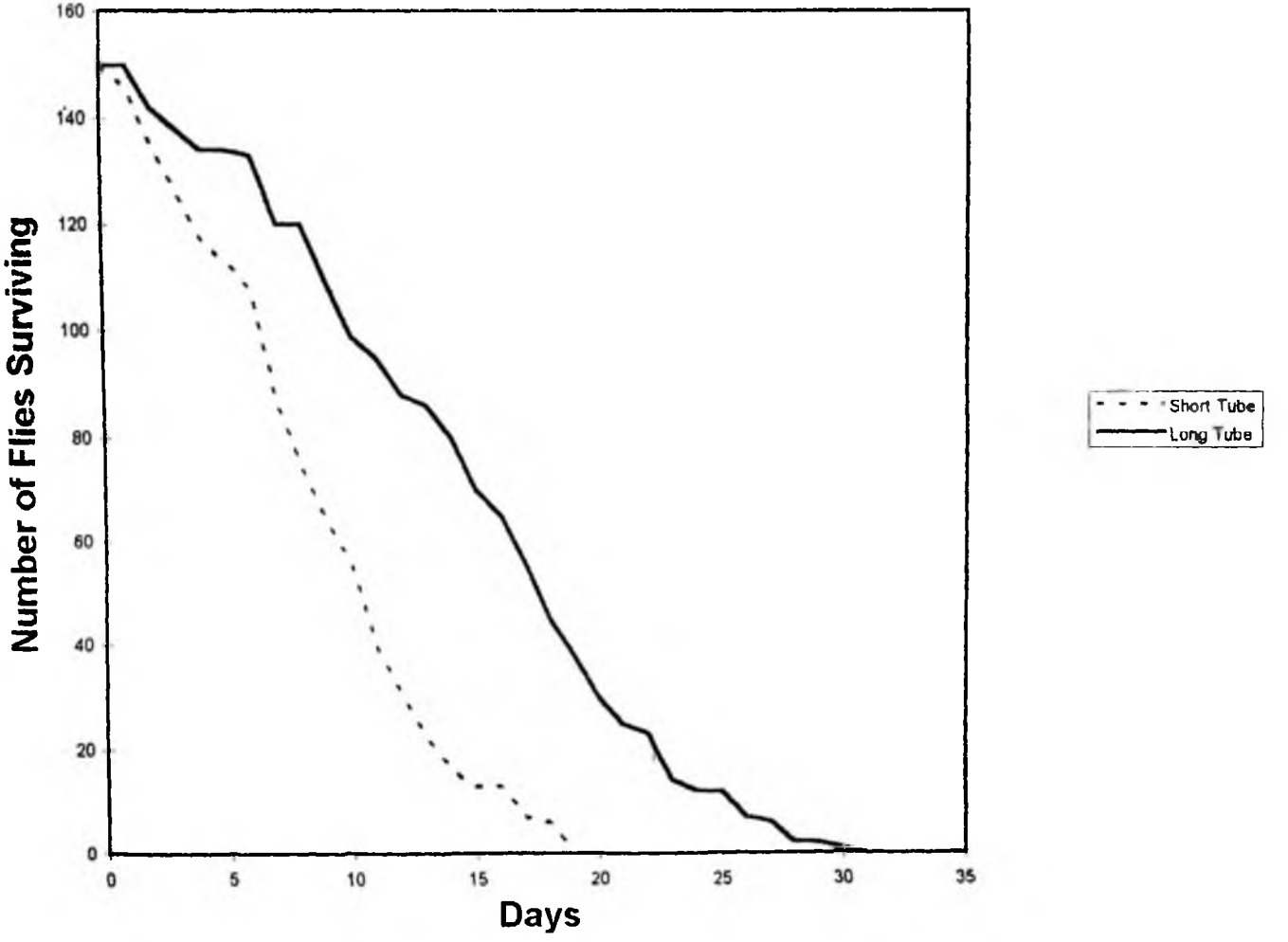
Mortalities of flies in the large tube were observed on the second day, while the last fly died on the thirty-first day. The mean number of days flies survived in the large tube was 14.6 days. There was a significant difference ($P < 0.001$) in the average number of days a fly survived between the two tubes. There was also a significant difference in the mortality rates observed ($P < 0.001$).

TABLE 1. Mean longevity of the flies for both tubes

VARIABLES	NUMBER OF CASES	DAYS (MEAN)	STANDARD DEVIATION
Short tube	150	8.7533	± 2.012
Long tube	150	14.5733	± 3.542

T-test for equality of means at 99% for 2-tail significance is P =0.001

FIG 24 The survival rates of adult female *Simulium damnosum* s.l. maintained in different tube types.



3.3.4 DISCUSSION

Wenk (1981) reported that *S. damnosum* s. l. females have a maximum longevity of one month, and that the savanna species had greater longevity than the forest species. Le Berre *et al.* (1964) and Le Berre (1966) reported that out of 100 eclosions on a given day, recorded in different bioclimatic regions, in the rain forest the number of females surviving on the ninth day were three, thirty-three in the Guinea savanna and 47 in the Sudan savanna. Adult blackflies kept in containers in refrigerator at 4°C survived well and better than those at room temperature of 28°C (Ham and Fleming, 1988).

Raybould *et al.* (1982) also observed that mortality fell at low temperatures but egg maturation slowed down. Although, flies kept in the large polypropylene tubes at a temperature range of 23-25°C could survive up to an average of 14.6 days, as against the 8.8 days for the smaller plastic tube, this average survival period of 14.6 days was considered to be an appropriate and better result considering egg oviposition as the main focus. *Simulium damnosum* s l. flies kept in the laboratory at a temperature range of 23-25°C can oviposit 10 days after bloodfeeding. The eggs obtained develop normally, but from about the thirteenth day, the eggs were either absorbed internally or disintegrated (unpublished observation).

Doucoure (1996) found that of the 3276 bloodfed female blackflies maintained in the small tube (4.5 x 1.5 cm) in the laboratory below room temperature, 53.5% survived up to the 10th day. For this study, 42% of the flies survived up to the 10th day at room temperatures for similar sized tubes. The difference in the survival rate may be accounted for by the differences in the experimental temperatures used and confirms that blackflies survive much longer at lower temperatures.

However, for the present study, 65% of the adult blackflies maintained in the large tubes survived up to the 10th day. This is significantly higher than both survival rates for the short tube at room temperature, and that maintained below room temperature, (Doucoure, 1966).

Furthermore, despite the fact that *S. ornatum* kept at 4°C survived for much longer periods, (60% of 100 flies survived after one month) while the last few flies lasted for 111 days (Ham and Fleming, 1988), the disadvantage is that of slow egg maturation at reduced temperatures. Therefore since the main focus of the present study was towards laboratory colonization of *S. damnosum* s.l., the much improved survivorship at room temperatures will enable normal development of eggs for oviposition. The other advantage is that the long term survival of adults for more than 14 days will make possible experimental crosses of progenies of different parents in the laboratory.

3.4. STUDIES ON OVIPOSITION METHODS AND FERTILITY OF CAPTIVE *SIMULIUM DAMNOSUM* S.L.

3.4.1 INTRODUCTION

Wild-caught females of all species of African Simuliidae tested so far have been found to oviposit in captivity, but the required conditions vary a great deal with the species (Raybould and Grunewald, 1975). Raybould (1967b) reported that *S. woodi* females had to be kept until they laid eggs of their own accord, since oviposition could not be induced either by carbon dioxide anaesthesia, decapitation or immersion.

Lewis *et al.* (1961) were the first to induce gravid *S. damnosum* s.l. females to oviposit by placing them in water and also by decapitation. Marr (1962) induced oviposition in a West African form of *S. damnosum* complex using CO_2 anaesthesia. Similar results were also obtained with the various forms of *S. damnosum* complex investigated in East Africa, although not all individuals oviposited their eggs when stimulated this way (Raybould and Grunewald, 1975). Cupp *et al.*(1981) and Simmons and Edman (1981 and 1982) developed techniques for oviposition inducement in *S. damnosum* s.l., which were improvements on the previous methods mentioned above. These methods involved a complex laboratory set-up with water dripping onto an artificial log.

The objective of this study was to modify and simplify the oviposition system used by Cupp *et al.* (1981), Simmons & Edman, (1981; 1982) and McCall *et al.* (1994), due to the fact that they occupied large laboratory space.

a) Having modified the oviposition system, to compare this oviposition method with that of the immersion method used by the OCP, in terms of their efficiency and fertility rates.

b) To investigate the effect of group oviposition and extracted egg pheromone (McCall *et al.*, 1995) on oviposition and fertility of captured *S. damnosum* s.l. females

Blood-fed adult flies were obtained at Sutri Rapids on R. Tano and Sehyere-Hemang on the R. Pra. The flies were kept individually in small polypropylene tubes with a push-in-cap, and fed on sucrose solution in the laboratory till they were fully gravid, usually three to five days after blood feeding.

To induce oviposition by immersion, each gravid female fly was placed in a fresh tube which was lined on the side with a rolled up piece of fine silk-netting material and filled with aerated water to a depth of about 1 cm. The tube was tilted and rotated to immerse the fly which induced it to oviposit on the netting material. Care was taken to ensure that flies did not oviposit on either the inside of the lid or at the bottom of the tube. The netting material with the eggs attached was removed and suspended in a rearing trough containing aerated water by means of a thread and a paper clip.

The eggs were kept in the rearing system until the fourth day, when the silk-netting was removed and eggs counted using the dissecting microscope. To facilitate the egg counting procedure, the silk-netting was soaked for one and half hours in 5% sodium hydroxide. This dissolved the sticky gelatinous material surrounding the eggs, thus freeing the eggs from the sticky gelatinous matrix (Fredeen,1959). Empty egg cases which denoted hatched eggs and unhatched eggs were counted separately. Hatchability was determined from the numbers of the egg cases and the eggs which still remained undeveloped With this method, only one fly could be processed at a time.

The drip oviposition system is shown in (Fig. 25). Basically, it consists of two Grenier tubes (11.6 x 3.0 cm) with both ends open and fitted with silk-netting between them. The net material served as a substratum for the flies to lay their eggs. A long rubber tubing (about three metres long with a diameter of eight millimetres) with one end fitted with a blue micropipette tip was inserted in the cap on the oviposition chamber. The other end of the tube was placed in a container of water which was continuously aerated by air pumps. A dim source of light provided by a torch light was

either directed up from beneath the oviposition chamber, or directed downwards along the oviposition chamber just below the position of the silk-netting material.

Gravid female flies were introduced into the oviposition chamber through the small hole in the cap using an aspirator. The water was then allowed to drip in continuously through the micropipette tip into the oviposition chamber and wetted the silk-netting which subsequently wetted the fly on it. For the fly to stay on the net, the light in the room was switched off and the only source of light was torch beneath the oviposition chamber. The flies were attracted towards the light source and thus stayed on the net material. The wetting of the flies thus induced them to oviposit. Where group oviposition was carried out, the flies were put in the chamber in groups of five and ten . The netting material with the eggs attached was placed in a rearing trough and then treated as described to free them after the fourth day. Both hatched and unhatched eggs were counted.

Five methods were developed out of the drip system, consisting of single oviposition, and multiple oviposition where flies laid together in groups of five and ten. The single ovipositors had three methods, namely drip single, where one fly was placed in the oviposition chamber together with extracts of pheromone, drip with pheromone, where one fly was placed in the oviposition chamber, and the control experiment where a single fly is placed in the oviposition chamber after a $1cm^2$ filter paper soaked with 10µl hexane has been placed in the oviposition chamber, serving as a control for the pheromone . For the drip with pheromone, 1 cm^2 filter paper soaked with 10µl volatile extracts of pheromone obtained from freshly laid eggs of *S. damnosum* s.l. (McCall,1995), was introduced into the chamber before the flies were placed in the oviposition chamber. For all the methods, immediately the flies were placed in the oviposition chamber, a timer was set and the time taken for each fly to start oviposition was recorded. All the oviposition procedures were performed in the evening.

In effect six different methods were used in the present study, the immersing method here as the "old single" and the five different methods of the drip system,

namely the "drip single", "drip five", "drip ten", "drip pheromone", and " drip hexane". The following variables were used to determine the effectiveness or the efficiency of the six methods:

a) time taken for the flies to commence oviposition; b) the total number of eggs laid; c) the proportion of eggs hatched; d) the proportion of retained eggs.

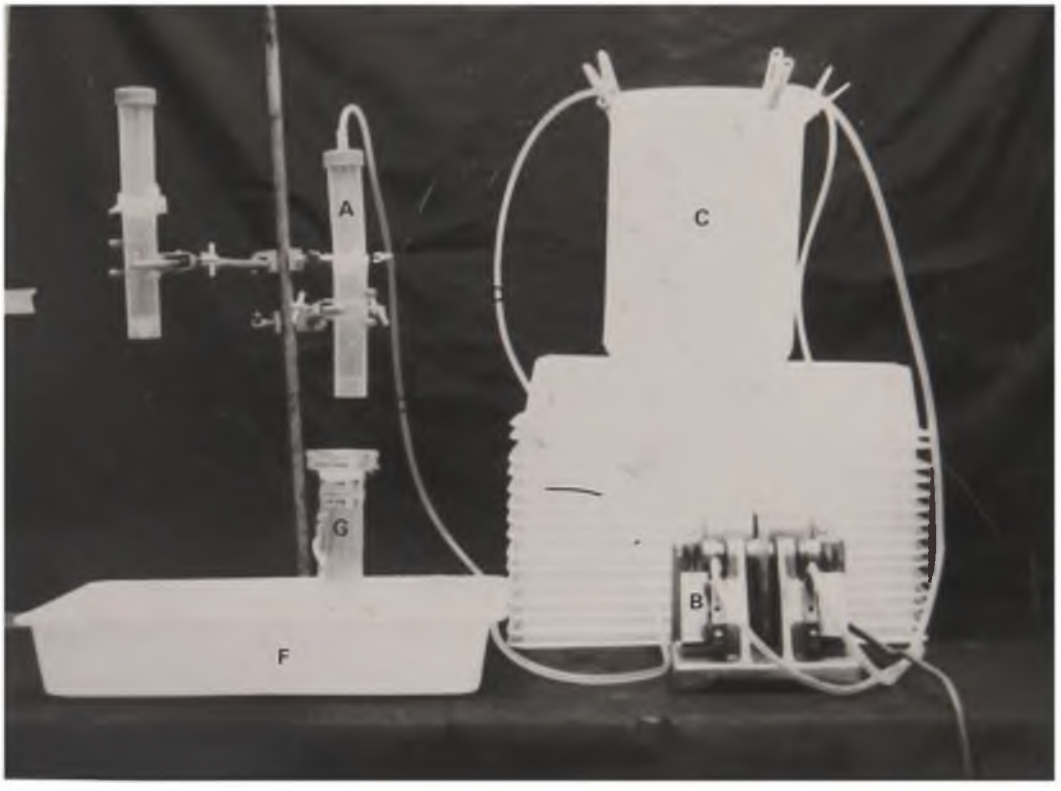


Fig 25. Diagram of drip oviposition system (front view) set for stimulation of female *Simulium damnosum* s.l.

The system consists of an oviposition chamber (A), constructed from two grenier tubes, an air pump (B), which is connected to airstone and used to aerate water in a 5 litre container (C). Aerated water is drawn from C into the oviposition chamber through a rubber tube (D) connected to a micropipette tip, which is inserted in a small hole made in the cap of grenier tube. The water drips continuously unto a nylon netting material (E) and collects in a receptacle (F). The oviposition chamber is held in place by a stand and illuminated from below with a water proof touchlight (G).

3.4.3 RESULTS

The results obtained using the six different systems are indicated in tables 2-4.

The old single and the drip single

Comparing the old single and drip single, there were significant differences in all the parameters that were measured. The average time to oviposition with the old single was 316. 50 seconds, but was 125.40 with the single drip. ($P< 0.001$). The mean number of eggs hatched, using the drip single was significantly high ($P < 0.015$). Likewise there was a significant difference in the average number of eggs retained ($P < 0.016$), with the old single, flies retained an average of 22% as against 7.1% in the case of the drip single.

Old single and drip five

Comparing the old single and drip five methods, there was a significant difference in time to oviposition ($P <0.001$), while there were no significant differences in eggs hatched ($P < 0.655$) and eggs retained ($P <0 .115$).

Old single and drip ten

Comparing the old single and drip ten, there was a significant difference in the time to oviposition ($P <0.001$), and retained eggs ($P < 0.001$). Number of eggs hatched was not significant, ($P <0 .869$), but the proportion of hatched eggs of the drip ten system was significantly higher (87.6%) than the old single (64.8%). The inconsistency between the numbers and the proportions of eggs was due to the fact that there was apparently not the same number of eggs in the ovaries of each fly.

Comparing the old single and drip pheromone, there were significant differences in all the parameters that were measured. Old single and drip pheromone shows significant differences in the time to oviposition ($P < 0.001$), and the number of eggs hatched ($P < 0.012$), with old single having 64.8% hatched eggs and drip pheromone method with 86.6%. and the retained eggs also shows significant difference. ($P < 0.014$).

Drip single and drip five

Comparing drip single and drip five, only time to oviposition showed significant difference, ($P < 0.002$). The number of egg hatched ($P < 0.579$), and eggs retained ($P < 0.567$) were all not significant.

Drip single and drip ten

Also a comparison of drip single and drip ten showed no significant difference in the eggs hatched, and retained. However time to oviposition was significant. ($P < 0.012$)

Drip five and drip pheromone

Drip five and drip pheromone showed significant difference in only time to oviposition ($P < 0.001$)

Drip ten and drip pheromone

Drip ten and drip pheromone showed significant differences in only time to oviposition ($P < 0.001$)

Comparing drip single and drip pheromone, drip single and drip hexane, drip five and drip ten, there were no significant differences in any of the parameters measured.

Drip pheromone and drip hexane

Both experiments did not show any significant difference in all the parameters that were measured.

Comparing drip single and drip pheromone, drip single and drip hexane, drip five and drip ten, there were no significant differences in any of the parameters measured.

Drip pheromone and drip hexane

Both experiments did not show any significant difference in all the parameters that were measured.

Results of oviposition response.

ɔn ɛnt	Total no. flies tested	Total no. flies responding	Time to oviposition (seconds)		
			Mean	Median	Range
mersion	32	20	316.5	285	30 - 900
le	24	20	125.4	114	18 - 360
p five	50	49	193.8	180	30 - 360
p ten	50	49	182.0	180	30 - 360
le + pheromone	21	20	103.2	93	12 - 240
le + hexane	25	20	114.9	93	12 - 330

Table 3 The effect of the different oviposition methods on fertility of female *Simulium damnosum* s.l.

Oviposition inducement method	Fecundity				Fertility			
	Total eggs produced	No. egg laid (% of Total)	Mean(median)	Range	Total no. hatched eggs	Mean	Hatchability	Range
Water Immersion	5,127	4033 (79%)	202 (130)	68 - 403	2614	131	64.80%	0 - 100%
Drip single	4,916	4568 (93%)	228 (185)	95 - 407	4166	208	91.20%	61 - 100%
Drip group five	11,014	9656 (88%)	193 (NA)	NA	8268	165	85.60%	70 - 99%
Drip group ten	9,032	8424 (93%)	169 (NA)	NA	7379	148	87.60%	82 -95%
Drip single + pheromone	4,827	4461(92%)	223 (194)	84 - 394	4149	207	93%	63 - 100%
Drip single + hexane	4,843	4489 (93%)	225 (190)	70 - 422	3949	197	88%	69 - 100%

4 Efficiency of the various inducement methods on oviposition by female Simulium damnosum sl

osition cement iod	Flies with retained eggs No. flies (% of Total)	Mean (+- S D)	Median	Range
ersion	11 (55%)	104(77)	109	1 - 224
single	4 (20%)	87 (99)	84	1 180
group five	8 (16%)	170 (67)	165	45 - 279
group ten	7 (14%)	87 (52)	66	34 - 170
single + pheromone	4 (20%)	92 (92)	77	2 - 210
single + hexane	5 (20%)	71 (94)	10	1 - 200

FIG 27 Graph showing the proportion of gravid female *Simulium damnosum* s.l. that oviposited using the various oviposition methods

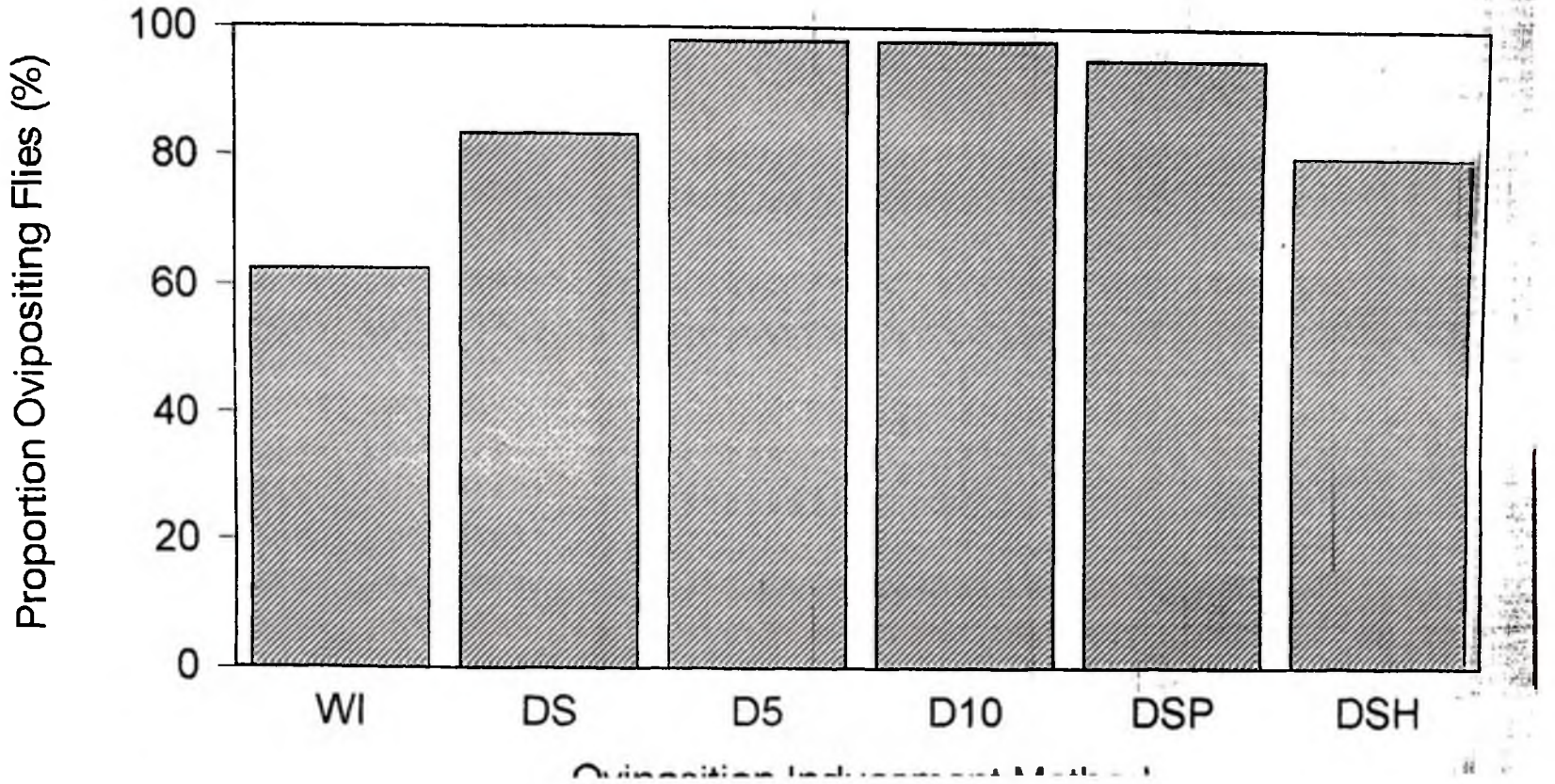
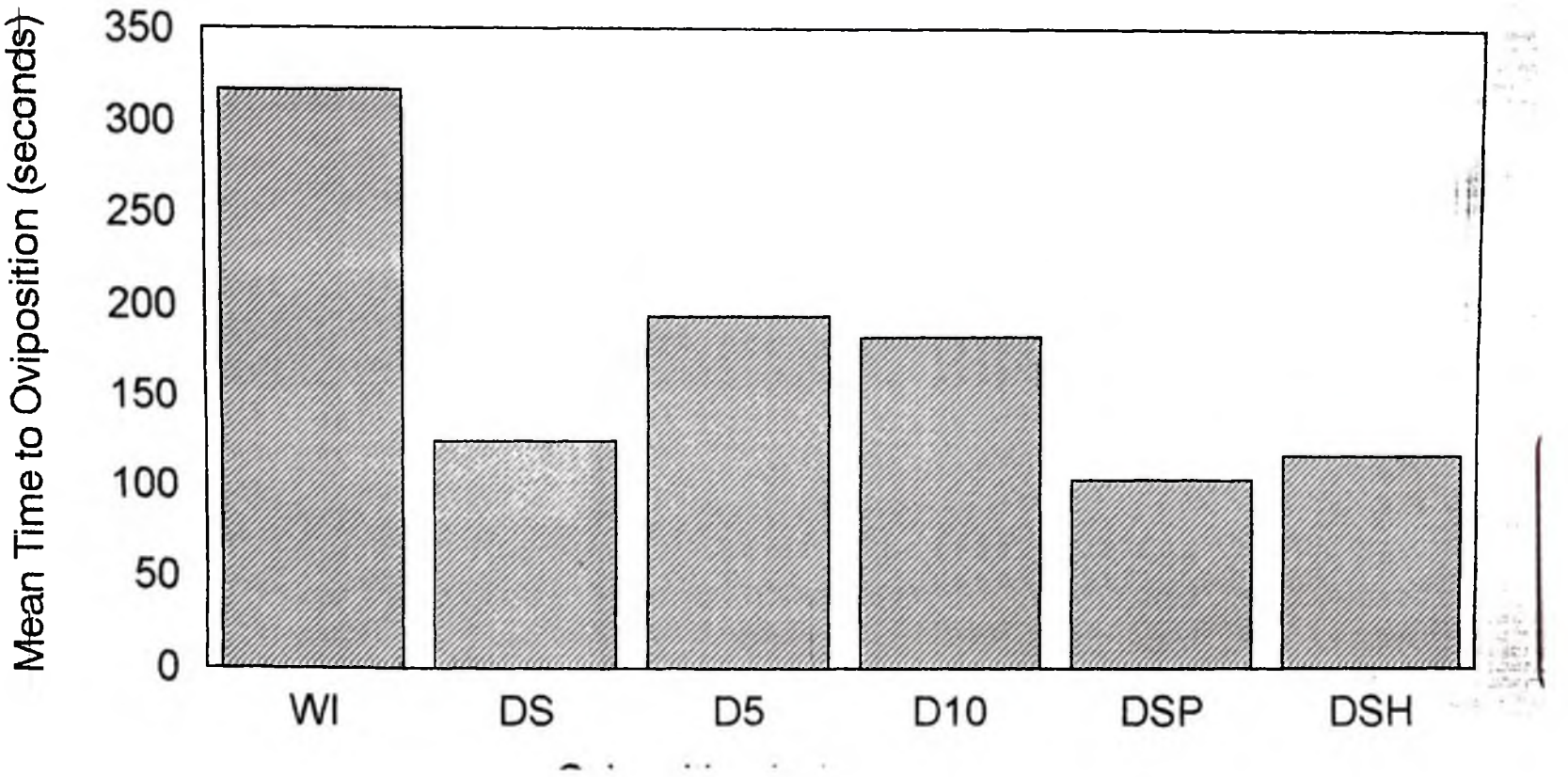
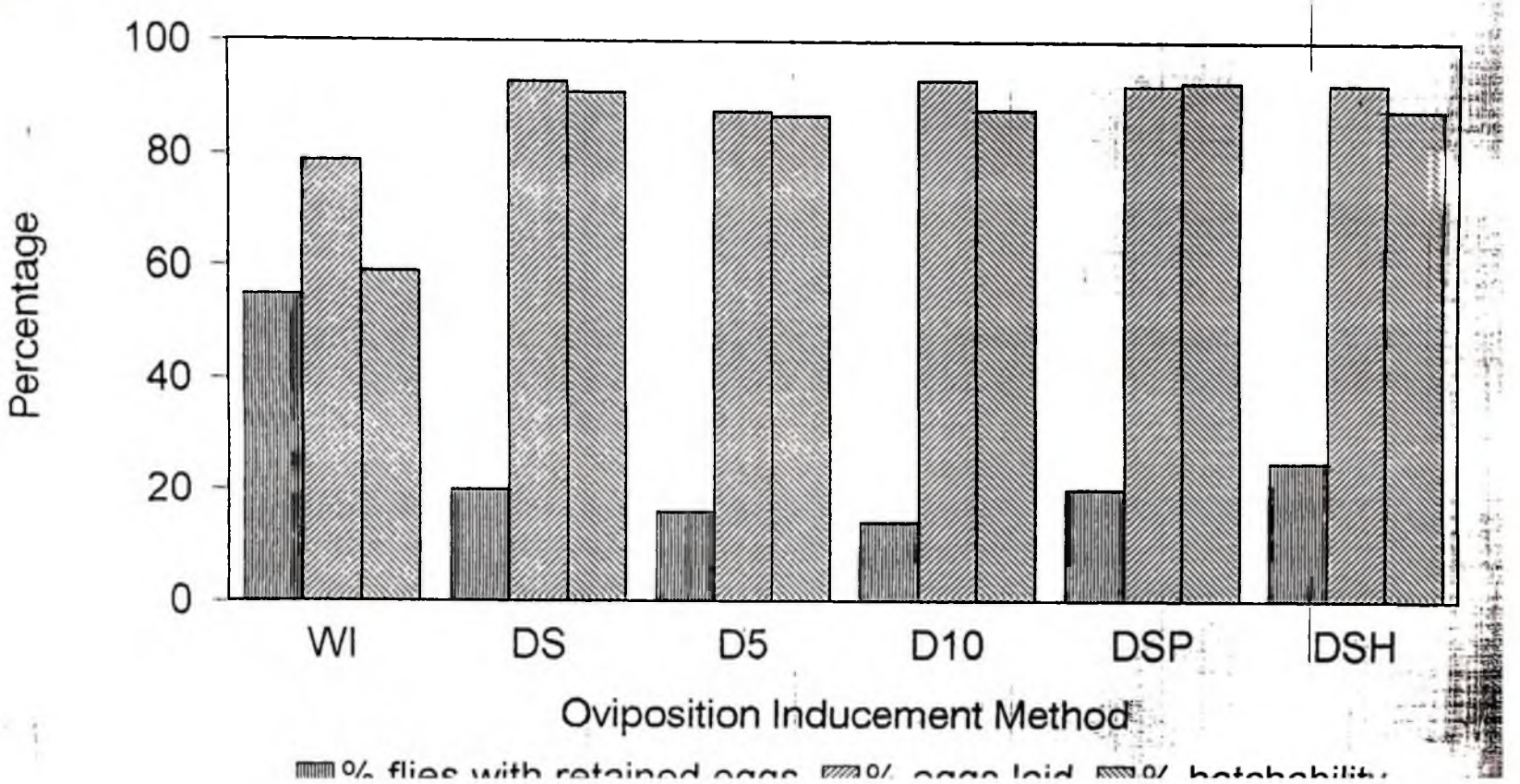


FIG 28 Response (Time taken to commence oviposition) by *Simulium damnosum* s.l. to various inducement methods



and effect of various oviposition inducement methods on fertility (hatchability) of females *Simulium. damnosum* s.l



The results obtained show that there were significant difference between the immersion method and the drip system in all the variables measured, i.e. time for oviposition inducement, number of eggs that were laid, proportion of eggs that developed and the proportion of eggs that were retained. In all these aspects the drip system proved to be a much better method than the immersion method.

In all the oviposition inducement experiments conducted with the drip system on single flies, (using drip single system, drip pheromone, and drip hexane), the drip system was found to be superior in all aspects to the immersion method, thus when the drip system was used, greater percentage of the flies that were stimulated took less time to oviposit, had better hatchability and retained less number of eggs.

All the three methods of the drip systems for single oviposition inducement however, did not show any significant difference between them, on time taken for oviposition inducement, number of eggs hatched , unhatched eggs and number of retained eggs. The implication is that the pheromone extract obtained from eggs did not show any effect on the parameters measured.

The drip five and drip ten methods were also found more efficient than the old system (immersion) considering the time used to initiate oviposition, the number of unhatched eggs, and the number of eggs retained. However, these two methods of group oviposition did not show any difference in their effectiveness to induce oviposition and hatchability. Although the drip methods for oviposition inducement of single flies were much faster in oviposition inducement than the group or multiple oviposition methods, the latter have the advantage of processing more flies at a time.

The study shows that the drip system when used for oviposition inducement, produce eggs that have a high fertility rate with relatively low egg retention in the adult female fly, and is therefore better than the immersion method when used for laboratory colonization of *S. damnosum* s.l.

modification of the twilight method of Simmons and Edman (1982) gives a higher egg fertility rate of 71% while the immersion method recorded 36% fertility rate during the evening time oviposition. In this study, the fertility rate, of the immersion method was 64.8% while that of the single drip system was 91%. Furthermore, the percentage of eggs retained by twilight method of Boakye and Raybould (1985) was 11% while that of the single drip system was 5.8%. The two studies go to confirm that twilight system is better than the immersion method. However, the results obtained in the present study were significantly higher than the two methods reported by Boakye and Raybould (1985). The difference might be attributed to the fact that different species of *S. damnosum* complex were used in the two studies. *Simulium squamosum* was used in the previous study (Boakye and Raybould, 1985) whilst *S. sanctipauli* s.s. was used for this study, and therefore suggest that there might be variation between the species of *S. damnosum* complex for the parameters measured. This needs to be studied further.

3.5 LABORATORY REARING OF *SIMULIUM DAMNOSUM* S.L.

3.5.1 INTRODUCTION

Simulium damnosum s.l. adults have been reared in the laboratory from eggs oviposited under artificial conditions to the adult stage (Wright,1957; Raybould and Grunewald, 1975) however, the apparatus employed had been either too large or cumbersome. For example, the rearing systems described by Brenner and Cupp (1980) and Cupp *et al.* (1981) for rearing *S. damnosum* s.l., though very successful, was expensive, too large and too elaborate for easy transportation or use in for example a temporary field laboratory. Some studies have attempted using a simple, small scale system with either compressed air (Muirhead-Thomson, 1957; Doby *et al.*,1959). or electro-magnetic stirrer to generate current (Raybould *et al.*,1979). However, these systems produce larvae whose development was usually slow, mortalities were high and only few undersized adults were produced. Another major obstacle which was usually encountered with all the earlier rearing systems, was pollution due to metabolic by-product of the larvae and decaying food particles, that led to high mortalities but this was subsequently rectified using self-purification systems (Grunewald,1973; Raybould and Grunewald, 1975; Grunewald and Grunewald, 1978). The self-purification system consisted essentially of a gravel filter and an activated charcoal filter through which water from the rearing system passes before returning to a rearing vessel via a container with submerged roots of *Monstera* sp. (Araceae). The roots acted by removing various ions, such as excess phosphates and nitrogen compounds. However, this self-purification system made transportation very difficult and took much time to assemble and to stabilize.

Subsequently a simple, cheap and easily transportable rearing system was designed and used by Raybould *et al.* (1982) and Raybould and Boakye (1986).

The system was essentially a closed system of water circulation comprising a 60 litre container from which water was pumped along tubing to the top of an inclined rearing trough down which it flows back into the container using an AC electrical centrifugal

that the water falling from the rearing trough above impinged upon it at the point where it penetrated the water creating an area of turbulence where larvae that have moved downstream could attach. Although this system has been used successfully on several occasions to rear *S. damnosum*. s.l., it is still essential to modify it, reduce the large sized container to a much smaller size and the bigger volumes of water to curtail water consumption and make it suitable for a small sized laboratory.

Large numbers of *S. damnosum* complex adults were successfully reared from eggs under artificial condition for the first time in 1966 (Raybould 1967). 5,023 adults were obtained from 28,640 eggs, which represented about 17.5% survival up to the adult stage. The system employed, however, was too large and cumbersome for normal laboratory use. The simple cheap and easily transportable rearing system developed by Raybould *et al.* (1982), also gave good results with the survival rate sometimes exceeding 90%. Nevertheless, much higher mortalities, especially of pupae were sometimes experienced.

The present studies therefore seek to modify if necessary, a simple, cheap and easily transportable rearing system developed by Dr. D. A. Boakye to enhance its efficiency.

3.5.2 MATERIALS AND METHODS

The rearing system used in this stud is a simple, cheap and easily transportable system that uses small volumes of water, but efficient enough to produce normal sized adults from laboratory induced eggs. It has been suggested that the critical factor of the rearing system is the strength of water current, and a similar system has been experimented with to produce larvae from eggs for cytotaxonomy (Boakye, unpublished data). The emphasis on this study was to develop and modify if necessary this system for rearing *S. damnosum* s.l. The rearing system consists of a four litre container, an air pump,

air stones attached at the ends, these rubber tubing passes through the grenier tubes. The grenier tubes creates a suitable surface for larval attachment. Egg batches laid on silk-netting material were placed inside the grenier tubes and attached to the rubber tubing by means of a thread. The grenier tubes were then placed in the container filled with about 3.5 litres of river water. When the air pump, was switched on, the water current generated was suffient to produce suitable conditions within the container, and inside the grenier tubes for the proper development of the larvae. The developing larvae were fed on Tetra® fish food every other day using the conditions described by Raybould *et al.* (1982). The water in the system was changed every day by siphoning off the polluted water and replacing it with fresh river water. Care was taken to ensure that larvae were not siphoned together with the water. The water temperature of the rearing system was monitored regularly between 22-26° C After most larvae had pupated, the container of the rearing system was covered at the top with a sheet of mosquito net held in position by a wire net. The container itself was sheathed with a dark material such that lighting was only from the top. Emerged adults were collected using an aspirator and were kept individually in tubes. Depending on the number of larvae and adults needed for a study, the rearing units were increased.

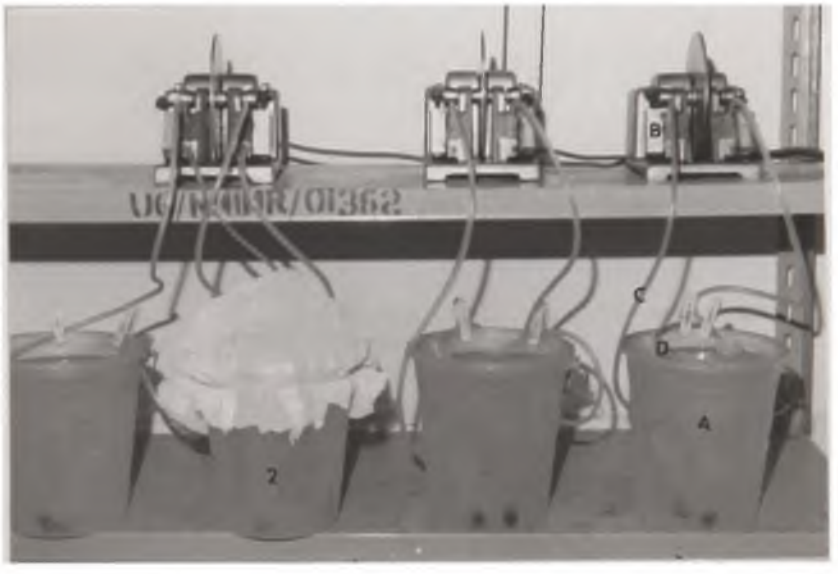


Fig 26 Diagram of laboratory rearing system of *S. damnosum* s.l.

The system consists of a 5 litre plastic container (A), an air pump (B), connected to airstones by rubber tubing (C), these are placed in larval attachment tubes (D) constructed from two grenier tubes), which creates a suitable surface for larval attachment.

The rearing trough (2) has a sheet of nylon netting over a loop of wire netting covering the entire rearing system.

3.5.3 RESULTS

It was observed that a few days after hatching, the larvae moved from the silk netting material to the inner surface of the grenier tubes. At room temperatures (24-28° C) most of the eggs were hatched by the third day. Larval development was usually rapid provided adequate food was supplied, with the late larval instars having a much faster growth rate. Full sized larvae were usually produced after seven days and these gave rise to full sized pupae and adults. At these temperatures, pupation normally commenced by the eleventh day and adults emerged usually from the second day onwards.

More than 300 individuals were usually obtained from a single egg batch, using this rearing system. Where single egg batches were reared, it was not uncommon to obtain about 95% of the hatched eggs developing to full size larvae. The larvae obtained by this method produced also good polytene chromosomes, that were used for cytotaxonomic identifications. Despite the small capacity of the rearing trough, it was quite common to rear more than 600 larvae in a rearing chamber. More than 4,000 larvae were reared during the period of study for insecticide susceptibility tests, and for cytotaxonomic analysis.

3.5.4 DISCUSSION

This rearing system though simple, cheap, and uses small volume of water, gave equally good results as the one developed by Raybould *et al.* (1982). It also has a special advantage of rearing single egg batches to a very high survival rate of about 95%. The system however has a small capacity which limits its capacity to about 600 larvae but it is very efficient. In addition, other systems used self-purification or large volumes of water and algae to prevent the rearing media from being polluted (Grunewald and Grunewald, 1978; Raybould *et al.*, 1982) but regular changing of the water medium was enough to prevent pollution. The use of relatively small amounts of river water for rearing, makes this system more advantageous, especially where the source of river is far from the laboratory. The grenier plastic tubes used were found to provide a good surface for larval attachment, and the presence of the airstone connected to rubber tube placed in the grenier tube provided a consistent water current and turbulence which was adequate enough to provide conditions suitable for larval survival, but not excessive enough to dislodge them.

IN THE LABORATORY.

3.6.1 INTRODUCTION

The failure to induce most African Simuliids to mate in captivity has proved to be one of the biggest stumbling-blocks to laboratory colonization for many years. But with much effort about 19 species of Simuliids have been made to mate in captivity (Edman and Simmons 1986). Hand-mating techniques have not been successful (Raybould and Grunewald, 1975). Specially designed rectangular shaped cages have been used as mating chambers for blackflies (Wenk and Raybould,1972; Raybould and Grunewald, 1975). The cages (40 cm x 40cm x 60 cm) were made of black cloth with a triangular opening about 40 cm high, on one side. This opening is covered with netting through which light enters. (Grunewald, 1972)

Wenk and Raybould (1972) obtained insemination rates ranging from 50-92% in mating trials with Kibwezi form of the *S. damnosum* species complex, using between 17 to 39 females. The authors also observed that the higher the proportion of males present in the mating chamber, the higher the percentage of Kibwezi females inseminated. Wenk (1981) stated that regularly more than 80% of the females of *S. damnosum* s.l. carried spermatophores 48 hours after emergence, when pupae were placed in the mating chamber.

3.6.2 MATERIAL AND METHODS

The mating cage was made of a paper box (40 x 40 x 30 cm) with a triangular opening about 25 cm high on one side. This opening was then covered with netting material on which the flies could settle to copulate. A total of 250 males and 200 female flies which were removed immediately they emerged and kept single in tubes were introduced into the mating cage. After 24 hours, the flies were removed and fixed in 80% alcohol. The female flies were then stained in a solution of 50% saturated fuchsin in 40% ethanol for 15 minutes. They were then washed several times in 80% ethanol. This removed the stain from all other tissues except the spermatophore. The flies were dissected in absolute alcohol to remove the spermatheca. The presence of sperms in the spermatheca was indicated by a translucent ball of cotton like appearance, as opposed to the loose granular material present in virgin females.

3.6.3 RESULTS AND DISCUSSION

Out of the 200 female flies that were placed in the mating chamber, 50% were inseminated after 24 hours in the mating cage. The 50% insemination of the *S. damnosum* s.l. females obtained in this study is lower than the figures obtained by Wenk and Raybould (1972) and those quoted by Wenk (1981). This may be due to the fact that the size of the mating cage used in the study was smaller than those used by the authors mentioned. Also, the lower rate of insemination may be due to the fact that, the flies were kept in the present study cage for only 24 hours instead of 48 hours. This time was chosen because it was found from the onset of this study that mortalities in the mating cage increased dramatically after the first 24 hours.

However, Cupp *et al* (1981) were successful in the production of F_1 adults of *S. damnosum* s.l. with 1% insemination rate which means that the rates achieved here will be enough for the establishment of permanent colonies, but this was not carried out due to the limitation of time.

CHAPTER FOUR

INSECTICIDE SUSCEPTIBILITY LEVELS OF TEMEPHOS (ABATE$^{(R)}$) IN *SIMULIUM SANCTIPAULI* S.S. POPULATIONS OF SOUTH WEST GHANA

4.1 INTRODUCTION

The use of insecticides in the control of vectors must satisfy a wide range of requirements. First the insecticide must be highly effective against the vectors, but safe for the rest of the environment, and also the constituents should be biodegradable. Since the vectors are under constant insecticide pressure in especially very extensive control zones, alternative insecticides should be made available in reserve, preferably belonging to different chemical classes, so that any resistance to one or more compounds could be avoided or dealt with promptly (WHO, 1995).

In the Onchocerciasis Control Programme area of West Africa, organophosphate insecticides, especially temephos, have been the preferred larvicide, because of their effectiveness, range (the distance over which they remains effective), and safety for non-target fauna. However, the appearance of temephos resistance since 1980 in the OCP areas in West Africa, required the adoption of a strategy of alternating insecticides with different modes of action, so as to forestall the appearance of new cases of resistance. The rotation of

insecticides has given ample proof of its worth. Because of the need for this method, six different insecticides are now being used in the OCP area, namely temephos, phoxim, pyrachofos, permethrin, carbosulfan and *Bacillus thuringensis* serotype H-14 (WHO, 1995). However, Kurtak, (1987) stated that these changes in insecticides usage have resulted in a significant increase in the cost of insecticide treatment. Therefore in areas where the use of different insecticides becomes increasingly difficult as a result of lack of funds, and especially where treatment is yet to commence, then the use of such a cheaper and cost effective insecticide like temephos, if found not to be resistant, is still very useful.

In large scale application of insecticides, the impact of larvicide treatments on the larval populations is checked by evaluating sites easily accessible by land along the rivers. This provides information concerning the response of the larval population to the insecticides applied. The appearance of resistant vectors may be of great consequence to eradication programmes, and insecticide susceptibility tests are of great importance in detecting the emergence of resistance at an early stage (WHO, 1963). In carrying out insecticide susceptibility test, batches of insects are exposed for a specified time to come into contact with papers impregnated with several concentrations of insecticide. Larvae may also be placed, for a specified time, in water into which have been added selected concentrations of the insecticide. Every test must include a control, i.e., a batch of insects subjected to all the procedures of the test, except that adults are exposed to untreated paper, and larvae are placed in water containing no insecticide. A high mortality rate in the control may indicate that

the insects have been handled carelessly, or that they may have accidentally come into contact with insecticide or have been exposed to adverse climatic conditions, perhaps during the 24-hours holding period subsequent to the exposure to insecticide. Control mortality rate of 20% or higher meant that the results for that set must be disregarded or test should be repeated (Swaroop, 1966).

In order to detect the appearance of an insecticide-resistant strain of insects, it is necessary to establish a baseline susceptibility level for the species, either before the wide use of insecticides or with specimens from an unaffected area. Thereafter routine checks should be carried out at regular intervals to detect any changes in susceptibility levels. The routine checks should be repeated at several locations and at different seasons of the year to discount possible effects of climatic changes.

A number of factors can influence the susceptibility of insects to poisonous chemicals, for example, temperature, humidity, age, sex and weight. Superimposed upon the effects of these factors are daily changes in physiology and behaviour which may also influence insecticide susceptibility levels (Carole *et al.*, 1982). Several studies have also shown that the response of an insect to insecticide exposure may vary over the 24 hour period, although not all studies have shown the same pattern of susceptibility changes (Carole *et al.*, 1982).

The problem of development and spread of insecticide resistance has necessitated the need for the frequent check of insecticide susceptibility levels of *S. damnosum* s.l., since the development of resistance can easily lead to the spread of

resistance to all river basins by all different species at one time or another within the OCP area (Kurtak, 1990).

It has also been observed that the use of agricultural insecticides in areas around river systems where farming is carried out extensively, could also affect the susceptibility levels of the vector population. If this is not noticed and reported earlier, it may subsequently produce resistant population and may result in the dependence on much expensive insecticides, should vector control measures become necessary. Hence it becomes necessary to carry out insecticide susceptibility test, to recognize and document the insecticide susceptibility levels for future work.

4.2 OBJECTIVES

The main objectives of this study are two-fold first, to determine the susceptibility levels of *S. sanctipauli* s.s. found in untreated areas of southern Ghana. And secondly to analyse the chromosomal karyotyes of the populations for any relationship between the surviving larvae and chromosomal inversions The specific objectives are

1) To determine the susceptibility levels of the organophosphate insecticide, temephos (Abate$^{(R)}$) in *S. sanctipauli* s.s. population found in the River Pra-Ofin and River Tano basins.

2) Compare the susceptibility levels of temephos in the two *S. sanctipauli* s.s. populations for differences and if found, analyse the karyotypes for chromosomal markers.

4.3 MATERIALS AND METHODS

Larvae were obtained from the large breeding sites of Sutri Rapids (River Tano) and Sekyere-Heman (River Pra). Larvae were collected from trailing vegetation and other submerged objects to which they were attached. When transportation of larvae to the laboratory was necessary, the larvae were kept moist in plastic bags, in a cooling box.

Fifth and sixth instar larvae were selected and placed 25 each in glass bowl (volume of 500mls) which was filled with 100 ml of distilled water. The bowls with larvae were allowed to stand for thirty minutes for the larvae to settle. When the larvae attached were well distributed the distilled water was gently discarded, and immediately replaced by 250 ml of insecticide solution. Serial concentrations of temephos (Abate), were prepared, including 1.5 ppm, 0.625 ppm, 0.3125 ppm, 0.156 ppm, 0.078 ppm, 0.039 ppm and a control which was without insecticide was used for each test. (WHO, 1981).

The test concentrations of the insecticide solution were prepared by pipetting 2 ml of the appropriate standard (or parent insecticide solution) into a 500 ml of distilled water that has been pre-oxygenated and stirred vigorously. Where the quantity of the calculated parent solution was not up to 2 ml, it was made up with absolute alcohol. In the case of the control experiment, 2 ml of absolute alcohol was added to 500 ml of distilled water. Depending on the number of larvae available, two or four replicates of each concentration were prepared for each test. The test was

allowed to stand for an exposure period of three hours at temperatures that ranged from 20-25°C. After the exposure period, the provisional mortalities were recorded. Larvae without any spontaneous movements were counted as dead, those with spontaneous movement but could not re-attach were considered as moribund and those that could re-attach considered survivors (Mouchet *et al.*, 1977; WHO, 1981).

The surviving larvae were preserved in Carnoy's solution (three parts alcohol and one part acetic acid) while the dead larvae were preserved in 70% alcohol. Microscopic examination of each larva was done to confirm their developmental stage. The developmental stage or instar of *Simulium* larvae was determined from the size of the larvae and the appearance of their histoblast.

A total of six tests were carried out at different times of the year at Sekyere-Heman, (R. Pra) whilst at Sutri Rapids, (R. Tano) four tests were carried out. Larvae surviving insecticide susceptibility tests, were fixed in Carnoy's solution and stored at 4°C. Larval silk gland polytene chromosomal preparations of the survivors were made using a modification of the techniques by Dunbar (1972), as described by Boakye *et al* (1993). Cytotaxonomic identifications were done using the criteria of Vajime and Dunbar (1975), Post (1986) and Boakye, (1993).

The analysis of the data was done using the OCP software for insecticide susceptibility analysis, called "Anabol."

4.4 RESULTS

In all the experiments carried out at Sekyere-Heman (River Pra), none of the larvae exposed to temephos insecticide at both the diagnostic concentration and the double diagnostic dose (1.25 mg/l) survived. Also at the diagnostic dose, none of the larvae survived out of the six tests carried out separately at different months of the year. The results of the insecticide susceptibility tests are shown in (Table 5 and 6, and Figs.30-33). There were no significant differences between the lethal concentrations, LC_{50} and LC_{95} of the susceptibility test carried out in both the dry and wet seasons. The average LC_{95} and LC_{50} of temephos for *S. sanctipauli* s.s. was 0.73945 mg/l and 0.2092 mg/l respectively.

At Sutri-Rapids (R. Tano), survivors were recorded for all the insecticide tests at both the double and diagnostic concentrations. At the double diagnostic concentration, at least specimens survived in each test, whilst at least two specimens survived at the diagnostic concentration in each test. The lethal concentration, LC_{95} and LC_{50} for *S. sanctipauli* s.s. was found to be 1.7448 mg/l and 0.3013 mg/l respectively. The LC_{95} of River Tano population is significantly higher than that of the River Pra population.

Ten *S. sanctipauli* s.s larval survivors obtained were found to be six males and four females. Besides the fixed inversions that characterise *S. sanctipauli* s.s, the polymorphic inversions recorded were the inversions 1S-A, 1S-21 and IS-25. Inversion IIIL-B was found in five (83.3%) males whilst inversion IIL-7 was found present in nine (90%) of the samples.

Tables 7 and 8 show the inversion frequencies and the proportion of inversion frequencies of larvae surviving the diagnostic and double diagnostic concentrations of temephos susceptibility test done on river Tano. A comparison of the inversion frequencies of the survivors and that of the general population are shown in Tables 9-14, and they indicate almost the same inversion frequencies and proportion of inversion frequencies scored.

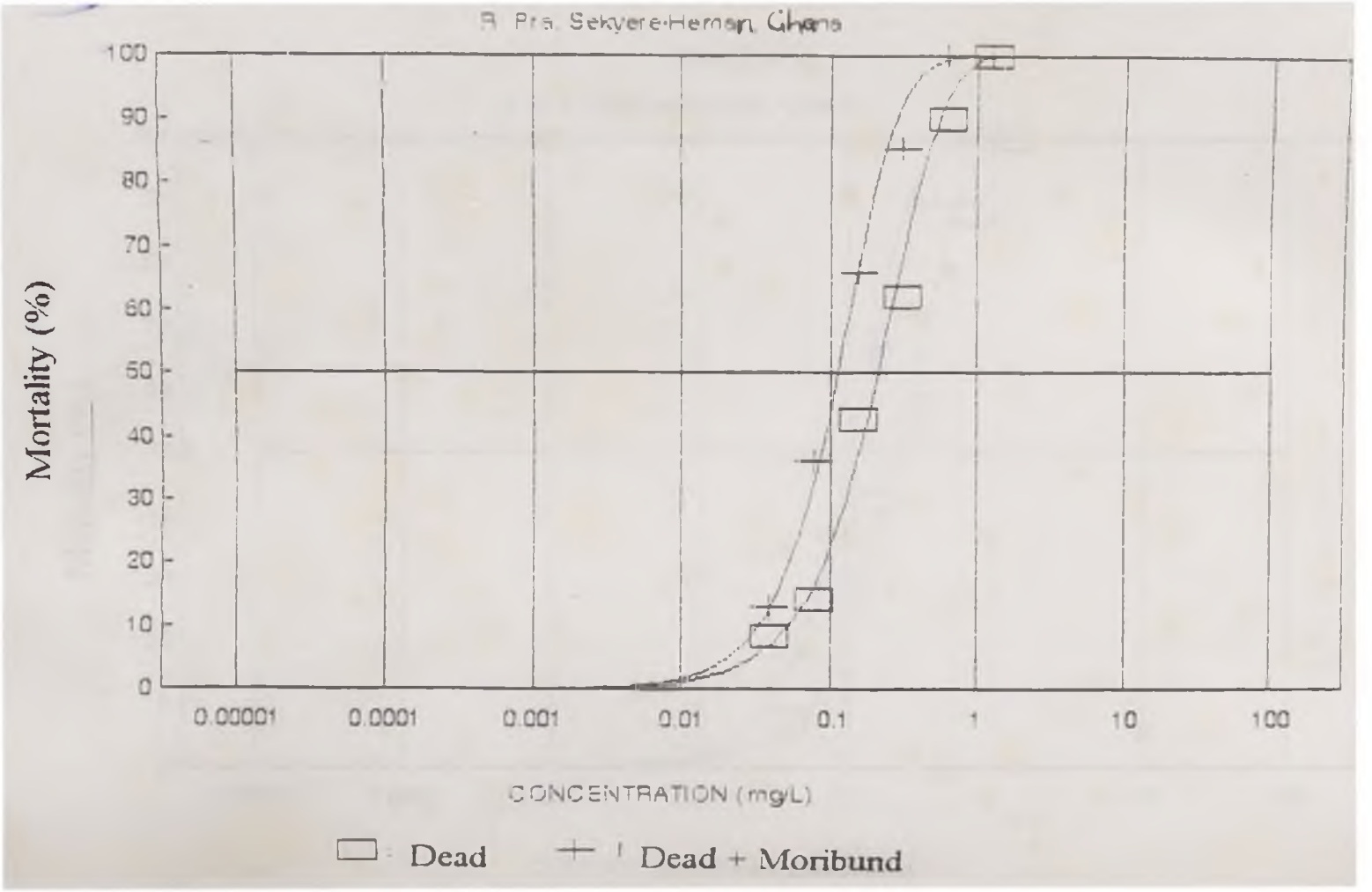
Table 5 Lethal Concentrations (LC_{50} and LC_{95}) of temephos susceptibility test of *S. sanctipauli* s.s carried out on River Pra at Sekyere-Heman.

Date	Lethal Concentration (LC_{50})	Lethal Concentration (LC_{95})
31/3/95	0.22775	0.73156
28/3/95	0.20978	0.66702
8/6/95	0.21289	0.70263
10/6/95	0.23081	0.71942

Table 6 Lethal Concentrations (LC_{50} and LC_{95}) of temephos susceptibility test of *S. sanctipauli* s.s carried out on River Tano at Sutri Rapids.

Date	Lethal Concentration (LC_{50})	Lethal Concentration (LC_{95})
8/6/95	0.3021	1.7171
10/6/95	0.3005	1.7725

FIG 30 Graph of temephos susceptibility of *Simulium sanctipauli* s.s. at, Sekyere-Heman, River Pra on 8/6/95, showing percentage mortalities at different concentrations.



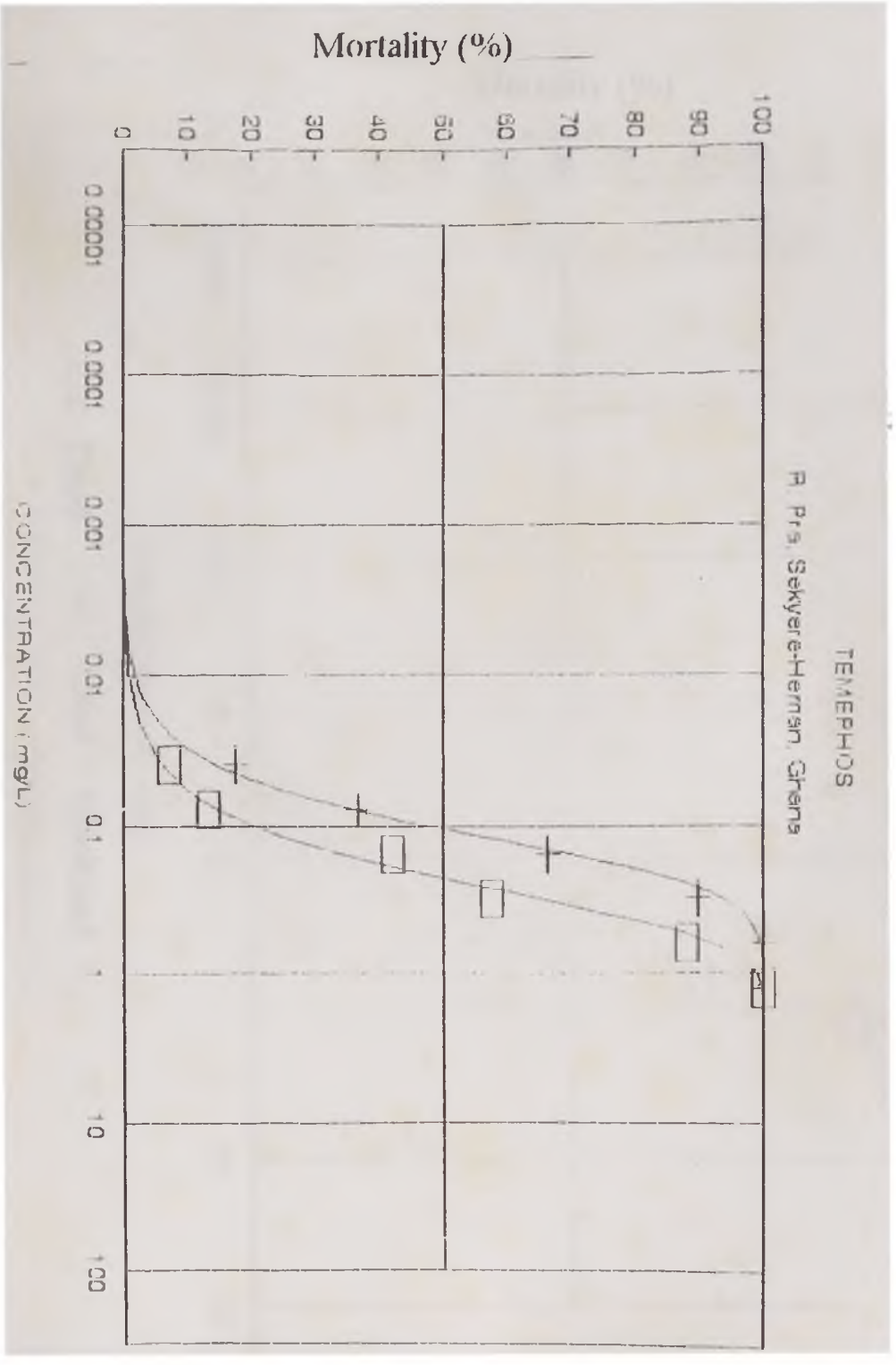


FIG 31 Graph of temephos susceptibility of *Simulium sanctipauli* s.s. at Sekyere-Heman, River Pra on 10/6/95, showing percentage mortalities at different concentrations

FIG 32 Graph of temephos susceptibility of *Simulium sanctipauli* s.s. at Sutri-Rapids, River Tano on 8/6/95, showing the percentage mortalities at different concentrations.

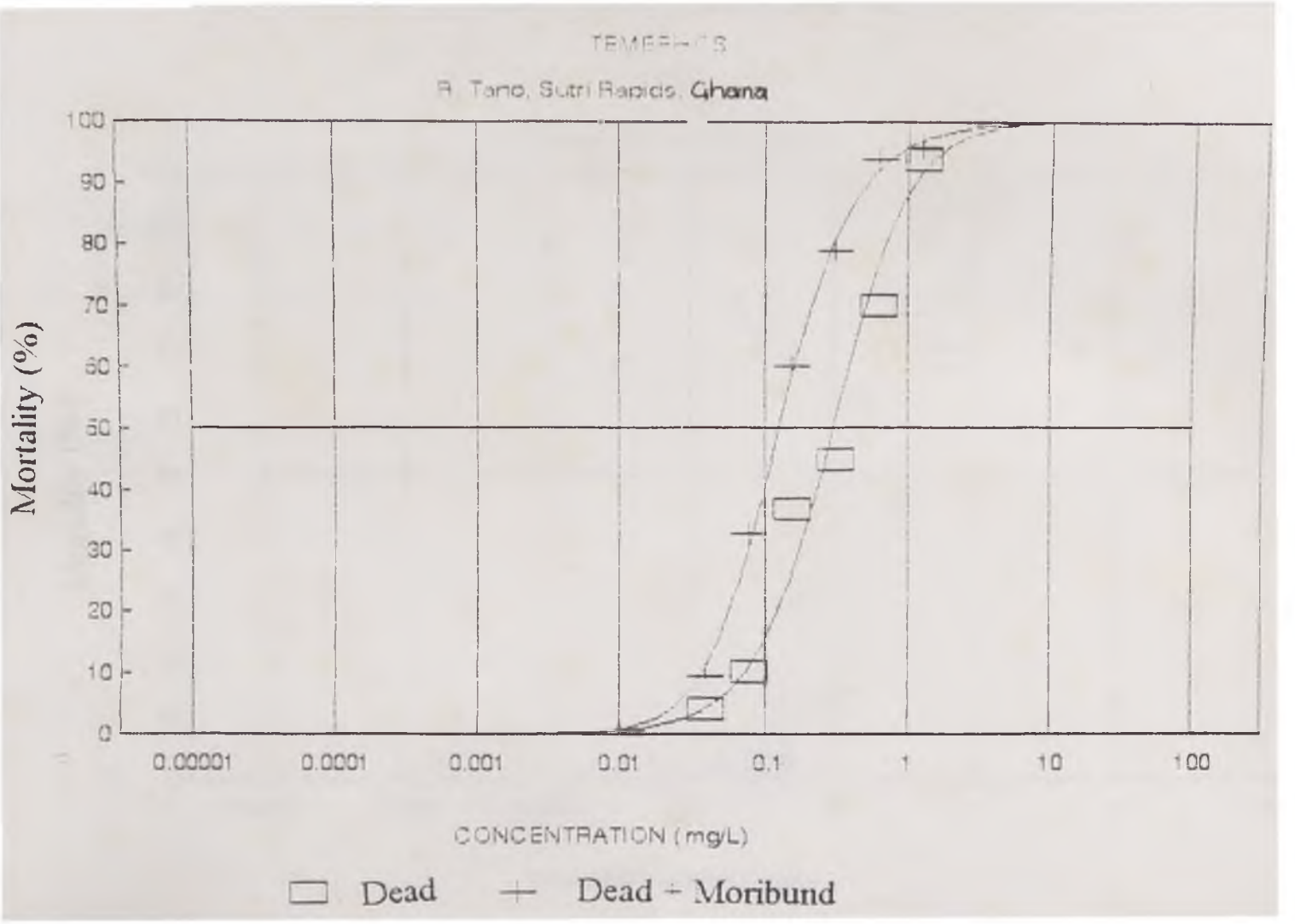


Fig 3.3 Graph of temephos susceptibility of *Simulium sanctipauli* s.s. at Sutri-Rapids, River Tano10/6/95 showing the percentage mortalities at different concentrations.

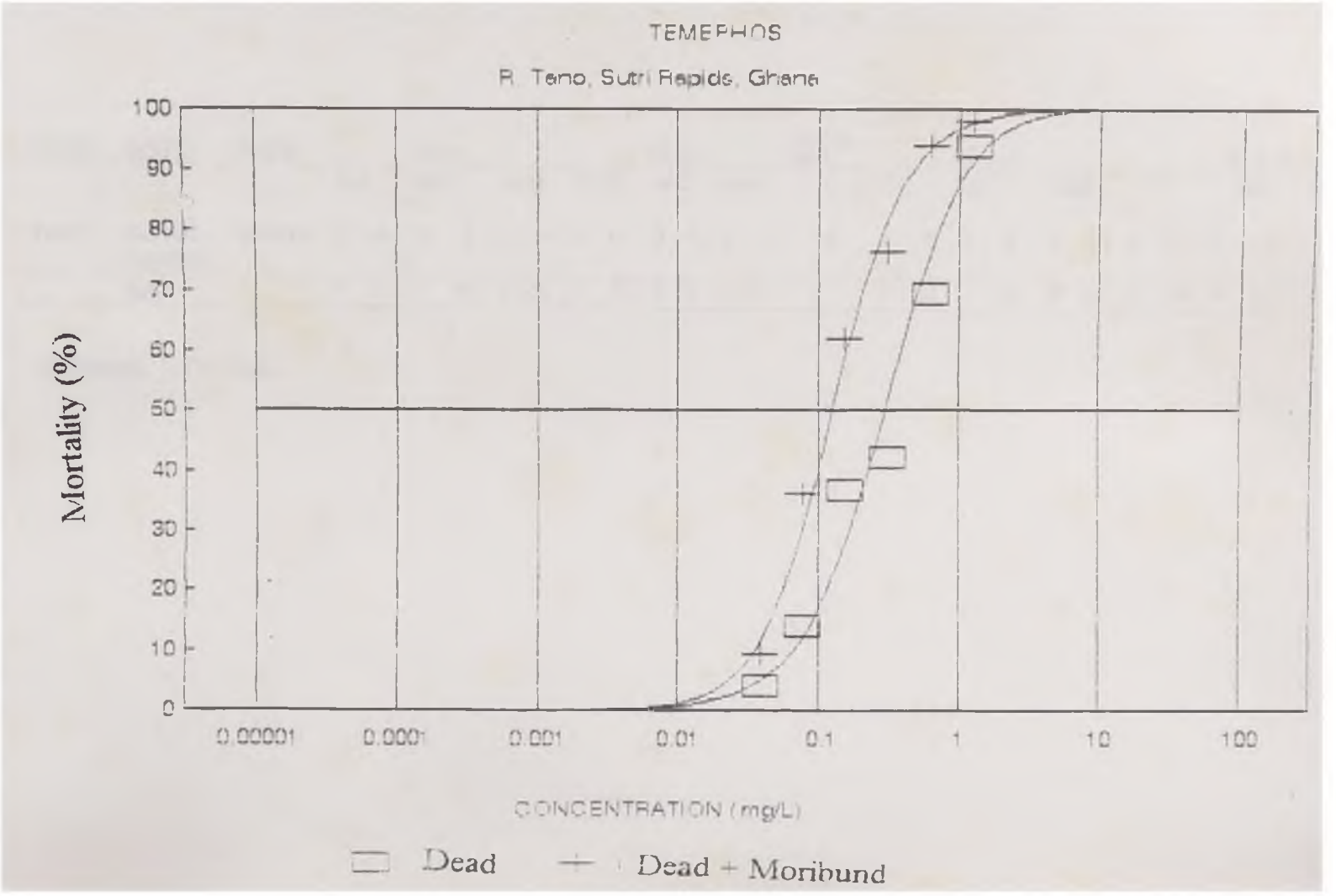


TABLE. 7 Inversion frequencies of *S.sanctipauli* s.s. larvae surviving insecticide susceptibility test at Sutri Rapids, Rivers Tano.

RIVER	SITES	DATE	1S-A						1S-21						SAMPLE SIZE	II L-7							III L-B				
			A\A		ot\A		ot\ot		21\21		ot\21		ot\ot			7\7		ot\7		ot\ot		B\B		ot\B		ot\ot	
TANO	SUTRI RAPIDS	10\6\95	0	0	2	1	3	5	2	0	0	1	4	3	10	4	5	0	0	1	0	4	1	1	5	0	0
	Sex		F	M	F	M	F	M	F	M	F	M	F	M		F	M	F	M	F	M	F	M	F	M	F	M

(F) Female (M) Male

TABLE 8 Proportion of inversion frequencies of *S. sanctipauli* s.s. larvae surviving insecticide susceptibility test at Sutri Rapids, Rivers Tano.

RIVER	SITES	DATE	1S-A	1S-21	II L-7	III L-B
TANO	SUTRI RAPIDS	10/6/95	0.152	0.301	0.90	0.70

4.5 DISCUSSION

In the Onchocerciasis Control Programme, the diagnostic concentration used for the insecticide susceptibility test of temephos (Abate®) is 0.625 mg/l. This concentration is supposed to give almost 100% mortality rate, if susceptible larvae of *Simulium damnosum* s.l. are exposed.

The lethal concentration killing 50% and 100% of insects exposed were determined and indicated as LC_{50} and LC_{100}. However the LC_{100} is a theoretically indeterminable value and is normally not estimated, rather the LC_{95} is usually determined. The LC_{50} is often used as a single index that summarizes susceptibility, because in statistical theory, it can be estimated with greater precision than lethal concentration at either end of the range. It has, however, been indicated that in malaria eradication, it was found to be less suitable as an index, because anopheline species that became resistant to high concentrations of insecticide may not show commensurable increase at the concentrations that kill 50% of the vectors (WHO, 1963; Swaroop, 1966), and hence the need to use LC_{95}.

In the evaluation of susceptibility tests, the responses of the vectors to insecticides have been categorized into three: susceptible, intermediate and resistant (WHO, 1963). The susceptible insects population, in response to temephos insecticides have an LC_{95} close to the normal base-line value, for the exposure period. However, there may be occasional survivors. The category intermediate, means that a substantial proportion of the test insects population, less than 50%,

survived the base-line concentration. The resistant category applies, when 50% or more of the exposed insects population survived the base-line (Swaroop, 1966).

WHO (1963) stated that the susceptible insects in response to the insecticides have an LC_{100} or LC_{95} close to the normal base-line susceptibility value. The diagnostic dose of temephos of 0.625 mg/l is used by the OCP, Therefore, the susceptible larvae in response to the insecticide should have an LC_{100} or LC_{95} close to this normal base-line susceptibility value. On the basis of this the *S. sanctipauli* s.s. populations on the River Pra are susceptible to temephos at an LC_{95} value of 0.739 mg/l.

However, the *S. sanctipauli* s.s. population from River Tano, were found to have a much higher LC_{95} than the River Pra populations, at 1.745mg/l, (about two and half times higher) and therefore resistant. Though both Rivers Tano and Pra are untreated, this detection of resistance and increased tolerance levels on River Tano is not surprising because Meredith *et al* (1986) had reported the detection of full resistance in *S. sanctipauli s.s.* at a site on River Tano in south-western Ghana where systematic larviciding has never been carried out. The authors suggested the probable migration of resistant population from rivers in southern Côte d' Ivoire as the possible reason. The use of agricultural insecticides in cocoa plantations situated along the banks of River Tano which is untreated might also account for the detection of resistance in *S. sanctipauli* s.s. at Sutri Rapids. Since insecticides like Deldrin are being used by farmers against pests of cocoa, it is likely that these have washed down into the river during the rains, thus selecting for resistance. Factors like temperature, humidity, age, sex, weight and nutritional status can also influence the susceptibility of insects to poisonous chemicals (Carole *et al.*, 1982). And also

superimposed upon the effects of these factors are daily changes in the physiology and the behaviour of the insects which may also influence insecticide susceptibility levels. However, some of these factors including age, temperature and humidity are not likely to be the reasons for the resistance, since they were controlled for.

The occurrence of occasional survivors at chosen diagnostic concentrations in a periodic test may be due to normal variation, but regular occurrence of survivors as was found at Sutri Rapids for all the tests carried out constitute a signal for future investigation.

Meredith *et al.*, (1986) had reasoned that the inversion IIL-A observed in the resistant *S. sanctipauli* populations was not responsible for resistance but may be only an indicator of its presence. The authors however, thought that this inversion revealed a distinct population and that it could be useful as a marker to trace movements of resistant populations of resistant *S. sanctipauli* s.l. However, Post (1986) in his revision of *S. sanctipauli* subcomplex recorded IIL-A as the diagnostic inversion for *S. sanctipauli* s.s. This means that Meredith *et al.* (1986) actually found resistance in *S. sanctipauli* s.s. populations. Susceptible populations which lacked the inversion IIL-A and termed the 'eastern form' (Meredith *et.al.*, 1986) were *S. soubrense* according to Post (1986). The *S. sanctipauli* s.s. population at Sutri Rapids is unique from the other populations of the same species on the River Tano, because of the presence of inversion IS-25, and the distribution of various inversion frequencies (see Table 14). Analysis of the chromosomal karyotypes of *S. sanctipauli* s.s. larvae that survived the insecticide susceptibility tests and that of the sampled wild population did not

reveal any significant differences in inversion frequencies. Therefore, it can be concluded that there was no new chromosomal inversion or unique inversion frequencies that could be related to resistance to temephos by *S. sanctipauli* s.s. populations.

CHAPTER FIVE

CYTOTAXONOMY OF *SIMULIUM SANCTIPAULI* S.S POPULATIONS IN SOUTH WESTERN GHANA

5.1 INTRODUCTION

Chromosome cytology has proved to be a useful tool in the identification of insect species complexes which are vectors of diseases, such as *Anopheles gambiae* complex (Coluzzi, 1968; Green and Hunt, 1980), polytene chromosomes are used for identification during cytological studies. Polytene chromosomes are formed from polytene nuclei, these are highly polyploid and develop in the nuclei of certain tissues of endodermal, mesodermal and ectodermal origin: these include gastric caecae, mid-and hindgut and papillae, fat body and malpighian tubules (Culham, 1957).

In Simuliidae, polytene chromosomes are found present in the larval salivary gland nuclei. Cytological studies done on Simuliidae mostly use larval polytene chromosomes, these are characterized by well differentiated banding pattern. Banding patterns are genetically determined and are consequence of variation in the packing ratio of the DNA; i.e. it reflects an ordered structure assumed by the DNA helix and the associated histone protein (Ashburner, 1980), constitute patterns of dense bands and less dense interbands.

The most important vectors of human onchocerciasis in Africa, *S. damnosum* s.l., is a sibling species complex (Dunbar 1969; Dunbar and Vajime 1972; Vajime and Dunbar, 1975). New biological information on the complex is of limited value unless it is known to which species it refers. It is known that the

members of the species complex show profound differences in their epidemiological importance (Post and Boakye, 1992) and so not all sibling species are equally important. For instance the savanna cytospecies transmit the more severely debilitating form of savanna onchocerciasis.

Species identification of *S. damnosum* s.l. commenced with the use of morphological characters, despite much progress in morphological taxonomy (Quillévéré *et al.*,1977; Garms, 1978; Dang and Peterson, 1980; Wilson *et al.*, 1993), chromosomal differences in the larvae remain the most reliable characters for species determination within the complex. For instance it has been found that certain morphological differences between adult flies are size, rather than species dependent (Garms, 1978; Townson and Meredith, 1979; Cheke and Harris 1980). Furthermore, Garms *et al.* (1980) have shown that although adult morphological characters are useful in classifying *S. damnosum* populations, individual flies are often unidentifiable. Wilson *et al.* (1993) have improved upon this by using multivariate statistical analysis to achieve 100% classification of the *S. damnosum* subcomplexes, but not the individual species within the complexes. Therefore cytotaxonomic determination of late instar larvae continues to be the only definite method of species identification.

Among the members of *S. damnosum* species complex, the *S. sanctipauli* subcomplex has been well studied, and the most reviewed species in most parts of West Africa. It is therefore important that wherever revision of this subcomplex has never been carried out, further studies may be needed to update the current status of the *S. sanctipauli* subcomplex.

5.1.1 CURRENT STATUS OF SPECIES OF THE *SIMULIUM SANCTIPAULI* SUBCOMPLEX

Cytotaxonomic studies for species identification in *S. damnosum* complex depend to a large extent, on the identification of specific segments of banding pattern of the larval salivary glands polytene chromosomes (Dunbar, 1969). The main structural rearrangements of the chromosomes used in cytotaxonomy of *S. damnosum* s.l. are fixed diagnostic inversions within each particular species. Sex chromosomal (X and Y) differences may also be used in the identification of the species, the inversions associated with these chromosomes may be in homozygous or heterozygous conditions.

The sequence of the banding pattern of *S. squamosum* polytene chromosome is used as the standard for the *S. damnosum* s.l., because it is the closest to the East Africa species (Vajime and Durbar,1975). The standard species *S. squamosum*, is characterized by a fixed inversion 1S-1 and 1L-3 (Vajime and Dunbar, 1975). However, 1S-3 is probably not very useful because it is polymorphic in some populations in West Africa (Boakye, 1993).

The *S. sanctipauli* subcomplex is separated from *S. squamosum* by the inversions II L-4, which is fixed in *S. sanctipauli* subcomplex but absent in the latter. Four sibling species have so far been described in the *S. sanctipauli* subcomplex namely *S. sanctipauli* s.s., *S. soubrense*, *S. konkorense* and *S. leonense*.(Boakye *et al*, 1993). *S. sanctipauli* subcomplex has only one fixed inversion IIL-4 diagnostic for all its members with each member of the subcomplex having its own fixed inversions (Boakye *et al*., 1993).

Simulium sanctipauli s.s. is characterized by inversion IIL-A as intraspecific (Post, 1986) and two other fixed inversions IIL-6 and IIIL-2. Besides these fixed inversions, there are various floating inversions that are found in various populations at different frequencies. And these inversion frequencies can be used to characterize *S. sanctipauli* s.s. populations. A geographical variant of this species *S. sanctipauli* "Djodji" form has been described in Togo and Ghana. This form has no fixed inversion difference from other populations of *S. sanctipauli* but rather a sex determination system associated with the inversion IS-21. Males of the Djodji forms are heterozygous for this inversion 1S-21 but absent in the females (Surtees *et al.*, 1988).

Simulium soubrense has been revised by Post (1986) and Boakye et al., (1993). They recognized all members of the *S. sanctipauli* subcomplex lacking the inversion IL-A and IIL-A as *S. soubrense,* the inversions IIL-6 and IIIL-2 are fixed in all populations. Recent observations have however shown that there are a lot of fixed inversion differences between various populations which are sometimes found breeding in sympatric situations. Hence some have been raised to specific status and others, are at present, considered as geographic variants designated as forms. Those regarded as different species are included in *S. konkourense* (Boakye *et al.*, 1993). The various forms described under *S. soubrense* are the Beffa form (Meredith *et al.*, 1983), the Farmington and St. Paul forms (Kashan and Garms, 1987), and the Chûtes Milo form (Boakye *et al.*, 1993).

The Chûtes Milo form seems to be typical *S. soubrense,* almost identical to Vajime and Dunbar's Type material from the river Leraba (Boakye *et al.*,

1993). It is recognized by the presence of fixed inversion IIL-6, IS-A, IIIL-4, IIIL-17 and IIIL-24, and the absence of inversion IL-A and IIL-A, these fixed inversions are homozygous in all populations. Also, IIL-D is present as floating inversion but the inversion IIL-7.D are absent. Specific to the Chûtes Milo form but different from other populations in Cote d'Ivoire and Ghana that share similar inversions, is the association of IIIL-B with sex determination.

The 'Beffa' form shows a distinctive sex- linkage which is associated with the inversion IIS-6b (Meredith *et al.*, 1983), this is present in the male in heterozygote condition but absent in females. Inversions IIL-D, IIIL-4, IIIL-17, and IIIL-24 are present as floating inversions.

The St. Paul form is characterized by the fixed inversions IIL-D.7 and IIIL-4.17. It also shows sex-linkage with the inversion IIIL-5. The inversions IL-1 and IIS-7 are present as floating inversions.

The 'Farmington' form differs by the absence of any sex-linked inversion and the inversion IIL-D. Present as floating inversions are IIS-7, IIIL-4, IIIL-17, IIIL-5, IIIL-26.

Simulium konkourense (Boakye *et al.*, 1993) includes two forms; the Konkoure form (Quillévéré *et al.*,1982) and the Menankaya form (Boakye *et al.*, 1993). These were previously considered as forms of *S. soubrense* because they lacked the inversions IL-A and IIL-A. These two forms show clinal variation in the proportion of floating inversions along their distributional area. The Menankaya form have the inversion IIL-7.D as fixed and the Konkoure form has the inversions IIL-4.X/4.X or IIL-4.X/4.6.D.7. They can be distinguished from

each other by the absence of the inversion IIL-X in Menankaya form but floating in the Konkoure form.

Simulium leonense (Boakye *et al.*, 1993) was originally thought to be a form of *S. soubrense*, known as *S. soubrense* `B' (sensu Post, 1986). However, it differs from *S. soubrense* by the presence of the inversion IL-A, as a diagnostic inversion and IIS-7 and IIIL-2 also present as fixed inversions. Sex-linkage is found to be associated with IIIL-4.17 in most populations (Boakye, 1993). This cytospecies occurs mainly in Sierra Leone and its border areas with Guinea.

The taxonomic description of *S. damnosum* s.l. still remains largely dependent on the larval chromosome data, which sometimes prove difficult to interpret. This is especially true for the *S. sanctipauli* subcomplex which has gone through series of revisions. More recently, Boakye *et al.*, (1993) have revised the cytotaxonomy of *S. sanctipauli* subcomplex found in Guinea and the adjacent countries in the Onchocerciasis Control Programme areas. Some of the forms of the cytospecies within *S. sanctipauli* subcomplex have been elevated to species status.

Revision of *S. sanctipauli* subcomplex has also been carried out in most parts of the OCP areas. However, in areas of Ghana outside the OCP, this has not been done, and it is likely that detailed studies might reveal new inversions and inversion frequencies unique to the populations in South West Ghana. In this study, a full karyotyping of all chromosomes of *S. sanctipauli* subcomplex was carried out. The area of study was the main river systems in the South-western Ghana where it has been previously reported that this species occurs.

5.2 OBJECTIVES

The main objectives of this study are as follows.

1) To karyotype fully, all the chromosomes of *S. sanctipauli* s.s. found in the populations occurring in the rain forest belt of south-west Ghana, with the view of detecting new inversions, inversion frequencies and sex-linked inversions.

2) To analyze the data to determine if there is seasonal and clinal variation in the inversion frequencies of *S. sanctipauli* populations.

5.3 MATERIALS AND METHODS

Larvae of *S. damnosum* s.l. were collected from major breeding sites on Rivers Pra, Ofin and tributaries and River Tano. Larvae collected from the wild, were immediately fixed in Carnoy's solution and stored at 4°C. At most sites, samples were collected in a month or two depending on the availability of materials and the frequency of visits to the sites. However, at Sekyere-Heman, samples were collected throughout the whole year for cytotaxonomic analysis.

With the aid of a dissecting microscope, the posterior aspect of the larval abdomen was split open along the mid-ventral line in Carnoy's fixative, and then rinsed three times in distilled water. They were blotted dry and hydrolysed in 10% hydrochloric acid at 60-65°C for seven minutes. After hydrolysis they were again rinsed three times in distilled water, blotted dry and stained in lacto-acetic orcein solution for two to three hours. Thereafter, each larva was sexed according to the shape of the gonadial rudiment, which is oval in males and slender in females. (Boakye, 1988). After sexing, the silk glands were removed and mounted on a microscope slide in 50% propanoic acid, gently squashing the coverslip between filter papers to spread the chromosomes. Some of the preparations were made permanent by carefully removing the cover slip, drying both cover slip and the slide in absolute ethanol and remounting in Euparol.

Chromosomal inversions were recorded and species were identified using the criteria of Vajime and Dunbar (1975), Post (1986), Boakye *et al.* (1993) and Boakye (1993). The cytotaxonomic differences between populations were determined by the occurrences of fixed inversions differences, differences in the sex chromosomes and differences in the arrays of polymorphic inversions (Vajime and Dunbar, 1975).

5.4 RESULTS

The distribution of *S. damnosum* s.l. species and their inversion frequencies in the main rivers systems of south-west Ghana are shown in Tables 9, 10, 11,12 and 13.

5.4.1 SPECIES DISTRIBUTION AND KARYOTYPES OF *S. SANCTIPAULI* S.S. POPULATION ON RIVER TANO

The list of breeding sites with their co-ordinates where samples were collected for identification on the River Tano is shown in Tables 9-14. When available, a minimum of 25 specimens were examined from each collection. All the specimens examined from the four sites namely Nsawora, Bopa, Ruru Rapids and Sutri rapids were identified as *S. sanctipauli* s.s.

The diagnostic inversion IIL-A and other inversions for *S. sanctipauli* s.s. were present. The inversions IIL-4.6 and IIIL-4.17.2 were found to be fixed in all the specimens examined. The following different inversions were also recorded: IS-A, IS-21, IIL-7 and IIIL-B, these occurred either as fixed or

polymorphic inversions in one population or another. These inversions were found to occur at different frequencies in different populations.(see Table 9-13).

At Nsawora, the inversion IS-A was fixed, whilst inversion IS-21 had a low proportion of inversion frequency of 0.28 but a high proportion of inversion IIL-7 of (0.82) (see Table 14).

At Bopa, the inversion IS-A was also found to be fixed, with IS-21 having a low proportion of inversion frequency of 0.238 and a high proportion of inversion IIL-7 (0.90). At Ruru Rapids, however, the inversion IS-A was not found fixed in the population, but occurred at a high proportion of inversion frequency of 0.80. Inversion IS-21 occurred at a low frequency of 0.20 whilst inversion IIL-7 had a high inversion frequency 0.90. The *S. sanctipauli* s.s. population at Ruru Rapids and Nsawora had a dominant number of females heterozygous for the inversion IIL-7. At Nsawora, 71.4% (5) of the heterozygous for the inversion were females, whilst at Ruru rapids 83% (6) of the heterozygous condition were females.

At Sutri rapids the situation looked quite different, inversions IS-A and IS-21 were present at low frequencies (0.176 and 0.312 respectively) whilst inversion IIL-7 occurred at a high inversion frequency 0.92. However, inversion IIL-7 was completely absent in 8% of the population. Also in the Sutri Rapids population, a new inversion was identified and named inversion IS-25, this inversion was peculiar to this site only, and occurred at a frequency of 0.56. Sex-determination was found to be associated with the inversion IIIL-B, at all the sites. More than 85% of the males were heterozygous for the inversion whilst

more than 90% of the females were found to be homozygous for this inversion. Analysis (Chi-square) showed sex-linkage in the inversion IIIL-B for the males.

5.4.2 SPECIES DISTRIBUTION AND KARYOTYPES OF *S. SANCTIPAULI* S.S POPULATION ON RIVERS PRA-OFIN AND TRIBUTARIES

On River Pra-Ofin and tributaries, samples were collected from six different breeding sites. The two breeding sites on the river Pra were Sekyere-Heman and Awisam. On River Anum a tributary of River Pra, samples were collected at Anwiaso Jumaku and Jankobaa and on River Ofin at Assin Asaman and Pokukrom.

Some of the populations examined were pure, whilst others were mixed populations, with other cytospecies living in sympatry with *S. sanctipauli* s.s. All the specimens examined from Sekyere-Heman were identified as *S. sanctipauli* s.s. At Anwisam, 28 specimens were identified as *S. sanctipauli* s.s., and one specimen as *S. yahense*. At Anwiaso Jumaku out of 30 specimens examined, only one was found to be *S. sanctipauli* s.s., seven were *S. yahense* and twenty-two were *S. damnosum* s.s. At Jankobaa, out of 25 specimens examined, 17 were *S. damnosum* s.s., 13 were *S. yahense* and four were *S. sanctipauli* s.s.

All the *S. sanctipauli* s.s. examined from the Pra-Ofin basin were identified using the criteria of Post (1986) and Boakye (1993).

The diagnostic inversion IIL-A was present in all the specimen . The inversions IIL-4.6 and IIIL-4.17.2 were also found to be fixed in all the *S. sanctipauli* s.s.

population examined from the Pra-Ofin basin. The following different inversions were also recorded: IS-A, IS-21, IIL-7, occurred as either fixed or polymorphic in one population or another, with the floating inversions occurring at different frequencies in different populations.(see Table 9-13).

On river Ofin, the *S. sanctipauli* s.s. population at Assin Asaman had both inversions IS-A and IIL-7 being polymorphic and at a high proportion of inversion frequencies of 0.92 and 0.98 respectively. Inversion 1S-21 was at a relatively moderate frequency of 0.50 at Assin Asaman. At Pokukrom the inversions IS-A, and IIL-7 were fixed. The inversion 1S-21, was at a relatively low frequency of 0.333 (see Table 14). Sex-determination was found associated with inversion III L-B. More than 92% of the males were heterozygous whilst more than 90% of the females were homozygous for the inversion.

The *S. sanctipauli* s.s. populations found at Sekyere-Heman (River Pra) also had different inversions frequencies. The inversion 1S-A had a high proportion of inversion frequency of 0.68, but 1S-21 occurred at a low inversion frequency 0.222. Inversion IIL-7 was found to be fixed. Also at Awisam, the population had a very high proportion of both inversion 1S-A (0.928) and IIL-7 (0.982), with inversion 1S-21 at relatively lower frequency (0.464). In these populations, sex-determination was also associated with the inversion III L-B. At least 73.3% of the males were heterozygous for the inversion whilst almost all females (91.8%) were homozygous for IIIL-B.

On river Anum, the population found at Jankobaa had inversion IIL-7 as fixed, inversion 1S-A occurred at a high frequency of 0.75 but inversion 1S-21 had a low proportion of inversion frequency of 0.25. In these populations, sex-

determination was also associated with the inversion III L-B. More than 85% of the males were heterozygous for the inversion whilst more than 90% of the females were found to be homozygous for this inversion. At Anwiaso Jumaku the only *S. sanctipauli* s.s. identified had a homozygous inversion for 1S-A, IIL-7 and IIIL-B, however, the inversion IS-21 was not present.

5.4.3 SEASONAL VARIATION

On river Pra at Sekyere-Heman, all the samples collected regularly throughout the twelve months of the year were identified as S. *sanctipauli* s.s. Samples examined were found to possess the diagnostic inversion IIL-A. The inversions IIL-6.4.7 and IIIL-4.17.2 were present as fixed inversions. The different floating inversions recorded were IS-A, IS-21 IIL-7 and IIIL-B. (See Table 14).

During the dry season, the proportion of inversion IS-A observed in the *S. sanctipauli* s.s. was high (0.61), there was also a high proportion of the inversion IIIL-B (0.733) whilst inversion IS-21 was at a low frequency (0.201). During the wet season, there was a high proportion of inversion IS-A (0.671), and IIIL-B (0.701), with inversion IS-21 occurring at a low frequency. (0.223). Using Chi-square, there was no significant difference found between the inversion frequencies of the various polymorphic inversions in both dry and wet seasons. Also sex-linkage was not found to be associated with the inversions IS-A or IS-21. In this population, sex-determination was associated with the

inversion IIIL-B, 73.3% of the males were heterozygous for the inversion whilst almost all the females (91.8%) were homozygous for the inversion IIIL-B.

Table 9 The frequencies of inversion IS-A observed in *S. sanctipauli* s.s. at the various sites on rivers Tano, Pra and Ofin in south-west Ghana.

RIVERS	SITES	COORD-INATES	DATE	1S-A						SAMPL SIZE
				A\A		ot\A		ot\ot		
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	10	11	1	3	0	0	25
OFIN	POKUKROM	01°49'W 06°01'N	13\1\96	2	1	0	0	0	0	3
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	8	7	3	1	2	2	25
PRA	AWISAM	01°32'W 05°52'N	13\1\96	12	12	2	2	0	0	28
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	1	0	0	0	0	0	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	1	1	1	1	0	0	4
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	14	11	0	0	0	0	25
TANO	BOPA	02°36'W 06°04'N	10\12\95	12	13	0	0	0	0	25
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	10	8	3	1	1	2	25
TANO	SUTRI RAPIDS	02°38'W 05°23'N	9\12\95	1	0	2	2	8	12	25
Sex				F	M	F	M	F	M	

(M) Male of *S. sanctipauli* (F) Female of *S. sanctipauli*

Table 10 The frequencies of inversion IS-21 observed in *S. sanctipauli* s.s. at the various sites on Rivers Tano, Pra and Ofin in South-West Ghana.

RIVERS	SITES	COORD-INATES	DATE	IS-21						SAMPLE SIZE
				2\21		ot\21		ot\ot		
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	2	1	8	7	2	5	25
OFIN	POKUKROM	01°49'W 06°01'N	13\1\96	0	1	0	0	1	1	3
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	0	0	6	5	6	8	25
PRA	AWISAM	01°32'W 05°52'N	13\1\96	3	3	6	8	5	3	28
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	0	0	0	0	0	0	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	0	0	1	1	1	1	4
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	0	0	11	14	0	0	25
TANO	BOPA	02°36'W 06°04'N	10\12\95	0	0	7	5	5	8	25
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	2	1	10	12	0	0	25
TANO	SUTRI RAPIDS	02°38'W 05°23'N	9\12\95	6	4	8	7	0	0	25
Sex				F	M	F	M	F	M	

(M) Male of *S. sanctipauli* (F) Female of *S. sanctipauli*

Table 11 The frequencies of inversion IS-25 observed in *S.sanctipauli* s.s. at the various sites on Rivers Tano, Pra and Ofin in South-West Ghana.

RIVERS	SITES	COORD-INATES	DATE	IS-25						SAMPLE SIZE
				25\25		ot\25		ot\ot		
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	0	0	0	0	12	13	25
OFIN	POKUKROM	01°49'W 06°01'N	13\1\96	0	0	0	0	1	2	3
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	0	0	0	0	13	12	25
PRA	AWISAM	01°32'W 05°52'N	13\1\96	0	0	0	0	13	15	28
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	0	0	0	0	1	0	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	0	0	0	0	2	2	4
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	0	0	0	0	10	15	25
TANO	BOPA	02°36'W 06°04'N	10\12\95	0	0	0	0	12	13	25
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	8	3	4	5	1	2	25
Sex				F	M	F	M	F	M	

(M) Male of *S. sanctipauli* (F) Female of *S. sanctipauli*

Table 12 The frequencies of inversion IIL-7 observed in *S. sanctipauli* s.s. at the various sites on rivers Tano, Pra and Ofin in South-West Ghana.

RIVERS	SITES	COORD-INATES	DATE	IIL-7						SAMPLE SIZE
				7\7		ot\7		ot\ot		
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	10	11	1	3	0	0	25
OFIN	POKUKRM	01°49'W 06°01'N	13\1\96	2	1	0	0	0	0	3
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	8	9	3	1	2	2	25
PRA	AWISAM	01°32'W 05°52'N	13\1\96	12	12	2	2	0	0	28
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	1	0	0	0	0	0	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	2	2	0	0	0	0	4
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	8	9	5	2	0	1	25
TANO	BOPA	02°36'W 06°04'N	10\12\95	10	10	3	2	0	0	25
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	7	12	5	1	0	0	25
TANO	SUTRI RAPIDS	02°38'W 05°23'N	9\12\95	13	10	0	0	1	1	25
Sex				F	M	F	M	F	M	

(M) Male of *S. sanctipauli* (F) Female of *S. sanctipauli*

Table 13 The frequencies of inversion IIIL-B observed in *S. sanctipauli* s.s. at the various sites on rivers Tano, Pra and Ofin in south-west Ghana.

RIVERS	SITES	COORD-INATES	DATE	IIIL-B						SAMPLE SIZE
				B\B		ot\B		ot\ot		
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	10	2	1	11	0	1	25
OFIN	POKUKROM	01°49'W 06°01'N	13\1\96	2	1	0	0	0	0	3
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	10	2	1	12	0	0	25
PRA	AWISAM	01°32'W 05°52'N	13\1\96	12	1	1	13	0	1	28
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	1	0	0	0	0	0	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	1	0	0	3	0	0	4
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	10	2	1	12	0	0	25
TANO	BOPA	02°36'W 06°04'N	10\12\95	10	2	1	12	0	0	25
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	11	2	1	11	0	0	25
TANO	SUTRI RAPIDS	02°38'W 05°23'N	9\12\95	12	1	1	11	0	0	25
Sex				F	M	F	M	F	M	

(M) Male of *S. sanctipauli* (F) Female of *S. sanctipauli*

TABLE. 14 The Summary of inversion frequencies observed in *S. sanctipauli* s.s. populations at the various sites on rivers Tano, Pra and Ofin in south-west Ghana.

RIVERS	SITES	COORD-INATES	DATE	1S-A	1S-21	1S-25	II L-7	III L-B
OFIN	ASSIN ASAMAN	01°30'W 05°54'N	12\2\96	0.920	0.50	0.00	0.98	0.72
OFIN	POKUKROM	01°49'W 06°01'N	13\1\96	1	0.333	0.00	1	1
PRA	SEKYERE HEMAN	01°35'W 05°11'N	4\12\95	0.68	0 222	0.00	1	0.74
PRA	AWISAM	01°32'W 05°52'N	13\1\96	0.928	0 464	0.00	0.982	0.714
ANUM	ANWIASO JUMAKU	01°16'W 06°18'N	11\1\96	1	0	0.00	1	1
ANUM	JANKOBAA	01°16'W 06°20'N	11\1\96	0.75	0 25	0.00	1	0.625
TANO	NSAWORA	02°36'W 06°09'N	23\2\96	1	0 280	0.00	0.82	0.72
TANO	BOPA	02°36'W 06°04'N	10\12\95	1	0 238	0.00	0.90	0.74
TANO	RURU RAPIDS	02°34'W 06°00'N	9\2\96	0.80	0 20	0.00	0.90	0.76
TANO	SUTRI RAPIDS	02°38'W 05°23'N	9\12\95	0.176	0 312	0.56	0.92	0.70

5. 5 DISCUSSION

The populations of *Simulium damnosum* s.l. identified from the major rivers in south-western Ghana (rivers Tano, Ofin and Pra) consist of pure populations of *S. sanctipauli* s.s., and mixed populations of *S. damnosum* s.s., *S. yahense*, *S. sirbanum*, *S. squamosum* and *S. sanctipauli* s.s. Pure populations of *Simulium sanctipauli* s.s. were found in the following breeding sites, Sekyere-Heman on R. Pra, Sutri rapids, Ruru Rapids, Bopa and Nsawora on R. Tano.

Of all the *S. sanctipauli* s.s. populations examined, the frequencies of the floating inversions in the various populations were different. With the exception of *S. sanctipauli* s.s. population at Pekukrom, the rest of the populations of *S. sanctipauli* s.s. on Rivers Pra, Ofin and Tano that were examined, showed a distinct sex-determining system related to the inversion IIIL-B. This confirms the observation made by Boakye *et al.*(in press). Sex- chromosome differentiation is considered to be important in population sub- structuring leading to speciation (Procunier, 1982b). Closely related members differ only by sex chromosomes. Another important observation made in this study, was the relationship found between the sex chromosomes and the inversion IIL-7. The *S. sanctipauli* s.s. population at Ruru Rapids and Nsawora had dominant number of females heterozygous for the inversion IIL-7, this was consistent across the samples. However, the occurrence of the heterozygosity was not very high, therefore sex-linkage could not be ascertained. It must be emphasized that whenever the heterozygous condition was observed in males, it was found present only in the population with sex-linkage to IIIL-B, a probable indication that IIL-7 heterozygous is related to sex-linkage.

Full karyotyping of all the samples has shown that two cytologically distinct populations of S. *sanctipauli* exist in south west Ghana. The Sutri Rapid population is cytologically distinct from the other populations obtained from the various sites in the major rivers in south-western Ghana. This is reflected in the proportion of the various inversions (see Tables 9-13).

The Sutri Rapid population also has a unique floating inversion, IS-25, which occurs at high frequencies. This is a newly identified inversion which has never been recorded in the *S. sanctipauli* s.s. populations. This population also had an extremely low frequency of inversion IS-A, and has the highest percentage of the population lacking inversion IIL-7.

Within the other population at the River Tano, there were differences with regard to the proportion of the floating inversions. Also the *S. sanctipauli* s.s. population found on River Ofin is though not cytologically distinct from the River Pra population, the proportions of their floating inversions were different.

On the River Tano, the differences in the frequencies of the floating inversions show a unique pattern. The populations from the different sites showed a south-to-north directional increase in the proportion of inversion frequencies of IS-A and IS-21, except in the cases of inversion IS-21 at Sutri rapids (see Table14). Furthermore, there was also a south-to-north directional decrease in the frequencies of the inversions IIL-7 and IIIL-B, (with the exception of Sutri rapid population breaking the trend again in the case of IIIL-B).

A close similarity in the frequencies of both fixed and polymorphic inversions have been observed in two populations from two different major

rivers. The *S. sanctipauli* s.s. population in Assin Asaman on River Ofin and Awisam on river Pra are closely related in the proportions of frequencies of both fixed and polymorphic inversions. This is probably due to the fact that these two sites on the two rivers are very close to each other, about five kilometers apart, and hence the likelihood of each population migrating to the other sites. Moreover, of all the Pra population of *S. sanctipauli* s.s. observed, the Awisam population is the only one where inversion II L-7 was polymorphic and showed the same inversion frequency like the population at Assin Asaman.

In all the populations examined, only inversion IS-21 was found floating in all the populations, with the proportion of the inversion showing much variation between the different sites of the same cytotype.

On Pra river, Sekyere-Heman, though the frequencies of inversions varied between collections, these frequencies did not show any significant difference in the inversion frequencies and hence there is no significant seasonal difference between the dry and wet season.

CHAPTER SIX

GENERAL DISCUSSION

The devastating impact of *Simulium damnosum* species complex in transmitting *Onchocerca vovulus*, the filaria causing river-blindness (onchocerciasis) has precipitated the largest vector-control programme ever attempted (Walsh *et al.*, 1981). This long-term disease control in West Africa relies on regular treatment of *Simulium* infested rivers using six different larvicides including *Bacillus thuringiensis* serotype (H-14) (WHO, 1995). To enhance effective control strategies and for classic genetic experiments to be conducted on them, there is the need for laboratory colonisation of *S. damnosum* s.l., to provide samples in the laboratory for research, identification of specific cytotypes causing the disease, to provide knowledge on the vectorial role of the various species of *S. damnosum* s.l. and their susceptibility to insecticides. These among others are important and crucial research needs, if better management of the vectors of onchocerciasis is to be achieved.

Laboratory colonisation of insects has proved to be an important tool for scientific research, in that, it enables experiments to be conducted with them under controlled conditions and also makes materials available when field collections are not possible. Even when field collections are possible, high mortality rates of captive *S. damnosum* s.l. flies can rob researchers of any advantage if distances to nearby sites are far. The results obtained have clearly demonstrated that using the long tube

the majority of flies can survive for more than ten days at room temperature. In fact 95% of flies kept at room temperature survive the fourth day. The significance of this is that most gravid female flies can be maintained at room temperatures thus overcoming the limitations of slow egg maturation in flies that are maintained in cold storage. The long term survival of flies at room temperatures also opens up the possibility of carrying out filial crosses and backcrosses of reared flies in the laboratory. This can be achieved if unmated males and females from the same egg batch are maintained long enough till the next generation reaches the adult stage for crosses to be carried out. From experience it can be estimated that a period of 15-17 days is needed to completion of a generation starting from bloodfeeding adult flies, through oviposition inducement, to rearing adults from the eggs. From the results obtained, 30% of flies (at least females) should survive till then if kept in the long tubes. However, it is not known whether unmated females can also survive that well in captivity and this needs to be investigated. Before the present study, other possibilities existed for maintaining developmental stages of *S. damnosum* s.l. long enough to enable such crosses to be made in the laboratory. Raybould and Grunewald (1975) maintained eggs at 1-4°C for up to 1 month and found that survival was 80% after one week and 20% after a month. The other possibility was arresting the pupal stage such that rearing of parental stock and filials can be synchronised (Boakye and Roberts, 1988). However as demonstrated by the authors, only a maximum of 10% of emergence was observed when pupae were exposed at either 4°C or 12°C. Moreover it is not even known if adults reared from such pupae will be fit and/or will mate in captivity. Since the main focus of the present study was towards laboratory

colonisation of *S. damnosum* s.l. the much enhanced survivorship at room temperatures will enable normal development of eggs in flies.

The 50% insemination of female *S. damnosum* s.l. obtained in the present study is about the minimum rates for large mating cages that were used by Wenk and Raybould (1972). It is worth noting that the size of the mating cage used in the present study was smaller than those used by these authors and that the flies were kept in cages for 24 instead of 48 hours. This period was chosen because it was found earlier in the study that mortalities increased dramatically in the mating cages after 24 hours. Wenk and Raybould (1972) observed that increasing the proportion of males to females in the mating chamber resulted in a higher percentage of inseminated females. Thus it is possible that 2-3 times of males over females might result in higher rates of mating. However, Cupp *et al.* (1981) were successful in rearing a complete generation, with an insemination rate of less than 1 % with flies which were most likely *S. damnosum* s.str. For the establishment of permanent colonies it is important that mating rates between filial populations and flies of different generations be investigated, but this was not carried out in the present study.

For the oviposition inducement studies, the results obtained clearly show that the drip method for inducing egg laying is much better than the water immersion method in all the aspects of the parameters that were measured. With the drip method it took less time to induce oviposition, a higher proportion of eggs were laid and a greater proportion of the laid eggs developed. It is also clear that with the drip method processing single flies, including the use of the pheromone extract, it takes less time to induce egg laying than the system that simulated mass oviposition. From the results obtained it seems that it was the dripping effect that improved the

efficiency of oviposition and fertility of captive gravid flies. Although on the whole it took relatively less time to induce egg laying when the pheromone extract was used, its significance was not demonstrated when compared with the control (P = 0.608) and with the drip single (P = 0.376). McCall *et al.* (1994) in a two choice bioassay have demonstrated that the presence of eggs significantly reduced the time to oviposition of gravid flies. Similar observation was made with volatiles collected from freshly laid eggs (McCall, 1995). The results obtained in the present study do not invalidate these findings because the design of the experiments did not set out to confirm them. To achieve that, pheromone extracts should have been used with grouped flies also and one oviposition chamber and netting should have been used to process several flies individually. The findings that, grouped flies took significantly longer time to oviposit than single flies might be due to the smaller space of the ovipostion chamber. It was observed that flies usually took longer time to settle and usually took a longer time for the first fly to start oviposition, but once the first fly starts to oviposit, it normally takes a much shorter time for the rest to complete oviposition. On the other hand, the smaller volume used for the oviposition chamber might be an advantage in that by reducing the space for agitated behaviour of introduced flies, flies are quick to move towards the light source and getting wet in the process. This reasoning might account for the 100% success response to oviposition compared to that of 22.9% obtained by McCall *et al.* (1994).

With the development of the drip system, enhancing higher proportion of gravid female flies ovipositing, and with increased egg oviposition and fertility rate, and also with the development of effective but small sized rearing system, these

improved systems produce better results and will increase the potential of permanent colonisation of *S. damnosum* s.l.

The temephos susceptibility tests carried out on *S. sanctipauli* s.s. populations on River Pra, Sekyere-Heman, revealed that they were susceptible both in the dry and wet seasons. The LC_{95} was found to be 0.739mg/l compared to the OCP diagnostic dose of 0.625 mg/l. The *S. sanctipauli* s.s populations from Sutri Rapids, River Tano were however, found to be resistant to temephos with an LC_{95} of 1.745mg/l, about two and half times higher that of the diagnostic dose for temephos.

All the *S. sanctipauli* s.l. sampled in south-west Ghana were identified as *S. sanctipauli* s.s. with the diagnostic inversion IIL-A (Post 1996 and Boakye 1993). The chromosomal analysis revealed that the frequency of occurrence of the floating inversions, IS-A, IS-21, IIL-7 and IIIL-B in the *S. sanctipauli* s.s populations of River Tano and River Pra-Ofm were different. Also a new chromosomal inversion, IS-25 was identified and thus named. This inversion which is unique to Sutri Rapids, occurs as a floating inversion with an inversion frequency of 0.56. Thus, two distinct populations of *S. sanctipauli* s.s were revealed, the population at Sutri Rapids and that of the rest of the population of south-west Ghana. Also in all the samples of *S. sanctipauli* s.s identified in the study area, sex-determination was found to be associated with the inversion IIIL-B. The author further speculates that there is the likelihood of sex-linkage of inversion IIL-7 at Ruru Rapids and Nsawora, however, because the 25 specimens worked on were not enough, the results could therefore not be authenticated. In all the identifications made, more than 85% of the males were found to be heterozygous for the inversion, while more than 93% of the females were homozygous for the inversion.

At the Sutri Rapids, where resistance to temephos insecticides was detected, a comparison of the chromosomal inversions of the general population at Sutri Rapids and the survivors of the insecticide susceptibility test shows no significant difference in the various inversions and the frequencies of inversions. That is, no inversion or inversion frequencies were found to be responsible for resistance to temephos insecticides in *S. sanctipauli* s.s.

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