PRELIMINARY STUDIES ON *CARDIOCLADIUS OLIFFI* (DIPTERA: CHIRONOMIDAE) AS A POTENTIAL BIOLOGICAL CONTROL AGENT OF *SIMULIUM DAMNOSUM* S.L. (DIPTERA: SIMULIIDAE) VECTORS OF ONCHOCERCIASIS IN WEST AFRICA

By

FOKAM ERIC BERTRAND (B. Sc. ZOOLOGY)

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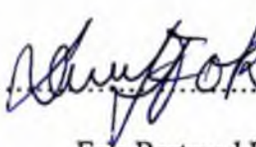
DECLARATION

I do hereby declare that the experimental work described in this dissertation was carried out by me and that except for references to other people's works that have been duly acknowledged, this thesis either in whole or in part has not been presented for any other degree elsewhere.

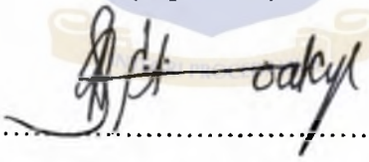


Eric Bertrand Fokam

(Candidate)

Dr. Michael D. Wilson

(Supervisor)



Dr. Daniel A. Boakye

(Supervisor)

DEDICATION

To my parents Takeugne Marcel, Tasse Pierre, Massad Genevieve and Tasse Rose Charlotte; my brothers and sisters: Louis, Lazare, Henri, Caroline, Rolin, Jules, Edmond, Leonie, Geraud, Nathalie, and my late friend Nsahlai Asife David

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LIST OF ABBREVIATIONS

Ag NO3	Silver nitrate
bp	base pair
dATP	deoxyadenosine triphosphate
ddw	double-distilled water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	Disodium ethylene diamine tetraacetate. $2H_2O$
EtBr	Ethidium bromide
EtOH	Ethanol
H_2O	Water
KAc	Potassium acetate
kb	kilobase
KOH	Potassium hydroxide
M	Molar
Mw	molecular weight
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
pH	Hydrogen-ion exponent
RNA	Ribonucleic acid

RNase	ribonuclease
rpm	revolution per minute
sddw	sterile double distilled water
s.l.	Sensu lato
s.s	Sensu stricto
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethyl ethylene diamine
Tm	melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3 propanediol
µl	microlitre
µM	micromolar

ABSTRACT

In the course of experiments aimed towards the laboratory colonization of *Simulium damnosum* sensu lato, egg masses of the flies were collected from the field and reared to adults. However, it was observed that with the samples obtained from Boti Falls (River Pawnpawn), the immature stages of *S. damnosum* were attacked and fed upon by some larvae belonging to the family Chironomidae (Diptera).

The presence of two different species of chironomid was suspected because of the occurrence of two peaks of emergence of adult flies from the rearing systems. The chironomid flies were subsequently identified as *Cardiocladius oliffi* Freeman, 1956 and *Cricotopus kisantuensis* Goetghebuer, 1934, by morphological means, but *C. oliffi* was incriminated as the predator. This is the first report of the genus *Cardiocladius* in West Africa. *C. oliffi* was then reared together with *S. damnosum* to quantify predation.

These predators caused high mortality among *S. damnosum* larvae as a result of disturbances, but the actual predation was on immobile pupae. It was observed that even at a ratio of one *C. oliffi* to five *S. damnosum*, the predators cause significant decrease in the number of adult *S. damnosum* emerging from the systems (treatments) as compared to the control (that had no *C. oliffi* present).

The polymerase chain reaction was used to positively incriminate *C. oliffi* as the predator of *S. damnosum.* Three sets of primers namely 16S, ND4 and P1-P2 were used to amplify DNA extracted from the *Simulium*-fed *C. oliffi,* but the sizes of the PCR products did not differ appreciably to separate by gel elctrophoresis. Then restriction analysis of the PCR products was carried out which resulted in different DNA band profiles for *S. damnosum* and *C. Oliffi.* However, the study also established that the chironomid flies could also develop successfully in the system on a fish food diet only.

We have not been able to understand the role of *C. kisantuensis,* whose egg masses are also attached to the egg masses of *S. damnosum.*

Chapter 1: Introduction

1.1 General Introduction

Human onchocerciasis is a filarial disease caused by the development in the human dermis of the filarial worm *Onchocerca volvulus* (Leuckart, 1893). *O. volvulus* is a parasitic worm that is "viviparous" and whose females for the greater part of their life emit embryos or microfilariae into the dermal tissue. These microfilariae cause itching and skin lesions. They may invade the eye, causing severe disorders culminating in blindness (Anonymous, 1995). Onchocerciasis commonly called "river blindness" is a severely debilitating blinding disease. It is the second cause of infectious blindness worldwide after trachomatis, and the second most devastating infectious disease in terms of economic consequences to afflicted communities after poliomyelitis (WHO, 1987). In affected communities, the mortality rate among blind people over thirty years of age is three to four times higher than that for sighted ones of the same age group (Prost and Vaugelade, 1981). Current estimations suggest that about 17.7 million persons are infected with the disease, of which some 270,000 are blind. Some 500,000 more are severely visually disabled (WHO, 1995). In Ghana, the data provided by the Onchocerciasis Control Programme (OCP) in West Africa reveals that some 123,000 people have been infected, out of which 7,400 are blind (WHO, 1995).

Onchocerciasis is a vector borne disease and is transmitted by the black flies of the genus *Simulium* (Diptera: Simuliidae). The disease is confined to the tropics because the climate and the behaviour of the vectors limit its transmission. The disease mainly affects small, isolated and remote rural communities and contributes to destroying of the already precarious balance of their subsistence economy. The decline in communities' living conditions is one of the factors responsible for villagers leaving the riverside lands and retreating to areas between rivers. Hectares of fertile land that could have been exploited in agriculture and livestock production to sustain the rural communities are therefore abandoned. While onchocerciasis is not always the initial cause of depopulation of valleys, with other disease such as trypanosomiasis and other factors playing a role, it nevertheless represents nowadays the only major obstacle to settlement in and development of valleys (WHO, 1995). Onchocerciasis control is an essential prerequisite both for bringing a very serious health situation to an end and for guaranteeing the success of development operations by freeing the valley abandoned because of the disease. The need for freeing the infested regions and assuring economic recovery of the abandoned regions was at the origin of the set up of the Onchocerciasis Control Programme in West Africa in 1974 by the WHO (Anonymous, 1995). This has appeared as the greatest vector control programme in the world (Anonymous, 1999a). This programme in its initial phase targeted the vectors of the disease in their most vulnerable stage (larvae) by the weekly application of insecticides in breeding sites in river. Later on mass chemotherapy became feasible because ivermectin a good filaricide was developed. Since 1984 both methods have been combined to control onchocerciasis. The control activities have permitted to free over 25 million hectares of land from the disease (Benton

and Skinner, 1990) and these are ready for resettlement of populations and agriculture and should enable the feeding of about 10 million people (Anonymous, 1995). The OCP operations will end in December of the year 2002 and the monitoring activities will be transferred to the participating countries that are expected to ensure that recrudescence does not occur.

However, two phenomena, re-invasion and biting nuisance of the flies pose a threat to the repopulation of oncho-freed zones. It has been known since the early 80s that the vectors, especially the savanna species could fly over tens of kilometres (at times more than 80 kilometres a day) along river-courses (Kassambara, 1987). This migration that is always in the direction of trade winds was responsible for the re-invasion (Traore and Hebrard, 1983) of the frontiers of the OCP's operation area that had been freed of the disease following control operations. The OCP activities did not cover Southern Ghana where savanna flies are known to occur (Wilson, 1999). It is therefore obvious that the Oncho-freed areas of Ghana are potentially re-invadable by the black flies carrying infections from untreated areas.

Members of the *S. damnosum* species complex are avid biters and apart from their importance in transmitting the parasite *O. volvulus*, can be a serious source of nuisance. The socio-economic importance of the disease has been well documented but that of biting nuisance due to the fly has not been that well studied especially that of its effect on productivity of afflicted communities. This has come about because of the importance given to its debilitating symptoms and blindness that result from chronic infections in humans. Thus, the Onchocerciasis Control Programme in West Africa (OCP) was

initiated to control blinding onchocerciasis only, until the disease no longer poses a public health problem. However, because of the high rates of blindness associated with onchocercal infections in savanna areas, only these geographical zones were included in its area of operations. Therefore, the southern areas of Ghana and Côte d'Ivoire for example that are endemic were excluded, because the disease in the forest area is associated with severe skin lesions but blindness is rare.

The magnitude of biting nuisance due to black fly in several communities in West Africa especially during the rainy season has long been recognized. In the early 1970s for example, it was so bad that in communities situated in the Asukawkaw and Menu river basins in Ghana children were obliged to wear trousers and long sleeve shirts to school. The situation improved when these rivers were included in the OCP theatre of operations that used aerial application of insecticides against *S. damnosum* larvae. Prior to the construction of the Akosombo dam and until the completion of the Kpong dam, the Akosombo Township and its surrounding villages were always at the mercy of the black flies, which were breeding in the Senchi Rapids. This forced the Volta River Authority (the owner of the hydro-electric projects) to embark on regular treatment of the river to control the flies and to protect its workers from the disease. The nuisance caused by the flies and the control activities ceased only after the resulting lakes inundated all the nearby breeding sites. Quite recently, incessant biting by black flies along the major rivers were reported in several communities throughout Ghana prompting the Ministry of Health (MOH) and the Noguchi Memorial Institute for Medical Research (NMIMR) to form a task force to study the situation and make recommendations for control

(Anonymous, 1999b). The flies even when free of infection constitute serious biting nuisance, as black flies are known as notorious nuisance pests (Crosskey, 1990). In areas of major developmental projects, these incessant bites could pose a serious threat to economic success (Jamnback, 1969). In Cote d'Ivoire, the nuisance around the Lereba, Bou and Foumbou Rivers is such that farmers can only work between 10.00h and 15.30h. In addition, this forces the farmers to work during the hottest periods of the day. In Ghana, the picture is quite similar as the National Disaster Management Organization (NADMO) reported increased *S. damnosum* biting nuisance over many areas during the raining season of 1999, with possible impact on economic activities including tourism (Anonymous, 1999b).

Twenty-six years on, the OCP still uses insecticides to control the flies, although it has recently adopted chemotherapy (using the drug ivermectin) to complement its efforts. The Programme has successfully controlled the disease such that for most of the original central area transmission was reduced to almost zero and prevalence of onchocerciasis also reduced from 70% to about 3% (WHO, 1995). Successful control has also led to re-population and increased economic activities in hitherto deserted fertile river valleys estimated at about 25 millions hectares of land, which potentially can feed about 10 million people (Benton and Skinner, 1990). OCP's operations end in 2002, at which time it is expected that the parasite reservoir will have been eliminated from most of the OCP area, including the extension zones. Thereafter, the 11 OCP participating countries are expected to maintain OCP's achievements and avoid recrudescence of the disease in controlled areas. However, one major phenomenon associated with *S. damnosum* fly populations, that of long distance

migration of mostly old flies originating from sources outside could lead to re-infestation and therefore, recrudescence of the disease in the controlled zone. In addition, biting nuisance could result in desertion of the river valleys by people who have come to know of the association of disease with the flies and the majority of whom have not experienced the nuisance on any scale.

It is therefore very obvious that the various countries would need to embark on some anti-black fly activities using insecticides to control the nuisance and the disease during the post- OCP era. However, the use of synthetic insecticides has many disadvantages including its usage and application by semi skilled and untrained persons. Synthetic insecticides are not cheap and they will also be very expensive since they would have to be used over a wide area and it is doubtful if their usage could be supported by the economies of the countries. Furthermore, the most important consideration would have to be the possible development of widespread resistance in the vectors to most insecticides. This particular problem is managed in the OCP through rotational use of different insecticides, which would also be beyond the capacities of the economies of the countries. Yet to do nothing would be disastrous in the long term. The morale and productivity of afflicted communities and of workers of large agro-industrial concerns would suffer; so would recrudescence of the disease undo the achievements of the OCP, although the later could be managed through mass distribution of ivermectin.

Rather unfortunately, no other effective alternative to the usage of insecticide to control black flies is currently available and if insecticides are to be used, then biological

insecticides such as *Bacillus thuringiensis* serotype H-14 (Trade name Vectobac®) and or use of natural predators are ideal. However *Bt* H14 is expensive and does not have a longer carry as the organophosphorus insecticide Abate® (temephos EC 20) the choice insecticide of OCP, which is cheaper and has a longer carry up to 20 kilometres and may be as long as 40 km in medium-sized rivers. Abate® is preferred because of its efficacy against *S. damnosum* s.l., its low toxicity and its relatively benign influence on non-target organisms, but VectoBac® has more because of its specificity against Diptera and Lepidoptera larvae including black fly and mosquito larvae. The other limitation of VectoBac® is its inconsistency in potency and efficacy, which is to be expected because no two different batches, can be produced under exactly the same conditions of fermentation. Scientists at Abbott Laboratories have achieved considerable improvement on the performance of VectoBac® since it was introduced in the OCP in mid 1980s, but more needs to be done to the product if the attributes of Abate® are to be reached and be affordable by the countries in West Africa. The need for control measures that are cheap in the long run, self-sustaining and self-propagating arises. This can only be achieved through biological control.

In the course of preliminary work that would have led subsequently to the establishment of laboratory colonies of the vectors of onchocerciasis *Simulium damnosum*, it was observed that egg batches collected from the field harboured some organisms that attacked *Simulium* larvae and pupae in the rearing system such that no adult flies were obtained in most cases. For example, from rearing systems, which at a point in time contained 69 and 111 larvae *S. damnosum* respectively with a number of chironomids, we

obtained 8.69% and 1.80% adult *S. damnosum* only. In addition to this, observation made in the field indicated that black flies were being controlled naturally on the River Pawnpawn on its sections that included Boti Falls and Huhunya where the egg masses were obtained. These sections of the river have numerous sites that are suitable for breeding *S. damnosum*. However, the inhabitants do not complain of biting nuisance. Moreover, trial catches mounted by teams of the NMIMR yielded very few flies (6 flies caught by 4 collectors in 3 hours), unlike on similar exercises on other rivers such as river Pra in Western Ghana or at Tsatsadu Falls in the Volta Region.

Following these observations, it was proposed to investigate the potential of the predator-organisms (which were later identified as chironomids) as biological control agents for *Simulium damnosum* by first establishing that *Simulium* was fed upon by the chironomids.

1.2 Objectives

The overall objective of this work was to evaluate the predation of these chironomids on black flies in order to provide preliminary data on the potential of their use as biological control agents of *S. damnosum* s.l.

The specific objectives were:

- To identify and provide sufficient taxonomic description of the chironomids that were found preying on *S. damnosum* in the laboratory;

  To detect *Simulium* material in the predator and thereby proving that these chironomids effectively feed on black flies;

  And to conduct some laboratory experiments to determine the predatory capacity of the identified chironomids on black flies populations.

Chapter 2: Review of Literature

2.1 Onchocerciasis: Symptomatology

Onchocerciasis commonly called "river blindness" was first described from three humans in Gold Coast (Leuckart, 1893). The main clinical manifestations of the disease are onchocercal nodules, skin lesions, ocular lesions and lymphadenopathy (Duke, 1990).

The nodules harbour the adult worms and when visible at the surface are found essentially over bony prominence on the head, shoulder, girdle, rib, trochanter, knee and ankles. The nodules are little medical importance, but when on the head they may erode the bone (Muller, 1975; Manson-Bahr and Apted, 1982; Duke, 1990).

The skin infection symptoms include pruritus, thickening (see Figure 1b) owing to sub-cutaneous oedema that leads to the "peau d' orange" effect. Changes in pigmentation further lead to the symptom commonly described as the "lizard or elephant skin". At this stage, the scarring of the dermal tissue are so extreme that little or no clinical improvement can be expected to follow even the most effective filaricidal treatment. A few microfilariae may invade the cornea in the eye.

The response to the local presence or local microfilariae death produces fluffy opacity in early and late infection. The symptoms at this stage are irritation, lacrimation and photophobia. Heavier and chronic infestation lead to sclerosing keratitis in the cornea and this is largely responsible for the exceptionally high onchocercal blindness (see Figure 1a) in the Sudan-Guinea savannah zones of West Africa. Inflammation of the iris and the ciliary body of the eye with secondary glaucoma are also a major cause of blindness. Progressive blindness takes place because the pulpit becomes occulted with attendant secondary glaucoma or cataract (Anderson and Fulgsang, 1977; Duke, 1990). There is however differences in lesions, which are more acute in savannah than in rain forest areas.

In heavy microfilarial infestations, the lymphatic system is affected when some parasites get to the lymph nodes where they produce lymhadenitits with subsequent fibrosis (Duke, 1990). The inguino-femoral nodes may enlarge grossly in hanging pockets of skin ("hanging groin") predisposing the patient to hernia and sometimes accompanied by scrotal elephantiasis. Here also, the scarring is so great that only surgery can be expected to provide relief.

(a) (b)

(c)

Figure 1: Some effects of onchocerciasis on patients and communities (Pictures from OCP/APOC slides). (a) An oncho-blinded patient, (b) Skin thickening characteristic of onchocerciasis, (c) An oncho-afflicted village deserted by its inhabitants.

2.2 Distribution of Onchocerciasis in The World

It has been estimated that 122.9 million people are exposed to the disease all over the world, of which some 17.7 million suffer from it, with the great majority living in Africa (Anonymous, 1989). The disease is restricted to the tropics because of the climate and the habits of the vectors. 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. Onchocerciasis is a serious problem in the Americas, the Arabian Peninsula and Africa (WHO, 1995). In Latin America, the foci are small or medium-sized and generally well defined (Lariviere *et al.*, 1985). The disease occurs in Mexico, Venezuela, Guatemala, Brazil, Colombia, and Ecuador. The total number of infected people in Latin America is estimated at about 140,455 with some 7,500 who have either gone blind or who have had their vision impaired (Anonymous, 1989).

In the Arabian Peninsula, onchocerciasis is endemic in Southern Yemen where the number of people infected is approximately 30,000.

Africa is the area where the prevalence of the disease is the greatest with more than 95% of the total number of infested people in the world (Anonymous, 1989). The largest and most severe foci of the disease were located in the Sudano-Guinean savanna regions of West Africa before the beginning of the Onchocerciasis Control Programme (OCP). There are equally some large and some isolated foci in Central Africa and East Africa.

2.3 Social and Economic Impact of Onchocerciasis on Communities

The disease generally affects remote areas up to about 10 kilometres from the watercourses where the vector breeds. In the worst affected villages of savanna zones of West Africa, up to about 15% of whole populations (including children) and more than 40% of males of working age may be blind (Duke, 1990). In these villages, the mortality rate among blind people over 30 years of age is 3-4 times as high as among sighted persons of the same age (Prost and Vaugelade, 1981) The disease therefore indirectly reduces the mean life expectancy of a significant part of the adult population. This results in the destruction of the already precarious balance of their subsistence economy. Inhabitants of these villages desert (see Figure 1c) the fertile river valleys to higher, but less productive lands further away (Philippon, 1977). Thus, in the context of the national economies of sub-sahelian countries of West Africa, the effects of the disease are further magnified.

2.4 The Transmission of Onchocerciasis

The parasite responsible for onchocerciasis is a Nematode, *Onchocerca volvulus.* The life cycle of *Onchocerca volvulus* uses both an insect (intermediate) and human (definitive) as hosts. Figure 2 shows the life cycle of the onchocercal worm. When the black fly bites an infested human being it picks up the microfilariae. The microfilariae then penetrate the stomach walls of the insect from where they migrate to various organs (Omar and Garms, 1977), but only those that reach the thorax develop successfully. Three larval stages occur within the vector and the 3rd stage is infective, motile and migrates to the head to wait their transmission during the next blood meal (WHO, 1995). The infective larva escapes by the proboscis into the bite wound into the human host, where they moult twice without multiplication to reach the adult stage. The adult worms live in fibrous nodules and the females emit millions of microfilariae (250-300nm in length) into the dermal tissue at the frequency of 500, 000 to 1,000,000 per year. The adult worms can live up to 13 or 14 years in the definitive host.

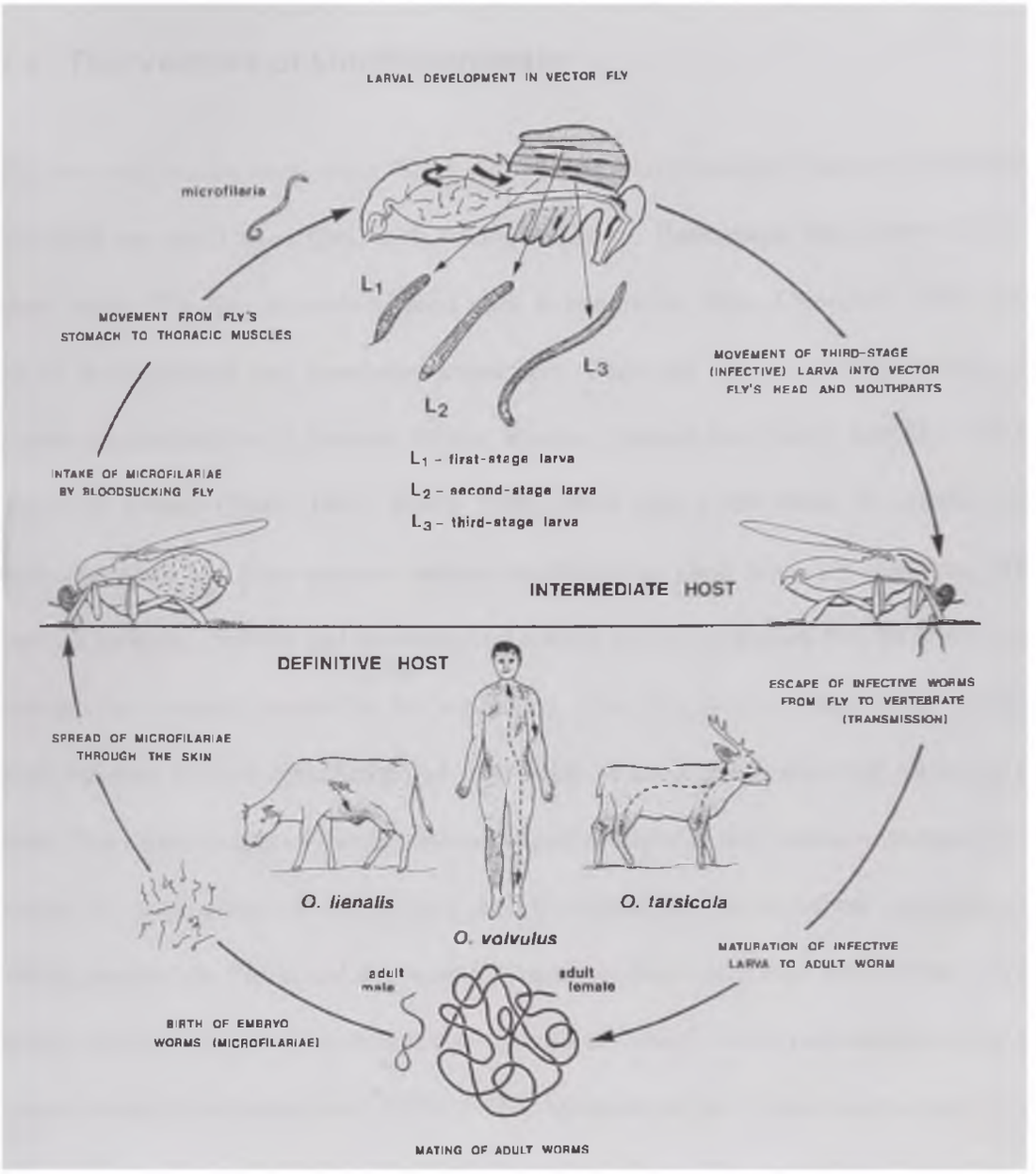


Figure 2: Life cycle and transmission of the filarial worms of the genus *Onchocerca*. The diagram shows the development of the parasite worms in the vector flies and the various vertebrate hosts they affect. After Crosskey (1990).

2.5 The Vectors of Onchocerciasis

The vector of onchocerciasis is a "black fly" of the genus *Simulium* (Diptera: Simuliidae). Simuliids are small stout flies, with a humped thorax. Both males and females feed on plant juices. The female needs a blood meal to mature her eggs (Crosskey, 1990). They are of both medical and veterinary importance. They are vectors of both human and animal onchocerciasis in tropical Africa, Mexico, Central and South America and the region of Yemen (Duke, 1990; WHO, 1995). More than 1,300 black fly species have been described and they possess various distributions. Their bites are injurious, often causing oedema, pruritus and secondary infections due to pathogens that enter the body through the wounds created by the scratching. The flies are also intolerable nuisance pests because of their abundance and their habit of landing and crawling about on the head. The main vectors of human onchocerciasis worldwide are *Simulium damnosum, S. neavei, S. onchraceum, S. metallicum,* and *S. exigum* and all constitute complexes of sibling species. In Africa and the Arabic Peninsula where more than 99% of the disease occurs, two species complexes (*Simulium damnosum and S. neavei)* are implicated in the transmission of onchocerciasis (WHO, 1995). Members of *the S. damnosum* complex are the main vectors of human onchocerciasis in Africa. This complex comprises *S. damnosum* s.s., *S. sirbanum*, *S. sudanense*, *S. dieguerense*, *S. sanctipauli* s.s., *S. soubrense, S. leonense, S. konkourense, S. squamosum, S. yahense, S. menguense* (Vajime and Dumbar, 1975; Boakye, 1993). Most members of the complex comprise both anthropophilic and non-anthropophilic forms. In East and Central Africa, the species of the *S. neavei* complex bite both man and animals (WHO, 1995); they include *S. neavei* s.s, *S. woodi* and *S. ethiopians*.

The pre-imaginal forms include the eggs, larvae and pupae. The eggs are laid in batches of 200 to 800 eggs in fast flowing waters (80-150 cm/s), coated with an adhesive, which makes them cling to varied submerged substrates 5cm below the surface of the water. Figure 3 gives the life cycle of *Simulium damnosum* s.l.

The eggs hatch in 36 to 48 hours and the larvae remain 30 cm below the water surface. The larvae feed by using their mandibular tufts indiscriminately to filter particles suspended in the water, from which they extract the nutrients as they pass through the digestive tract. Feeding is continuous. The larval life lasts 8 to 10 days and may become longer or shorter depending on the temperature. Then the larva weaves a cocoon within which it pupates. After some 2 to 4 days an adult emerges. Both sexes of adult black flies feed on juices of plants. The female fly takes a blood meal immediately after mating needing blood to develop the eggs. The eggs are laid 3 to 5 days later and less than 24 hours after oviposition, the female takes another blood meal and a new gonotrophic cycle begins. The activities of black flies slow down below 17°C and above 30°C and during strong wind and rain. They bite close to ground and a stationary person receives 95% of the bites below the waist level. Black flies do not have any preferred location and occupy the entire gallery forest. Even though they are very small, black flies are very good flyers and can travel many dozen of kilometres, up to 80 km in 24 hours, along watercourses. With the aid of the monsoon, they can even migrate for several hundred kilometres from one river basin to another during the first part of the rainy season and this behaviour is responsible for the re-invasion of the treated areas (Traore and Hebrard, 1983).

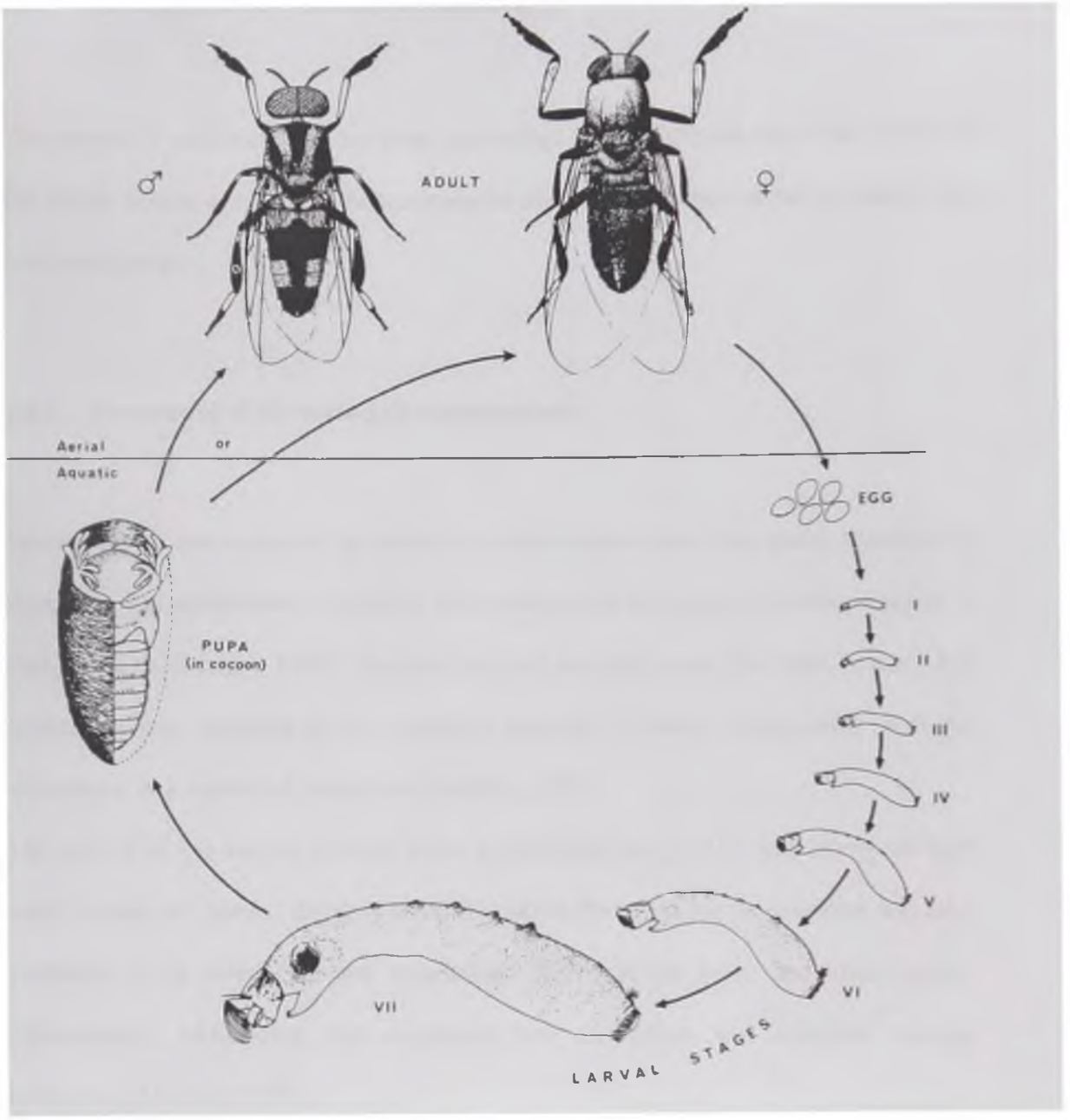


Figure 3: Life cycle of *Simulium damnosum* (Theobald, 1903) s.l. showing the aquatic and the aerial stages. After Crosskey (1990).

2.6 The Control of Onchocerciasis

The control of onchocerciasis has been approached in two different ways: the control of the vector aiming at reducing the transmission and the destruction of the parasite in the host using drugs.

2.6.1 The control of the vectors of onchocerciasis.

Several approaches to control the vectors of onchocerciasis have been made. Clearing the vegetation has successfully controlled *Simulium neavei* the vector of onchocerciasis in East Africa (McMahon, 1940). The area involved was very small (less than 42 km^2) and eradication was achieved by the complete removal of dense undergrowth with the elimination of a number of small trees (Buckley, 1951)

The control of the vectors through aerial adulticiding using DDT was attempted with some success in Uganda (Barnley, 1958). However, the effect of this operation was later attributed to the insecticide that accidentally drifted to the water and killed larvae. Subsequently, adulticiding was suspended and eradication was achieved through larviciding (McCrae, 1978).

The Onchocerciasis Control Programme was launched in 1974 with the aim of reducing transmission of Onchocerciasis through vector control, until it was no longer a disease of public health importance in the control area. It has mandate to ensure that there is no recrudescence of the disease. Control operations started in February 1975 and involved

seven countries and were gradually extended to cover 11 countries with a total area of 1,235,000 km^2. The countries covered by OCP are Benin, Togo, Ghana, Cote d'Ivoire, Niger, Burkina Faso, Mali, Sierra Leone, Senegal, Guinea, and Guinea Bissau (Figure 4a).

Control in the OCP is achieved by weekly application of insecticides upstream of the breeding sites in the rivers where the species of the *S. damnosum* complex that are associated with severe forms of the disease occur. The breeding sites in rivers were treated every week to prevent any larvae reaching the pupal stage at which it is no more vulnerable Studies conducted before the beginning of the Programme led to the selection of temephos (Abate®) as the choice insecticide to be used (Dejoux and Elouard, 1975; Dejoux and Venard, 1976). Temephos is an organophosphorous compound that is biodegradable and is of very low toxicity to non-target organisms. It was exclusively used until 1979 when resistance was detected in Cote d'Ivoire. Other insecticides were then gradually introduced including other organophosphorous, Carbamates, and Pyrethroids. The performance of the other chemicals however was inferior to those of the temephos and their toxicity to non-target organisms was very high. In addition to these limitations, cross-resistance with Temephos was developed.

The biological insecticide *Bacillus thuringiensis* (*Bt.*) was also introduced in the OCP in 1982 (Anonymous, 1995) to add to OCP's arsenal of insecticides. The serotype H-14 *Bt.* was formulated as liquid concentrate and marketed as Teknar® (Sandoz, USA) was subsequently replaced with Vectobac® (Abbot Laboratories, USA). However, even after

the improvement of the formulation of the *Bt.*, its use still remains limited to rivers of low water discharges. A strategy of rotating insecticides (Table I) for treatment was adopted to avoid resistance developing in the vectors (Akpouba, 1993).

The combined strategy of vector control and chemotherapy has led to a high reduction of the infestation of the flies and flies in the OCP area. The microfilarial loads of communities that are an index of the intensity of human infestation has dropped by about 60-80% as compared to the level before the start of OCP. The entomological indices show that in 85-90% of the original area the target species namely *S. damnosum* s.s and *S. sirbanum,* have been reduced to densities that are incapable of maintaining transmission (WHO, 1988). It is estimated that about 11 million children born in the OCP original area have been spared the risk of onchocercal blindness (Anonymous, 1999a). Thirty five million people are protected from infection by the disease, 500,000 prevented from going blind or visually handicapped and 1.5 million have lost their onchocercal infection. The control activities have freed a total 25 million hectares of land that have been made available for agriculture in the Programme area (Benton and Skinner, 1990). This area is ready for resettlement by people and it is expected that the total number of people that could be fed by the products of the traditional agriculture of this area is about 17 million.

The vector control activities are expected to end in 2002 by which time, after more than 20 years, all the adult worms would have died off. One of the major problems met in the control activities was that of the re-invasion of treated areas by flies that had migrated over long distances from outside the OCP area (Traore and Hebrard, 1983). *Simulium*

damnosum is known to migrate over 150 kilometres in West Africa (Kassambara, 1987; Plaisier *et al.*, 1995), therefore avoiding recrudescence after the OCP will be difficult. Considering the serious biting nuisance constituted by *Simulium* even if the flies are free of parasites they will still constitute a problem for the populations of endemic areas.

Table I: List of Insecticides used in rotation by the OCP showing the concentration (active ingredients) formulation and the conditions for their use (From Anonymous, 1995).

Common name	**Active ingredient/formulation**	**Quantity (ml per m^3/s)**	**Carry at $15m^3/s$**	**Carry at $100m^3/s$**
Temephos	20% emulsifiable concentrate	From 150 to 300 according to turbidity	12 km	17 km
Phoxim	50% emulsifiable concentrate	160	3 km	5 km
Pyraclofos	50% emulsifiable concentrate	120	12 km	18 km
Carbosulfan	25% emulsifiable concentrate	120	Not used	9 km
Permethrin	20% emulsifiable concentrate	45	Not used	7 km
Etofenprox	30% emulsifiable concentrate	60	4 km	7 km
B.t. H-14	<2% liquid concentrate	720	1.5 km	Not used

2.6.3 Control of onchocerciasis using Chemotherapy and the African Programme for Onchocerciasis Control (APOC)

The most effective method of controlling onchocerciasis is eliminating the adult worm from the body. The standard drugs that were used were diethylcarbamazine (DEC) that kills microfilariae and Suramin, which kills the adult worms. However, both drugs elicit severe and dangerous reactions in the skin and the eyes where they kill microfilariae and therefore could not be used on a large scale for the treatment of the disease.

In 1982, the Onchocerciasis Chemotherapy Project (OCT) was established with the aim of finding effective non-toxic filaricidal drugs. The research conducted by Merck, Sharp and Dohme (MSD) led to the development of a drug, Ivermectin that was efficient, well accepted and tolerated without any particular toxicity (Aziz *et al.*, 1982; Awadzi *et al.*, 1985; Awadzi *et al.* 1986). Ivermectin is a highly effective gastrointestinal antihelminthic and ectoparasitocide that was widely used in the veterinary field and has been brought up for use in human onchocerciasis under the name Mectizan®. It is the sole drug currently used by the OCP and the APOC (African Programme for Onchocerciasis Control) to control onchocerciasis morbidity. It is given at single annual dose and it causes 90% reduction of the microfilarial load and this reduction is maintained over six months (Dadzie *et al.*, 1991). In trials, it was found that treatment using Ivermectin achieved reduction of 96-99% of the microfilarial load, but had no destructive effect on the adult worm (Prost and Vaugelade, 1981; Aziz *et al.*, 1982; Awadzi *et al.*, 1985). A reduction of 60% in transmission was also recorded in the first year after treatment, even though no

further reduction was recorded later (Prost and Vaugelade, 1981).

The African Programme for Onchocerciasis Control (APOC) was set up in 1996 with the aim of establishing within a period of 12 years, an effective and self-sustainable, community-directed treatment with Ivermectin throughout the remaining onchocerciasis endemic areas in Africa, and to eliminate the disease by vector control in selected foci (e.g. Bioko Island). Attainment of this objective will ultimately realize the goal of elimination of onchocerciasis as a disease of public health and socio-economic importance throughout Africa. The APOC area covers the following countries; Burundi Cameroon, Central African Republic, Chad, Congo, Democratic Republic of Congo (former Zaire), Equatorial Guinea, Ethiopia, Gabon, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Sudan, Tanzania and Uganda (Figure 4b). The main methods currently employed in ivermectin distribution programmes in endemic African countries are; (a) large-scale, active distribution and (b) clinic-based or "passive" distribution. The former is carried out either by mobile teams of para-medicals or by trained individuals living in endemic communities, so called Community-based Distributors (CBDs), who usually have minimum direct supervision though the activity is strictly monitored. Cost implications with distribution by CBDs and clinic-based treatment are far less than with mobile teams.

A Mectizan® Expert committee based in Atlanta (Georgia) has been charged by MSD to manage the programme of Mectizan® donation.

(a) OCP area (b) APOC area

Figure 4: Map of Africa showing the areas (shaded) covered by the Onchocerciasis Control Programme in West Africa (left) and the African Programme for Onchocerciasis Control (right).

2.7 Natural Enemies of Black Flies

A number of naturally occurring organisms including pathogens, parasites and predators are known to adversely affect black flies. A good account of these organisms is given in (Laird 1981; Weiser and Undeen, 1981; McCreadie and Adler, 1999). Among the pathogens are viruses, bacteria and fungi. The parasites comprise protozoans and memerthid nematodes. The predators include vertebrates, insects and non-insect invertebrates.

2.7.1 The Pathogens

2.7.1.1 Viruses

Three groups of baculoviruses have been described from black flies. These are; iridescent viruses (IVs), densonucleosis viruses (DVs) and cytoplasmic polyhedrosis viruses (CPUs). IVs are also known from other Diptera and Coleoptera and cause a bluish-green or violet iridescence of the infected larvae of the host.

The infection by DVs causes the hypertrophy of the mid-gut epithelial cells and the fat body nuclei. However, the pathogenicity of this virus to black flies is still to be established (Weiser and Undeen, 1981).

The CVP-infected *Simulium* larvae are easily recognizable with the naked eye because of the white spots in the gut wall in the region of the cardia and the blind sacs at the insertion of the malpighian tubules. The role of viruses however in the limiting natural black flies populations is unknown (Weiser and Undeen, 1981).

2.7.1.2 Bacteria and Fungi

Black flies are usually infected when the water current is sharply reduced or ceases altogether. Bacteria that infect the gut pass through the intestinal wall, causing septicemia and larval death within 2-3 hours. The most common sources of larval septicemia in black flies during water fluctuation are virulent strains of the heavily pigmented ***Serratia marcescens*** (Weiser and Undeen, 1981). However, the most common bacterium known to be fatal to black flies is the *Bacillus thuringiensis* var. *israelensis*. Although initially known to be efficient in the control of Lepidoptera, it was later found (Lacey and Mulla, 1977) to be pathogenic to the larvae of *S. vittatum*. It was later demonstrated to be toxic to several species of black flies in laboratory tests (Undeen and Nagel, 1978). Formulations of *Bt* serotype H-14 are now used in the OCP area to manage resistance to organophosphorous insecticides. It is safe to non-target organisms (Anonymous, 1995).

Fungal diseases of black flies are more common than those due to viruses and bacteria. Infection takes place during the larval stage and may persist into adults. Members of the Saprolegniaceae infect stressed *Simulium* larvae (Nolan and Lewis, 1974). Several genera of the Trichomycetes affect the gut or the skin of black flies, although they harm them occasionally (Jekins, 1964; Roberts and Strand, 1967). Beard and Adler (2000) have also reported the occurrence of the Trichomycetes *Smittium megazygosporum* in a black fly

host. Some other Phycomycetes were already known from the ovaries of the female black flies (Undeen and Nolan, 1977).

2.7.1.3 Parasites

Protozoans

Some ciliates have been recorded from adult female *Simulium damnosum* in West Africa (Lewis, 1960, 1965) and other species of *Simulium* from Latin America (Garms, 1975). Some obligate Microsporidians have been discovered in different species of Simuliidae (Jekins, 1964; Roberts and Strand, 1977). Some of these genera of Microsporidians include *Nosema, Thelohania, and Pleistophora.* Adler *et al.* (2000) recently characterized and described a new species, *Caudospora palustris* (Microsporidia: Caudosporidae) from some North American species of black flies. The external signs of infection are rare. Larvae harbouring Microsporidae are characterized by white, irregularly lobate cysts at the posterior end. The parasites could invade the silk glands and the malpighian tubules (Weiser and Undeen, 1981). It was believed that infection reduced longevity. However, there is no information on the effect of protozoa on populations of black flies.

Mermethids nematodes of black flies

A summary of the information available on the parasitism of black flies by Mermethidae Poinar (1981). The first recorded mermethid-infested *Simulium* larva was by Von Linstow in 1898 and since then they have been found to be the most common parasite found in black fly larvae throughout the world. Over 60 species of black fly are known to be affected by mermethids and the actual number is even said to be several times greater (Poinar, 1981). Most of the mermethid parasites of black flies belong to the genera *Isomermis*, *Gastromermis*, and *Mesomermis (Neomesomermis)* (Poinar, 1981). Mermethids reduce the fat body and inhibit histoblast formation of Simuliid larvae. In the majority of cases, the parasitized black fly females are sterilized although they will maintain their normal behaviour (blood meal, attempt to oviposit).

2.7.2 Predators

These doubtless exert an important natural control on simuliid populations, especially on the immature stages, but there is little understanding of their impact on black fly populations (Davies, 1981). Davies (1981) and Crosskey (1990) summarized the information that was available on predation upon black flies by various authors. Davies broadly classified the predators of black flies either as vertebrates, insects and invertebrates other than insects.

2.7.2.1 Vertebrates

Bats are suspected to be predatory on adult black flies, however, there is no confirmation of such activity (Davies, 1981). The insectivorous North American water shrew may feed on larvae and pupae of black flies. In Australia, the platypus *Ornithorhyncus anatinus* is known to have a dominance of dipterans in its diet, including a high proportion of black flies (Faragher *et al.*, 1979).

Most birds are simply opportunistic feeders on black flies. However, appreciable numbers of black flies are ingested by only a few of them. They consume black flies either as adults (Dalmat, 1955) or as larvae and pupae (Carlsson, 1962; Dinulescu, 1966; Snoddy, 1967; Bengston, 1972).

Some Amphibians have equally been observed to be feeding on black flies. These include the shovel-nosed salamander (Martof and Scott, 1957; Martof, 1962) and the northern wood frog (Hocking, 1950).

Of all predation upon black flies, predation by fishes seems to be the most important and the most widely documented. Davies (1981) and Crosskey (1990) provide a checklist of fish predators of black flies. Predation by Salmonids is the most documented in the literature because of the economic importance of this group of fish. The juveniles feed more on the benthic fauna and thus their diet contains more larvae than adults (Gibson and Galbraith, 1975). Predation by fishes may be selective, not representing the prey availability. For example, Elliot (1967) reported that the brown trout less than one year old selected Simuliid larvae 25 times their availability. In Ghana, *Phractura ansorgei* is an important predator of simuliid larvae (Crisp, 1956a). It appears that under conditions

where black fly larvae are abundant and concentrated or where many adults are emerging or ovipositing, fish such as salmon and trout may eat large numbers and exert important natural control.

2.7.2.2 Insects

These include species belonging to the orders Trichoptera (caddis-flies), Odonata (dragonflies and damselflies), Ephemeroptera (mayflies), Plecoptera (stoneflies), Hemiptera, Thysanoptera (thrips), Neuroptera (lace wings), Lepidoptera (butterflies and moths), Coleoptera (beetles), Hymenoptera (bees and wasps) and Diptera (true flies) (Davies, 1981; Crosskey, 1990).

The larvae of caddis-flies are widely reported as the best insect predators of immature black flies (Miall, 1895, 1912; Davies 1981). The hydropsychids exert the greatest biological control on larvae and pupae of Simuliidae in many countries and regions (Burton and McRae, 1972). Burton and McRae (1972) found that 10% of 50 larvae of *Hydropsyche sp.* fed on larval *Simulium damnosum* in Ghana, while Service and Lyle (1975) reported 31.5% predation by hydropsychids in Ivory Coast. However, the relative extent to which trichopteran larvae control Simuliids by predation or dislodging them to drift to unsuitable habitats is not well understood (Kurtak, 1978).

Nymphal dragonflies and damselflies are also known to consume immature stages of black flies. These have been reported from Ivory Coast (Service and Lyle, 1975) and Brazil (Gorayeb and Pinger, 1978). Adult dragonflies have equally been reported to devour adult black flies swarming around vertebrate hosts (Twinn, 1939; Service and

Lyle, 1975; Pritchard, 1978). Crisp (1956a) referred to Odonates as probably the most important predators of adult *Simulium* in Ghana. However, it seems that even at the best of times, nymphal and adult Odonates exert minor influence on Simuliid populations (Davies, 1981).

Among stoneflies, the Perlidae and the Perlodidae are the families most predatory on black flies (Davies, 1981). Crisp (1956a, b) considers *Neoperla spio* as the most formidable enemy of immature black flies in the Red Volta River in Ghana. Hynes and Williams (1962) also reported this same organism from Uganda in 1962.

The most unusual predators of immature black flies are three African species of *Drosophila*. They prey on eggs and newly hatched larvae (Tsacas and Disney, 1974; Disney, 1975). Tsacas and Disney (1974) found the potential of these *Drosophila* flies to control populations of black flies to be superior to that of larval trichopterans.

Among the Diptera, Crosskey (1990) considers that the Simuliidae are their own worst enemy. Larvae have no compunction about preying on one another, the older and larger often consuming the smaller and younger of their own or of a different species. In very dense aggregation, self-predation among black flies in its aggregate will have more importance on the population than predation by any other animal, invertebrate or vertebrate.

Several reports have been made of larval chironomids have been observed feeding on egg masses and larvae of black flies (Wu, 1931; Grenier, 1945, 1953; Hocking and Pickering, 1954; Dalmat, 1955; Peterson and Davies, 1960; Dinulescu, 1966; Kureck, 1969). In

1931-1932, 1200 larvae of *Cardiocladius* larvae, a genus well know to prey on Simuliids (Zwick, 1978) were transported from Australia to New Zealand for simuliid control (Anonymous, 1932). Many other Orthocladiinae are also known to destroy black flies. Chironomids, by spinning their silken tubes within and between black flies pupae, may promote the accumulation of algae around their respiratory filament, thus suffocating them (Grenier, 1943, 1953). They also cause eggs to detach and drift to less suitable habitats and be washed away.

Many other Dipterans are also quoted as predators of black flies. They include Empidids (Sommerman, 1962) and Dolichopodid (Twinn, 1939). The other insect orders reported to feed on black flies exert a more or less important control action.

2.7.2.3 Non insect invertebrates

Some *Hydra* sp. were observed feeding on 1st instar larvae of *Simulium*. Some Tubellarians, Annelids, Arachnida (especially spiders) and Crustaceans have all been reported to cause significant reduction of simuliids larvae or adults whichever one is present (Davies, 1981).

Chapter 3: Materials and Methods

3.1. The Study Site

The *S. damnosum* egg masses and larvae used for this study were collected from Boti Falls and breeding sites near Huhunya (about 7 kilometres downstream of Boti Falls), on the River Pawnpawn in the Eastern Region of Ghana. The river lies in moist semi-deciduous forest. The Global Positioning System was used to determine the geographical coordinates of the area as being 6°12'N and 0°13'W. The river is fed by a number of springs and side streams in this area. The river Pawnpawn flows southeast for 17 kilometres and then 60 kilometres eastwards to join the Afram arm of the Volta Lake, draining an area of about 160 km^2. The collections were conducted during the rainy seasons of 1999 and 2000.

3.2 The Insect Material

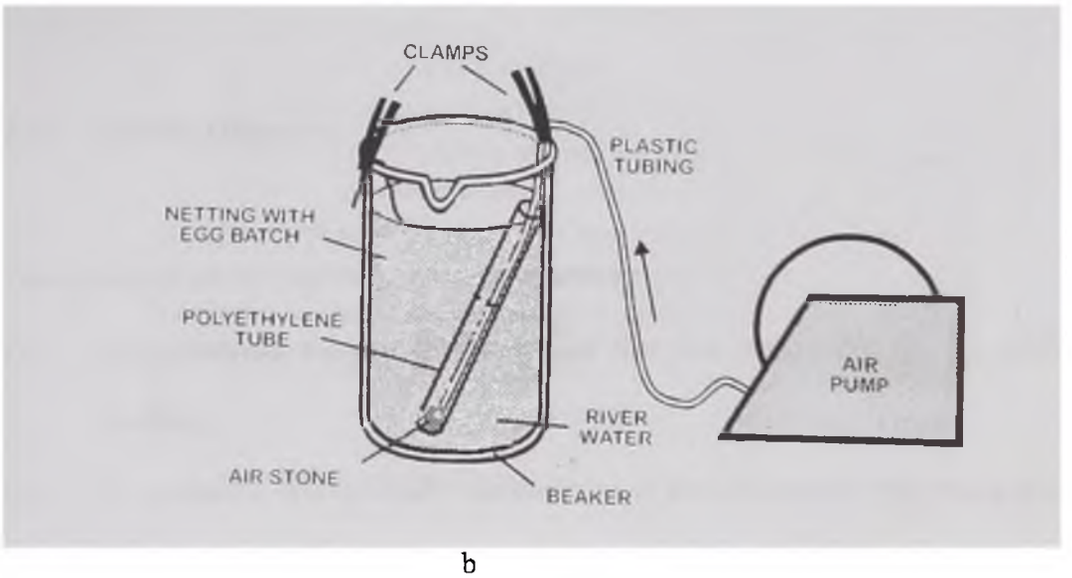
3.2.1 Field collection and transportation of *S. damnosum* egg masses

The river course was prospected for *S. damnosum* egg masses. The egg masses attached to vegetation were kept in plastic bags and stored in a cool ice chest as recommended by Boakye and Raybould (1986). They were then transported to the laboratory at base where the rearing was carried out.

3.2.2 Laboratory rearing of *S. damnosum* s.l.

The laboratory system and methods for rearing *S. damnosum* developed by Boakye et al (2000) was used for this study (Figure 5). Each leaf (substrate) was attached using a thread and reared in a separate rearing system. The leaves on which the egg masses were numerous were cut to smaller pieces and placed in individual systems. The water of the system was changed, the system cleaned every day and the larvae were fed on Tetra® fish food. Some of the chironomid larvae that were observed feeding on black fly pupae were collected and preserved in isopropanol for molecular studies. On observing pupation of either larvae (simuliids or chironomids), the rearing systems were covered with a piece of silk netting. All adult flies emerging were trapped in the net, collected daily using an aspirator and counted. The adult chironomids were collected, sorted and preserved in 70% ethanol for identification.

a



b

Figure 5: The set up of the rearing system used for rearing the black flies and the chironomid in the study (a); schematic diagram of the rearing set up. After Boakye *et al.* (2000).

3.3 Morphological Identification of the Chironomids

3.3.1 Introduction

The vectors of onchocerciasis have been properly and definitively identified as *Simulium damnosum* (Theobald 1903) complex and the literature related to it is very wide. Larvae and pupae were attacked and fed upon by some chironomid larvae in laboratory experiments. This chironomid needs to be properly identified in order to pursue further research on its potential as biological control agent of black flies.

This chapter describes the procedure of identification of the chironomids predator of black flies.

3.3.2 Specific Objectives

The objectives set for this study were the following:

a)- To incriminate the chironomid species that was responsible for the predation (feeding).

b)- To proceed to the complete identification of the Chironomid that was positively incriminated as the predator on black flies.

c)- To provide a good identification of the other Chironomid whose eggs were also found associated with those of *Simulium damnosum*.

3.3.3 Preparation of the Chironomids for morphological identification

The procedures described by Wiederholm (1983 and 1989) were used for the preparation of the larvae and the adults respectively.

The preserved larvae were first washed in distilled water and then transferred into a 10% potassium hydroxide solution. A small tear was made behind the head capsule to allow the KOH to penetrate into the body. The KOH was warmed to just below boiling point and the larvae were kept in there for 10 minutes. When muscles and fats in the body were totally cleared, the carcasses were transferred to glacial acetic acid for at least 10 minutes to neutralize KOH. Then the larvae were transferred to absolute alcohol, then to a mixture of Cedar wood oil with a layer of absolute ethanol and finally in Cedar wood oil for at least 10 minutes at each stage. The carcasses were finally mounted in Canada balsam on slides, covered with cover slips and the slides dried in the oven.

With adults, the antennae, wings and the legs of the right side of the body were dissected and placed in glacial acetic acid. The rest of the body was put in 10% KOH and heated for about 10 minutes till the muscles and fats were all cleared. Then the body was also transferred in glacial acetic acid and all the steps were followed as described for larvae. The rest of body was dissected during mounting; wings, legs of right side of body, head and antennae, thorax with legs of the left side attached to it and abdomen were mounted separately from one another. The male abdomen was mounted with dorsal side up and the head with eyes facing up.

3.3.4 Morphological identification of the chironomids

The identification keys of Wiederholm (1983, 1989) and of Freeman (1956) were used. The male fly specimens of chironomids were used for identification. The characters of the head, antennae, eyes, legs and the hypopygium (genital structures) were used. An Olympus BX50 microscope with an attached camera lucida was used to draw some important structures of the mounted specimens.

3.4 Positive Implication of the Chironomid as Predator on *S. damnosum*

3.4.1 Introduction

There are many records of predation upon black flies in nature. A variety of techniques have so far been used to detect *Simulium* material within digestive tract of their predators. These include the direct observation of the feeding on simuliids, the morphological identification of *S. damnosum* remains in the open alimentary canal (Davies, 1981) and immunological and serological tests of the gut content of the predator (Service and Lyle, 1975; Service and Elouard, 1980).

With the advent of molecular techniques, it is now possible to detect the presence of very little amount of DNA from a particular source (nature), even if it is surrounded by very important quantities of DNA from different sources. This has been used to identify the gut content of blood-fed female mosquito (Gokool *et al.*, 1993; Boakye *et al.*, 1999)

The polymerase chain reaction (PCR) was developed for *in vitro* amplification of DNA sequence (Saiki *et al*,. 1985). It is now used to characterize and differentiate organisms. Oligonucleotide primers that are highly specific for groups of living organisms and designed to flank variable regions of genes are used to amplify the target DNA sequence. In the course of a PCR, a target DNA fragment is replicated exponentially (Saiki *et al.*, 1985; Mullis *et al.*, 1986). The reaction involves repeated cycles of heat denaturation of

the DNA, annealing of the primers to their complementary sequence at lower temperature, and extension of the annealed oligonucleotide by the DNA Polymerase. The DNA polymerase in this case is a thermo stable enzyme isolated from *Thermus aquaticus*. The extended segments (products) are complementary to and can bind primers; therefore, subsequent cycles of amplification result in doubling the amount of the target segment amplified in the previous cycle. The annealing temperature of the primers is generally as high as possible to promote specificity. However, under standard conditions, it should be 5 °C below the melting temperature (Tm) of the primer that is calculated using the formula of Thein and Wallace (1986):

$$\mathbf{Tm = [4\ (G+C) + 2\ (A + T)]}$$

where G, C, T and A are guanine, cytosine, thymine and adenine.

The sizes of PCR products are estimated by comparison with the mobility of standards of known molecular weights on agarose gel electrophoresis. Differences are identified by the size of the amplification products. Sometimes, when it is not possible to differentiate between sizes of different PCR products from the amplification of DNAs from different sources, restriction analyses of the amplified DNA can reveal differences. These involve the digestion of DNA (or fragments of DNA) using restriction enzymes that cut nucleic acids at specific sites. The sizes of the restricted products are checked on agarose gel or polyacrylamide gel.

3.4.2 Specific Objectives

In this part of the study, the objectives were the following:

1. To extract DNA from three sources: *S. damnosum* larvae, chironomid larvae and larvae of chironomid that have fed on *S. damnosum*
2. To use the polymerase chain reaction (PCR) to detect *S. damnosum* DNA in the DNA extracted from chironomid and therefore implicate the chironomid as predator upon *S. damnosum* in order for it to qualify for research as a potential biological control agent of the vector of onchocerciasis

3.4.3 The Extraction of genomic DNA

The various buffers and solutions used were prepared as described in appendix I

The DNA was extracted from the larvae as described by Flook *et al.* (1992). Using this method, the larvae preserved in isopropanol were washed with Tris-EDTA and transferred into an eppendorf tube (1.5 ml). They were then crushed in 100 µl of bender buffer (Appendix I). They were incubated for 30 minutes at 65°C, then 25µl of Potassium acetate was added and the mixture incubated on ice for 20 minutes. The mixture was spun for 10 minutes at 14,000 revolutions per minute (rpm), the supernatant transferred to fresh tubes and 250µl of pre-chilled absolute ethanol added. The mixture was mixed gently and then incubated at -50°C for one hour, and spun at 14,000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-dissolved in 125 µl TE, 125 µl of phenol was added, vortexed and spun at 14,000 rpm. The supernatant was transferred into

fresh tubes, 125 µl of phenol/chloroform (1:1) added, vortexed and treated as above. The supernatant was transferred to fresh tubes, 250 µl of chloroform was added and spun at 14,000 rpm. At the last stage, the supernatant was transferred to fresh tubes, 250 µl of pre-chilled absolute ethanol and 31.5 µl of potassium acetate were added. The preparation was kept overnight at -20°C, spun at 14,000 rpm for 10 minutes, the pellet rinsed with 200 µl pre-chilled 70% ethanol, and spun for 5 minutes at 14,000 rpm. The washed pellet was then re-dissolved in 25 µl of TE RNase (50 µg/ml). After one-hour incubation on ice, the DNA was kept in the freezer (-20°C) until ready for use.

3.4.4 Detection of *Simulium damnosum* DNA from the chironomid

3.4.4.1 The Synthetic oligonucleotides (primers)

Three sets of oligonucleotide primers 16S, ND4 and P were used to detect *Simulium* DNA in the gut of *C. oliffi*. The sequence details of these primers and the expected sizes of PCR products for *Simulium* are given in Table 2.

The primer set 16S is made up of the forward primer 16s-5' and the reverse primer 16s-3' designed to amplify a section of the large sub-unit (16S) of ribosomal RNA (rRNA) gene called 16s/556.

The ND4 primer set comprises the forward primer ND4-1 and the reverse primer ND4-2, which are designed to amplify a section of the NADH dehydrogenase sub-unit 4 of the mitochondrial DNA.

The third primer set, which is universal, is made up P1 (forward) and P2 (reverse) whose sequences were designed from published sequences of the large sub-unit 28S rDNA of *Drosophila melanogaster* (Tautz *et al.*, 1988), and *Caenorhabditis elegans*. P1 primer is positioned 106 bp upstream of the expansion segment located on the rRNA genes, whilst P2 is positioned 228 bp downstream from the 3' end (Wilson, 1994).

3.4.4.2 The Polymerase Chain Reaction

The polymerase chain reaction was used to detect *S. damnosum* material from *C. oliffi's* gut. The amplification was carried out using a Hybaid PCR thermal cycler. The reaction mix of 50 µl contained 5µl of the PCR buffer supplied by the manufacturer (Sigma, USA), 100µM of each of the 4 deoxyribonucleotide triphosphates (dNTPs), 0.25 µM of each oligonucleotide primers, 1.25 units of Taq polymerase enzyme (Sigma, USA). One microlitre of the genomic DNA served as template for the amplification reaction. Sterile double distilled water (sddw) was used to make the volume up to 50µl. The reaction mix was spun down briefly at 10,000 rpm and overlaid with 50 µl of mineral oil to avoid evaporation and refluxing during thermocycling.

The cycling parameters for the reaction were as follows: 94°C for 2 minutes (initial denaturation) over one cycle, followed by 29 or 35 cycles of 94°C for 1 minute, 50°C for 1 minute (annealing), 72°C for 3 minutes (extension), and a final cycle of 94°C for 1 minute, 50°C for 1 minute, 72°C for 10 minutes. The annealing temperature used in various reactions depended on the primer set. For each reaction a positive control with PCR products of *Simulium* of the same primer set, and a negative control that contained no DNA template were included.

3.4.5 Analysis of PCR products

Following the PCR, the products were electrophoresed on a 2% agarose gel stained with 0.5 µg/ml ethidium bromide. Ten microlitres of each sample was added to 1 µl of Orange G (5X) gel loading dyes for the electrophoresis. The gel was prepared and run with IX TAE buffer using a maxigel system (BIORAD). The gels were run at 100 volts for one hour visualized and photographed over a UV transilluminator (UPC, USA), at short wavelength using a Polaroid camera type 667 film. The size of the PCR products was estimated by comparison with the mobility of a standard of known molecular sizes (Appendix II).

3.4.6 Restriction analysis of the PCR products

On the basis of the work done by Wilson (1994), the restriction enzyme *AluI* was used to digest the PCR products obtained using the primer set P1-P2. *AluI* was used in that study to analyse restriction site differences between species of Diptera at the family level, including Simuliidae and Chironomidae. For the present study, the digestion was performed in 20 µl volumes containing 2 µl of the enzyme, 2 µl of the buffer, 8 µl of the amplicon and sddw to make the volume. The reaction mix was incubated at 37 °C for at least four hours. The restriction products were electrophoresed on a polyacrylamide gel.

Table 2: DNA sequence of the synthetic oligonucleotides (primers) used for the amplification of *S. damnosum* material inside the chironomid. Each primer is given with its annealing temperature and the size of the expected PCR product for *Simulium*.

Name of primer	Annealing temperatures (°C) for set	Sequences (5'-3')	Expected size of PCR products (bp)
P1	55	TAGTGACGCGCATGAATGGA	774
P2		GACGTCGCTATGAACGCTTG GC	
ND4-1	45	ATCAGTTGTTGCTCATATAG	238
ND4-2		TATATTAGCAGAACACAATAAAA	
16s-5'	45	CGCCTGTTTATCAAAAACATG	556
16s-3'		CTCCGGTTTGAACTCAGATC	

3.5 Potential of the Chironomid As Biological Control Agent of *Simulium damnosum* s.l.

3.5.1 Introduction

After the identification of the chironomid as potential biological control agent, it was necessary to study how important it could be at reducing populations of *S. damnosum* in the laboratory. Therefore, experiments were carried out to determine the predatory capacity of the chironomid upon black flies in the laboratory.

3.5.2 Specific Objectives

The specific objectives of this study were as follows:

1- To evaluate the potential of the chironomid as control agent of *S. damnosum;*

2- To establish if the predatory behaviour was obligatory, that is if the chironomid could be reared or develop normally on other food sources such as fish food in the absence of a prey.

3.5.3 Design of the experiment

The leaves with attached egg masses collected from the field were first introduced into big rearing systems (pools) and reared as described in section 3.1.3. After about 10-11 days, when the larvae of both *Simulium* and chironomid had developed well enough to be visible, specific numbers of each were transferred to fresh rearing systems. The *Simulium* larvae were introduced first and allowed to settle in the new environment, which was usually 6 hours before the chironomid were added. The number of larvae used in each experiment was chosen such that it facilitated the cleaning and monitoring without any death of the larvae. The rearing systems were cleaned every day and the number of larvae dead or alive, pupae dead or alive and adult emerged was recorded.

Whenever *S. damnosum* larvae were needed for the system, fresh ones were collected from Bosomase (River Pra), where these chironomids had not been recorded after many samplings. *Simulium sanctipauli* is the only species that is known to breed there (Boakye *et al.* 1999b) whereas *S. yahense* and *S. squamosum* are the species breeding on the River Pawnpawn at Boti Falls (Fiasorgbor *et al.*, 1992) where the chironomids are found. The experiment consisted of five different regimes with the following ratios of chironomid to *S. damnosum;* i) 0: 25 control, ii) 1: 5, iii) 1: 2, iv) 1: 1 and v) 25: 0, introduced together into the rearing systems and then monitored until all had emerged as adults. This experiment was repeated six times except for the last unit (i.e. 25 chironomids to 0 simuliids), which was repeated four times.

3.5.4 Statistical analyses

The data collected were analysed using the SPSS for windows statistical software package. The ANOVA test was performed to determine if there was any significant difference between the treatments regimes and the control. The ANOVA was also used to determine if there was any difference between the numbers of adult chironomid emerged in the treatments and in the rearing system that had no *Simulium* larvae. Multiple comparisons using the Scheffe and the LSD (Least significant difference) were conducted to compare the performances of the various treatments. All differences were tested at an $\alpha = 0.05$ level of significance.

Chapter 4: Results

4.1 Laboratory Rearing of the Chironomids

The chironomids were reared in the rearing system developed by Boakye *et al.*, (2000) for *S. damnosum*. In scoring experiments, 87.94% of the 358 larvae introduced in the rearing system completed their growth and emerged as adult.

Two peaks were noted for the pupation and the emergence of chironomids. These appeared about two and three weeks after introduction of egg masses into the rearing system (Figure 6). These revealed the presence of two species of chironomid in the systems. *Simulium* larvae and pupae were still available after the first peak of emergence of the chironomids, but decreased and almost disappeared from the systems thereafter. The latter condition was therefore attributed to predatory activity of the second group of chironomid emerging from the rearing system

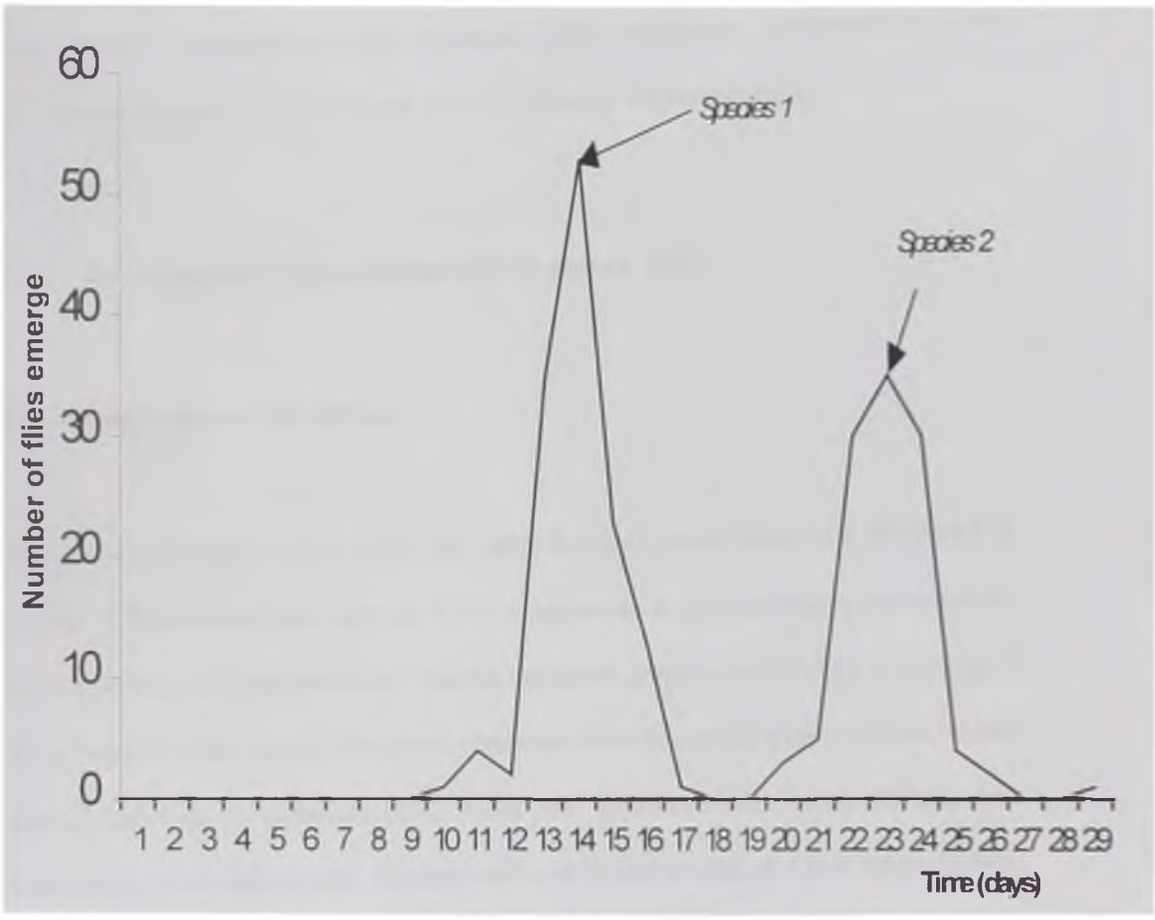


Figure 6: Emergence of adult chironomid flies from the rearing system showing the peak of emergence for flies of the two different species. The first insect emerges about two weeks after eggs have been laid and the second after about three weeks.

4.2 Morphological Identification of the Chironomids

All the specimens belonged to the Family Chironomidae. Two species of chironomid were found, *Cardiocladius oliffi* Freeman 1956 (subfamily Orthocadiinae) and *Cricotopus kisantuensi* Gotghebuer 1934 (subfamily Orthocladiinae)

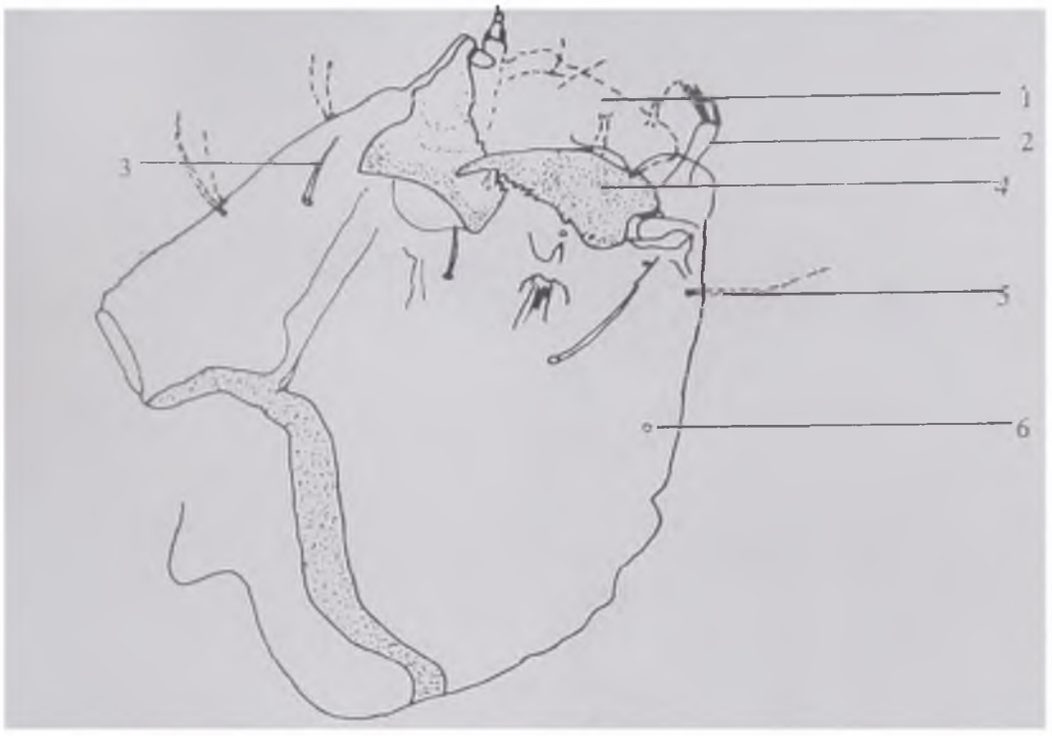
4.2.1 Description of *Cardiocladius oliffi* Freeman, 1956

The identification of the larvae

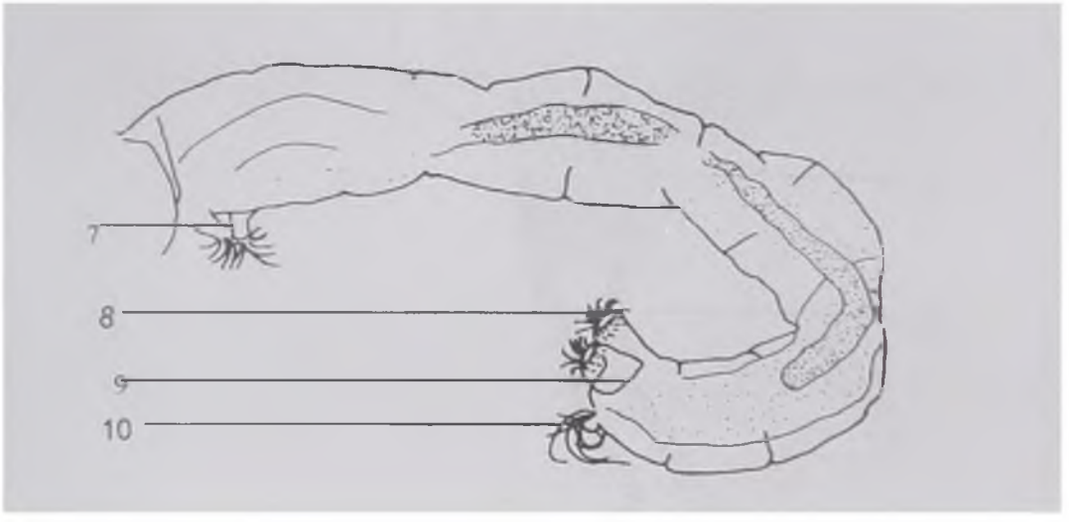
The main characters upon which the identifications were made are illustrated in Figure 7. The descriptions were as follows: antennae with 5 segments consecutively shorter. S setae of labrum simple, chaetae branched, pectin epipharyngis consisting of three simple scales apically rounded, chaetulae laterals simple, premandibles heavily sclerotized with a triangular inner tooth and premandibular brush absent. Seta subdentalis short and pointed. Mentum with one broad median and five pairs of lateral teeth, ventromental plates triangular, beard absent. Palpiger with leaf-like lamellae, galea with seta-like lamellae and without pectin galearis. Anterior parapods basally fused, each with an apical crown of claws, posterior parapods well developed, each separated with an apical crown of claws, procercus very short. Body setae simple.

The identification of the adults

Some of the main features of the adults used for the identifications are illustrated in Figure 8. The descriptions of the characters were as follows: macropterous with crossveins distinct; wing with cross vein MCu absent; tarsomere 5 not trifid (divided into three parts); gonostylus folded inwards, fore tarsomere shorter than fore tibia. All this combination of characters places the specimens in the subfamily Orthocladiinae. Wing membrane without macrotrichia; Vein R2+3 running close to R4+5 and ending distinctly in Costa; Eyes bare; Fourth tarsal segment cordiform. The specimens belong to the genus *Cardiocladius* Kieffer. Dorso-central bristles uniserial; Legs dark, pale only at the base, male styles broader: *Cardiocladius oliffi* Freeman.

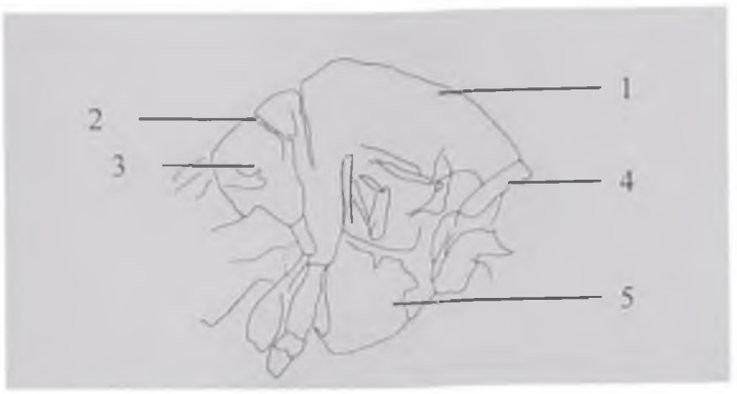


(A)

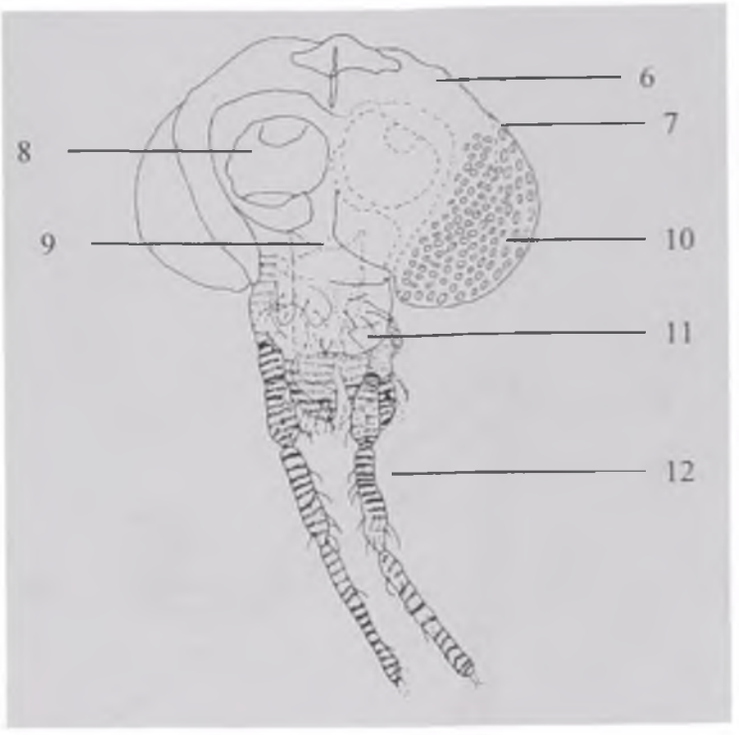


(B)

Figure 7: *Cardiocladius oliffi* larva; head (A) and body (B); 1 (labrum), 2 (antenna), 3 (seta submenti), 4 (mandible), 5 (frontal seta), 6 (eye spot), 7 (anterior parapods), 8 (claws), 9 (posterior parapods), 10 (anal setae). Drawing not according to scale.

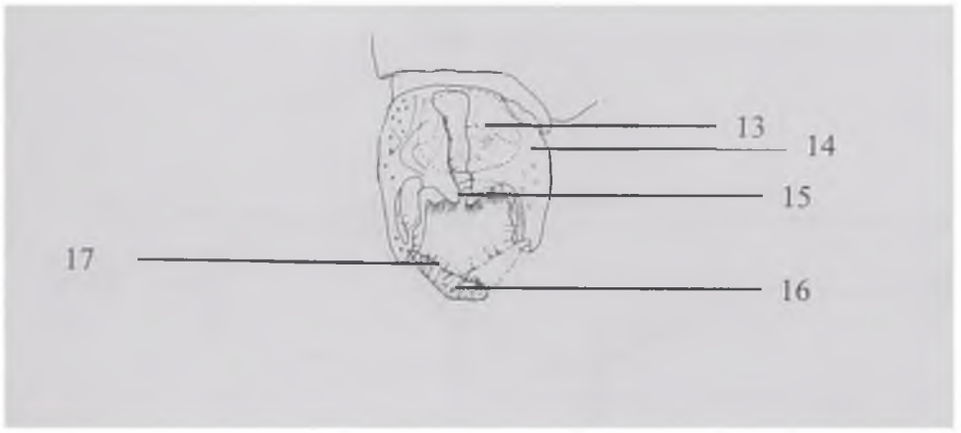


(A)

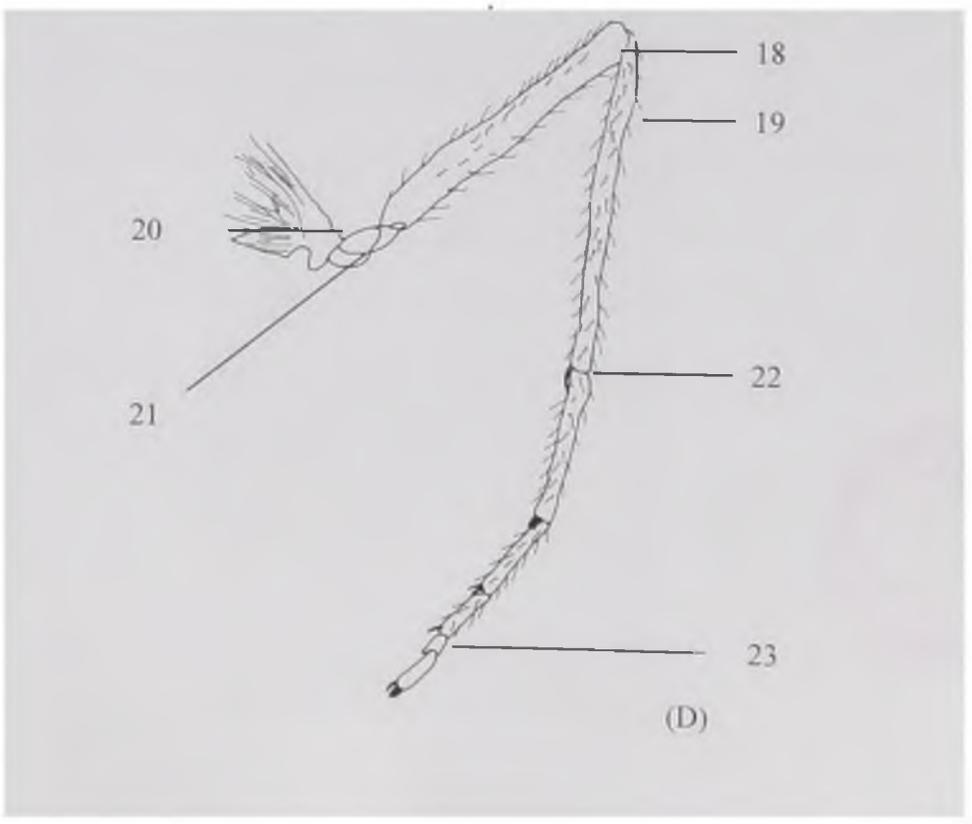


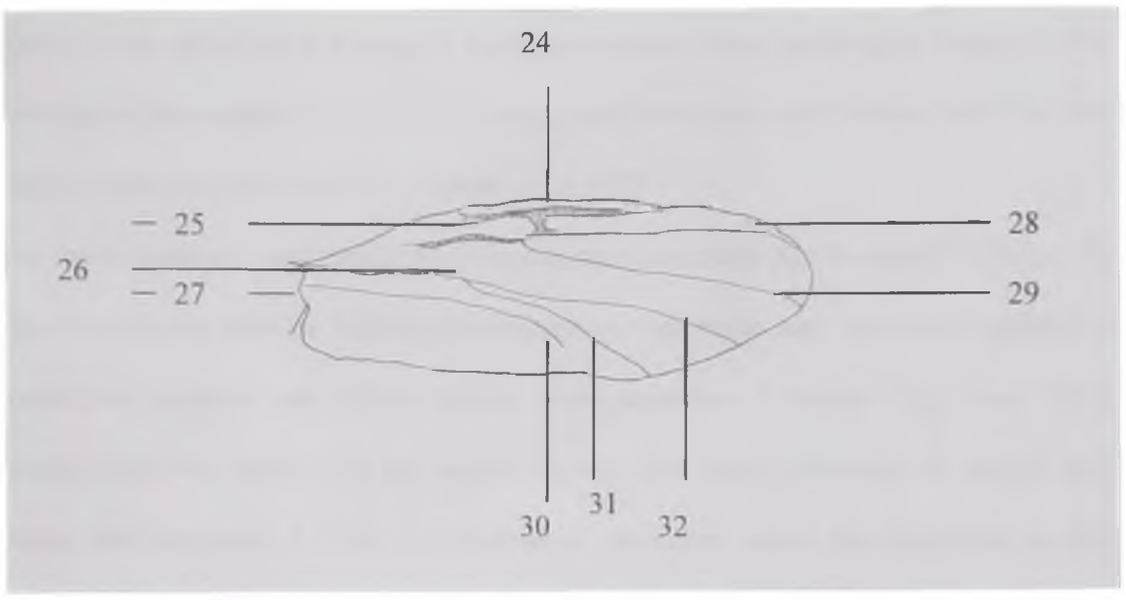
(B)

Figure 8: *Cardiocladius oliffi* Adult: thorax (A), head (B), hypopygium (C), leg (D) and wing (E); 1 (scutum), 2 (scutellum), 3 (postnotum), 4 (antepronotal lobe), 5 (preepisternum), 6 (orbitals), 7 (dorsomedial extension of eye), 8 (scape), 9 (cybarial pump), 10 (ommatidium), 11 (clypeus), 12 (labial palp), 13 (sternapodeme), 14 (gonocoxite), 15 (inferior vorsela), 16 (megaseta), 17 (gonostylus), 18 (femur), 19 (tibia), 20 (coxa), 21 (trochanter), 22 (tibial spur), 23 (4^{th} tarsomere), 24 (costa), 25 (radio-median RM), 26 (medio-cubital MCu), 27 (arculus), 28 (radial R_{4+5}), 29 (median M_{1+2}), 30 (anal An), 31 (cubital Cu_1), 32 (median M_{3+4}). Drawing not according to scale.



(C)



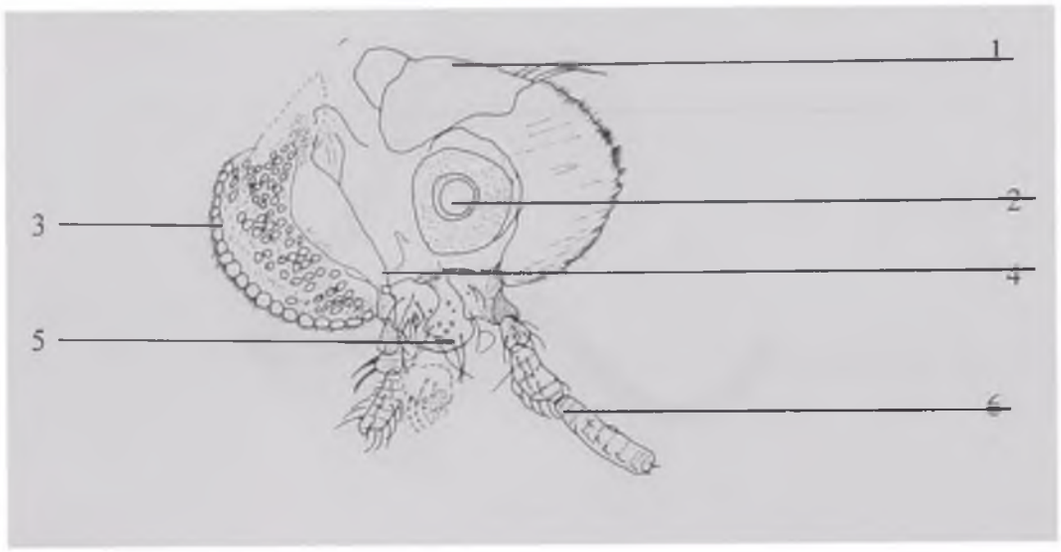


(E)

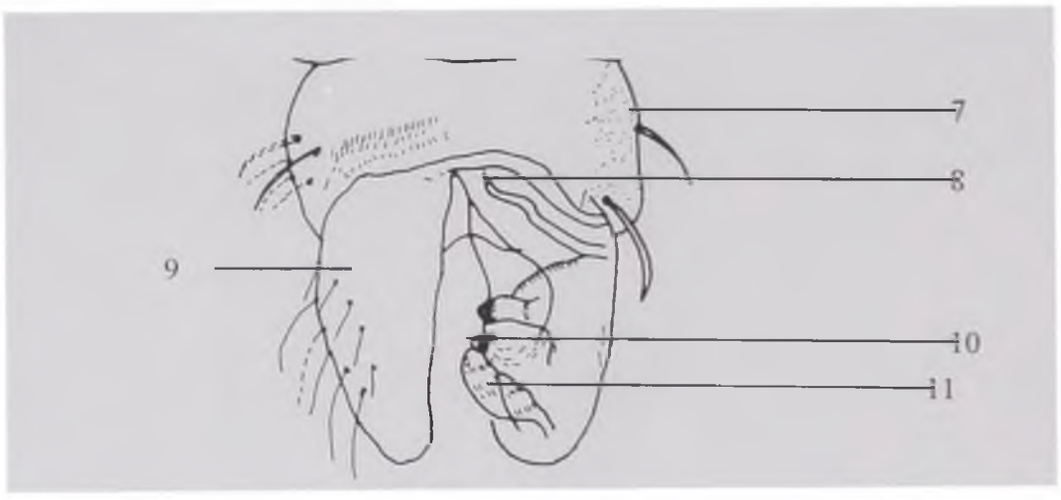
4.2.2 *Cricotopus kisantuensis* Goetghebuer , 1934

C. kisantuensis was described from a male specimen from Kisantu in the Belgian Congo and the holotype is kept in the Museé Royal du Congo Belge. The distribution throughout Africa South of Sahara is known. It has been reported from the Belgian Congo, many localities of the republic of South Africa especially from the Cape Province and from the Senchi rapids in Gold Coast (now Ghana) since 1951.

The main characters upon which the identification was made are illustrated in Figure 9. The descriptions were as follows: brachypterous with veins and crossveins indistinct; gonostylus movable and folded inward, fore tarsomere 1 shorter than fore tibia; hypopygium with endomeres not spoon-shaped; eyes hairy, tarsomere 4 simple and longer than tarsomere 5. This combination of characters places the specimens in the subfamily Orthocladiinae. Wing membrane without macrotrichia; vein R2+3 not running close to R4+5, ending distinctly in costa; squama with at least a partial fringe of 2-3 hairs; eyes pubescent (hairy); dorso-central hair minute and decumbent, tibia with white ring, humeral pits small. These characters put these specimens in the genus *Cricotopus* v. d. Wulp. Anterior tibiae with white ring; abdomen with brilliant steely blue bands; legs black except for white ring on front tibiae; white ring only on fore tibiae; abdomen with steely shining bands: *Cricotopus kisantuensis* Goetghebuer.

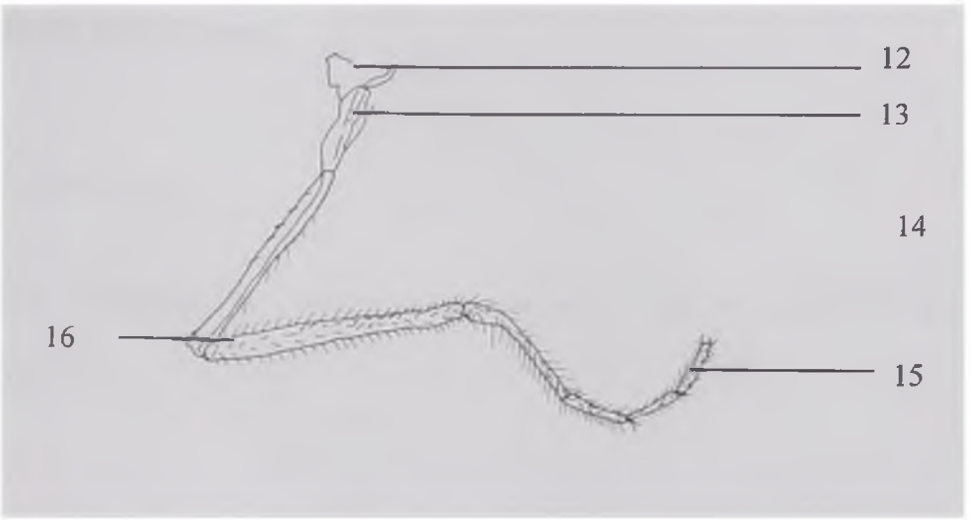


(A)



(B)

Figure 9: *Cricotopus kisantuensis* Adult: head (up left), hypopygium (up left), leg (down)1 (orbitals), 2 (scape), 3 (ommatidium), 4 (cibarial pump), 5 (clypeus), 6 (labial palp), 7 (laterosternite IX), 8 (sternapodeme), 9 (gonocoxite), 10 megaseta), 11 (gonostylus), 12 (coxa), 13 (trochanter), 14 (femur), 15 (tibia), 16 (claw). Drawing not according to scale



4.3 Positive Implication of *Cardiocladius oliffi* as Predator upon *Simulium damnosum*

4.3.1 DNA Extraction

The extraction of genomic DNA from *S. damnosum* and *C. oliffi* was successful in all cases (Figure 10).

4.3.2 PCR Assay

The PCR amplifications of all the DNA templates were successful, using all the primer sets (Figures 11 and 12). However, the sizes of the PCR products of *S. damnosum* and *C. oliffi* were about the same using all three sets of primers. It was therefore not possible to separate the PCR products of the *C. oliffi* larvae that had fed on *Simulium* and those that were unfed.

4.3.3 Restriction Analysis

The restriction digest (*AluI*) of the PCR products of *S. damnosum* and *C. oliffi* from the amplification using P1-P2 primer set is shown in Figure 13. For *S. damnosum* the restriction gave two fragments. The size of the smaller fragment was estimated at about 160 bp and the bigger fragment about 610 bp.

The restriction of PCR products from the amplification of DNA from *C. oliffi* resulted in three fragments of about the following sizes: 400 bp, 180 bp and 150 bp.

For the *C. oliffi* larvae that had fed on *S. damnosum* four fragments can been seen including the *Simulium* fragment of smaller size (about 160 bp) quite sharp in one (C) of them and fainter in the other one (B).

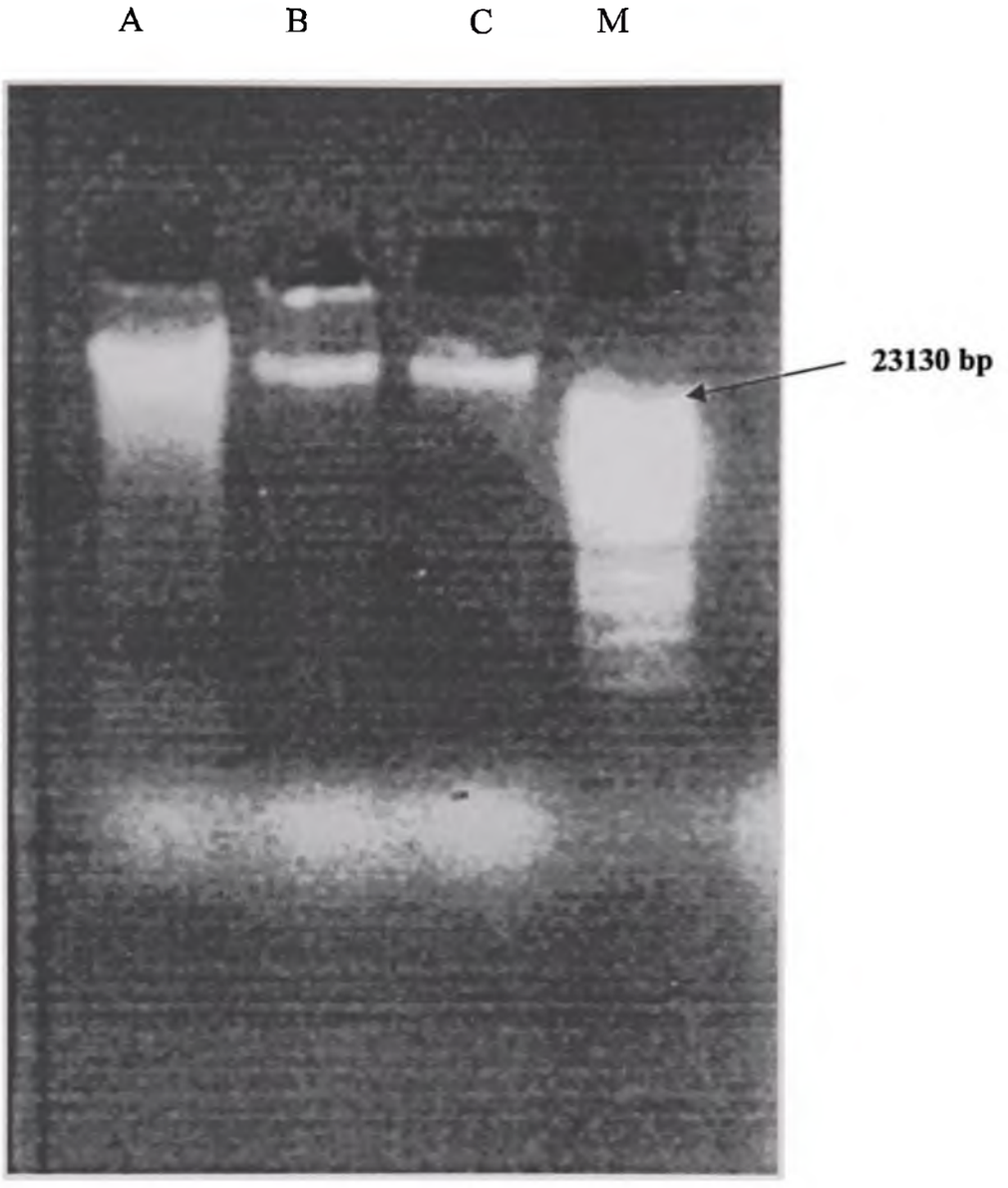


Figure 10: Agarose gel electrophoresis of genomic DNA extracted from larvae.

(lane A= *S. damnosum,* lane B= *C. oliffi* unfed larva, lane C= *Simulium*-fed larva of *C. oliffi,* lane M= λ-Hind III digest molecular weight marker).

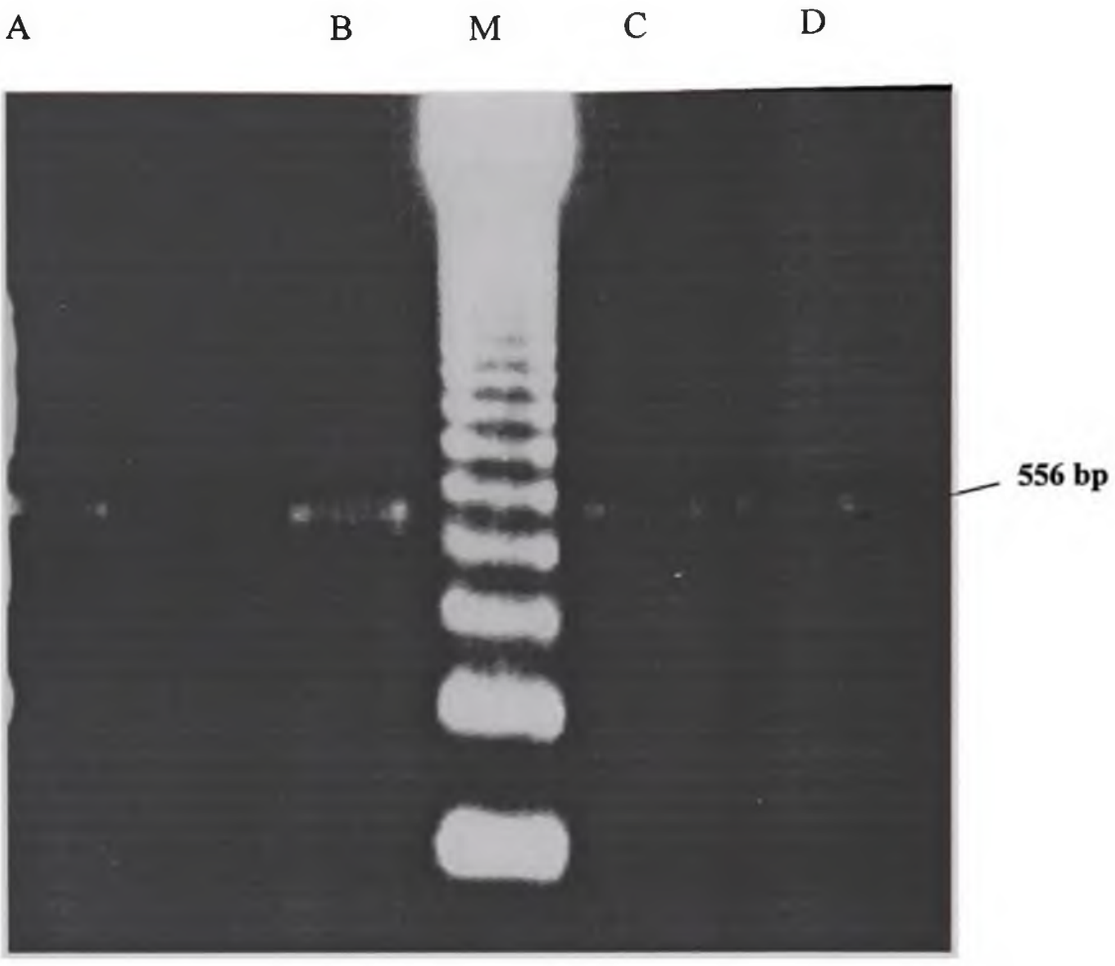


Figure 11: Agarose gel electrophoresis of PCR products from amplification of *S. damnosum*, *C. oliffi* unfed and *Simulium*-fed *C. oliffi* DNAs with primer set 16S.

(Lane A= *S. damnosum,* lane B= *C. oliffi* unfed, lanes C & D= *Simulium*-fed larvae of *C. oliffi,* lane M= 123 molecular weight marker).

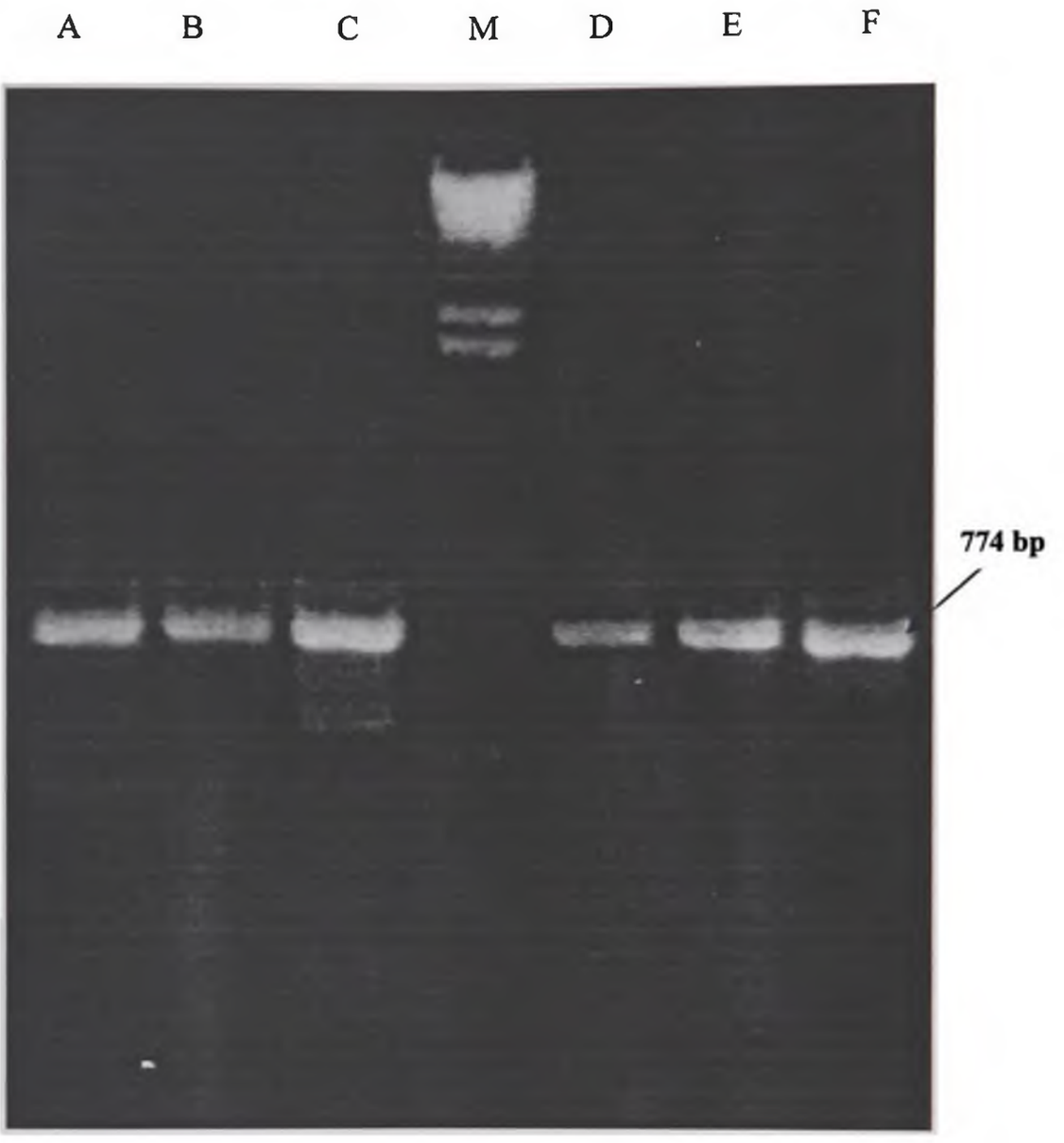


Figure 12: Agarose gel electrophoresis of PCR products from amplification of *S. damnosum*, *C. oliffi* unfed and *Simulium* fed *C. oliffi* DNAs with primer set P1-P2. (lane A= *S. damnosum,* lanes B & C= *C. oliffi* unfed, lanes D, E & F= *Simulium*-fed larvae of *C. oliffi,* lane M= lane M= λ-Hind III digest molecular weight marker).

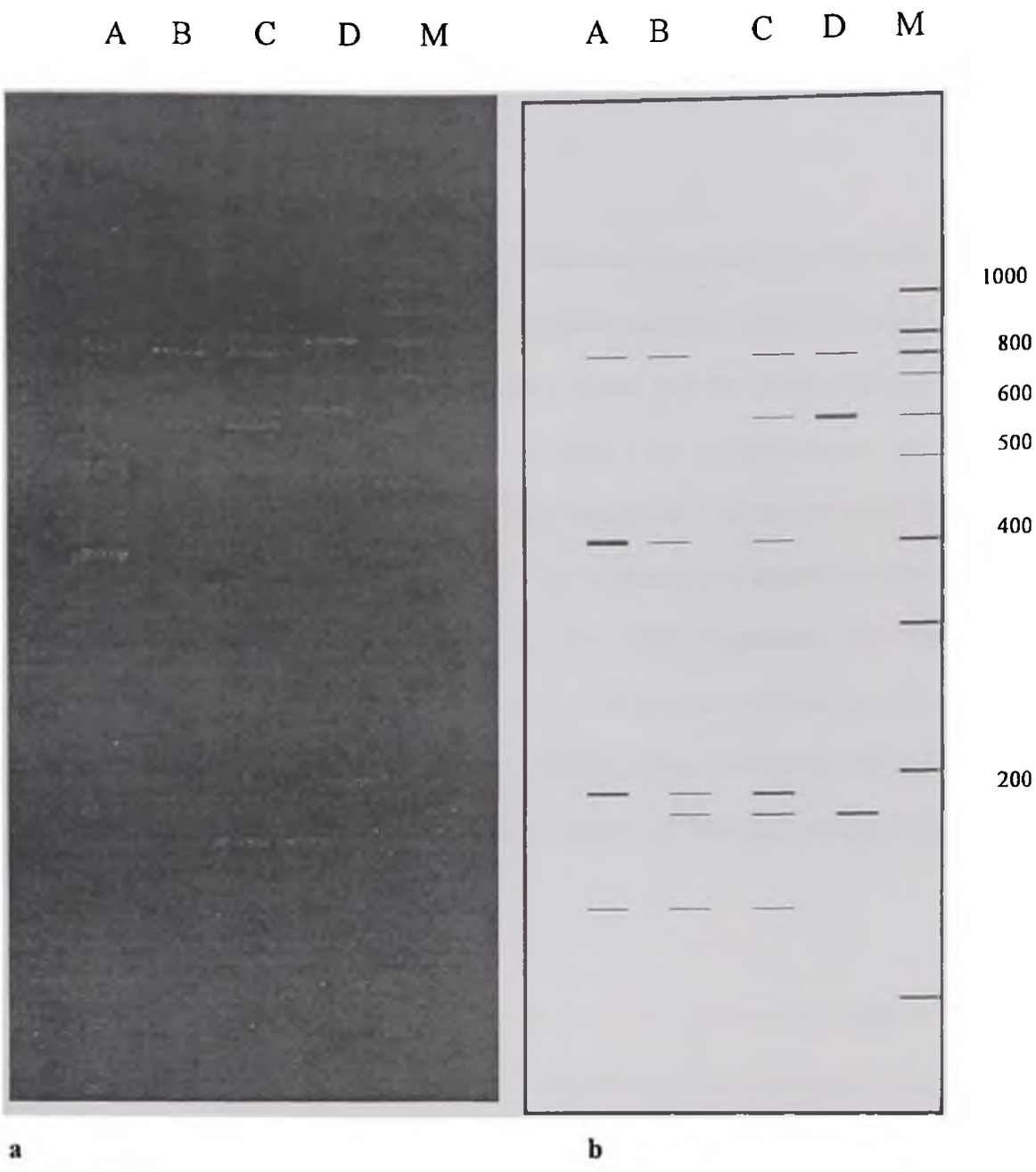


Figure 13: (a) Polyacrylamide gel electrophoresis of restriction digest products from the amplification of *S. damnosum*, *C. oliffi* unfed and *Simulium* fed *C. oliffi* DNAs with primer set P1-P2. (b) Schematic representation of the gel shown in (a)
(lane A= *C. oliffi* unfed, lanes B & C= *Simulium*-fed larvae of *C. oliffi*,=, lane D = *S. damnosum*, lane M= 100 bp ladder molecular weight marker). (Lines in bold represent sharp bands)

4.4 Potential of *Cardiocladius oliffi* as Biological Control Agent of *Simulium damnosum* s.l.

A total of 600 *S. damnosum* and 358 chironomids larvae were used for the experiments. It was found that 83% of the egg masses of *Simulium damnosum* also harboured *C. oliffi* eggs. Tables 3 and 4 summarize the number of larvae and the results obtained for the experiments carried in the rearing systems and Tables 5 and 6 summarize respectively the ANOVA and multiple comparisons of the mean number of *S. damnosum* adult emerged, larvae dead, pupae dead and eaten in the different experiments. A greater number of adult *Simulium* flies were observed to emerge from the control experiment. The difference between the mean number of adult *S. damnosum* flies emerged from the control and the mean number of adult *S. damnosum* flies from the various treatments was significant ($P<0.05$). The difference between the mean numbers of flies that emerged from the various treatments was not significant

There was no significant difference between the number of *S. damnosum* pupae that died, however, their numbers seemed greater in the treatments than in the control. The number of dead larvae though apparently greater in the treatments than in the control was not significantly different.

There was no difference between the proportion of adult *C. oliffi* that emerged from the control (25: 0) and the proportion of adult of the same species emerging from the various treatments. The *C. oliffi* in the control were fed only on fish food. This shows that the predator is not a specific predator to *Simulium* preys. Furthermore, this shows that in nature, it could sustain its development on other particulate matter.

Table 3: Number of *C. oliffi* and *S. damnosum* larvae used with the number of *S. damnosum* adults emerged and pupae eaten in the experiments.

Experiment (*C. oliffi: S. damnosum*)	Number of larvae used		Number of adults *S. damnosum* emerged	Number of pupae *S. damnosum* eaten
	C. oliffi	*S. damnosum*		
Control (0:25)	_	150	81	0
Treatment ii (1: 5)	30	150	22	28
Treatment iii (1: 2)	78	150	15	42
Treatment iv (1: 1)	150	150	10	55
Treatment v (25: 0)	100	/	/	/
Total	358	600	128	125

Table 4: Mean number of *S. damnosum* (adults and pupae eaten) that were observed in the various treatments regimes.

Experiment (Ratio *C. oliffi: S. damnosum*)	Number of adults *S. damnosum* emerged (sd)	Number of pupae of *S. damnosum* eaten (sd)
Control (0:25)	14.00 (3.94)*	0.00*
Treatment ii (1:5)	4.00 (2.94)	5.00 (3.82)
Treatment iii (1:2)	3.00 (2.16)	7.00 (7.45)
Treatment iv (1: 1)	2.00 (1.03)	10.00 (6.58)
Treatment v (25: 0)	/	/

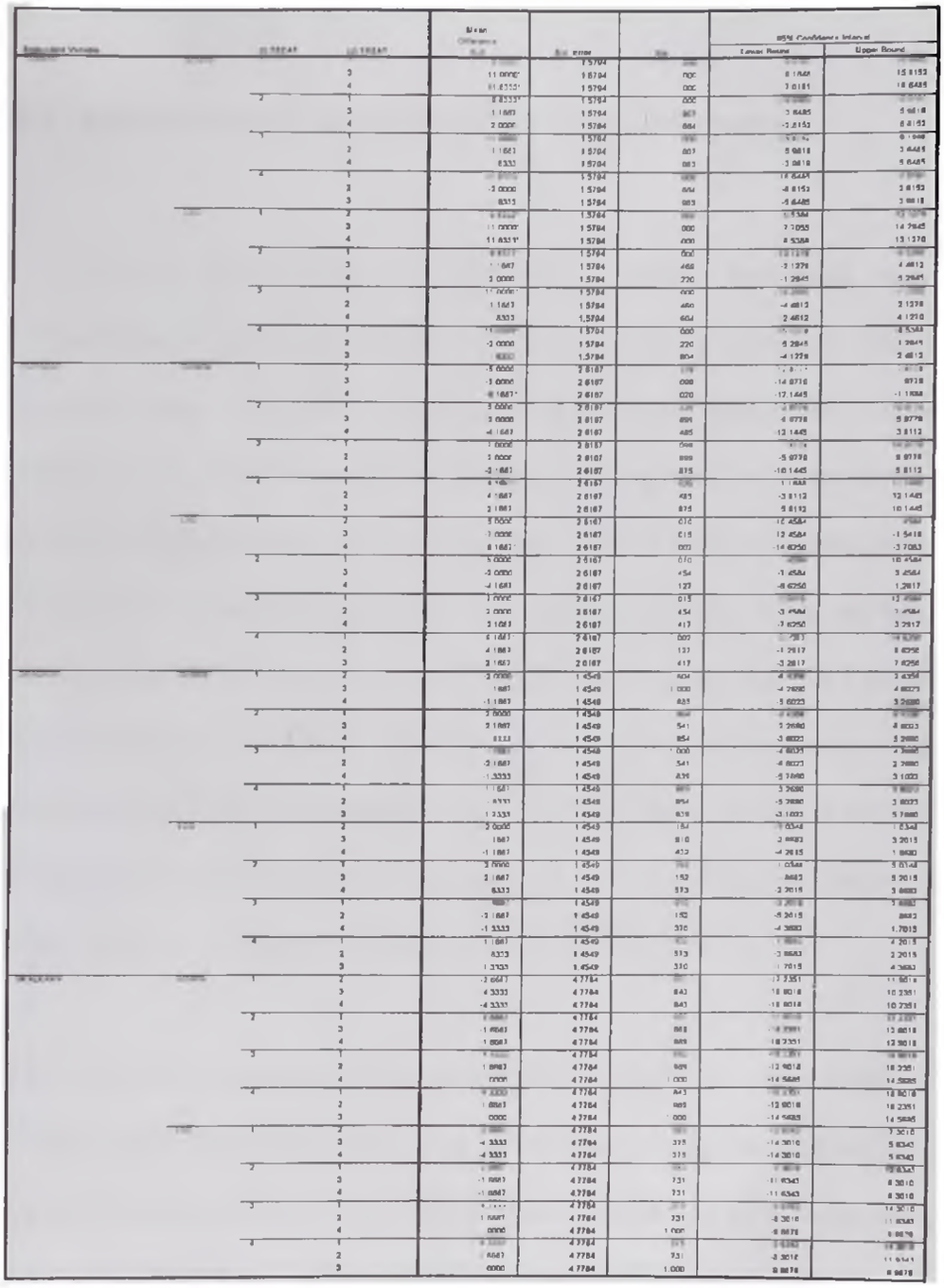
*indicates statistically significant differences at $p<0.05$

Table 5: ANOVA table of the for the comparison of the number of *S. damnosum* adults emerged (Adults.), dead pupae (Deadpu), pupae eaten (Pupaet) and dead larvae (Deadlarv)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ADULTS	Between Groups	545.667	3	181.889	24.306	.000
	Within Groups	149.667	20	7.483		
	Total	695.333	23			
PUPAEAT	Between Groups	276.125	3	92.042	4.481	.015
	Within Groups	410.833	20	20.542		
	Total	686.958	23			
DEADPUP	Between Groups	18.833	3	6.278	.989	.418
	Within Groups	127.000	20	6.350		
	Total	145.833	23			
DEADLARV	Between Groups	75.333	3	25.111	.367	.778
	Within Groups	1370.000	20	68.500		
	Total	1445.333	23			

Table 6: Multiple comparison of the number of *S. damnosum* adults emerged (Adults.), dead pupae (Deadpu), pupae eaten (Pupaet) and dead larvae (Deadlarv) in the different treatment regimes.



Chapter 5: Discussion and Conclusion

5.1 Morphological Identification of the Predator

Cardiocladius oliffi Freeman was described from male specimens from the Natal National park in South Africa, and the type specimens are kept in the British Museum. On his web page; "A checklist of the non-biting Midges (Chironomidae) of South Africa" Harrison A. D. (1999) provides some information on the distribution of *C. oliffi* in the South of Africa. However, its distribution throughout the African continent is not known. The flies are reported to breed in stones in rapids from Eastern Cape and Kwazulu-Natal (South Africa) to Ethiopia. So far, none of the four African species of *Cardiocladius* has been recorded in West Africa. This is the first report of their presence in Ghana and in the Onchocerciasis Control Programme (OCP) in West Africa. To date, a number of insects predacious on *Simulium* have been recorded from Ghana. These include the trichopteran *Hydropsyche* and *Cheumatopsyche* (Burton and McRae, 1972).

The larvae of Chironomidae of the genus *Cardiocladius* have a long history as predators of black flies. Their larvae are known to live among colonies of *Simulium* larvae and pupae on which they feed in rivers and streams (Thienemann 1954, reported by Freeman, 1956). In 1932, there was an attempt to import the chironomid *C. australiensis* from Australia to control the nuisance pest of cattle *Austrosimulium* in New Zealand.

5.2 Detection of *Simulium damnosum* DNA in the gut of *Cardiocladius oliffi*

The molecular detection of *S. damnosum* material in the gut of *C. oliffi* using the PCR products only was difficult because their sizes were close and therefore, separation on agarose gels could not be achieved in a satisfactory manner. This is certainly due to the fact that the regions from which the primers were designed in *S. damnosum* are also conserved in Chironomids. In fact, Chironomidae and Simuliidae are very close in phylogeny as they both belong to the super-family Chironomoidea. However, the restriction enzyme *AluI* that had already been used by Wilson (1994) showed that though the PCR products using the primer set P were close, the two differed in the sequence detail that could be exploited. The differential migration of restriction products on the polyacrylamide gel showed beyond any doubt that some *C. oliffi* larvae had *S. damnosum* material inside them.

5.3 Potential of *Cardiocladius oliffi* as Biological Control Agent of *Simulium damnosum* s.l.

These results showed that there was no significant difference between the number of *S. damnosum* dead larvae and dead pupae in the control and in the treatments regimes. However, *C. oliffi* larvae were observed on several occasions attempting to seize *S. damnosum* larvae, but generally failed to hold on to them firmly since the later could detach and move to change their location in the system. This continuous irritation of the *S. damnosum* larvae by the predator generally caused them to drift to occupy less suitable habitats. This was essentially true for young *Simulium* larvae, which were more vulnerable to the power of the water current generated by the aerator. This displacement by *C. oliffi* should have been expected to contribute significantly to larval mortality as a result of *S. damnosum* larvae drifting to unsuitable environment and floating in the fast water current. The results obtained however clearly indicate that this is not the case.

The experiments also revealed that the numbers of dead *S. damnosum* pupae in the various systems did not differ significantly. This finding therefore does not support the observation made by Grenier (1943, 1953) that chironomids by spinning their silken tubes within and between *S. damnosum* pupae promote the growth and accumulation of algae around their respiratory filaments, suffocating them and therefore reducing their populations. As shown by this study, such mortality on its own will not justify a drastic reduction of the number of adult *S. damnosum* flies emerging, but together with other factors such as active predation might contribute to successful control.

The results obtained have clearly shown that a significantly greater number of adult *S. damnosum* was obtained from the controls than from the treated rearing systems ($P<0.05$). This difference was obviously due to heavy predation of the *C. oliffi* larvae on *S. damnosum* pupae.

Effectively, the numbers of *Simulium* pupae that were eaten in the various systems by the predators were found to be significantly different. The predation on the *S. damnosum* pupae (evident by empty cocoons cases) therefore seems to be the only likely cause of significant reduction in the yield of adult *S. damnosum*. As expected, at the pupal stage, the prey cannot evade its predator since they were not mobile and therefore were easy target for *C. oliffi*.

The experiment also showed that *C. oliffi* and *C. kisantuensis* could complete their development successfully in the absence of *Simulium*. One might be tempted to think that predation on *Simulium* is facultative because of their confinement together. However, since in all the rearing systems *C. oliffi* larvae were fed on fish food and in the treatments (where they were with *S. damnosum*), they still went hunting *S. damnosum*, it is reasonable to assume that *S. damnosum* was preferred. Furthermore, it was observed that *C. oliffi* that were hitherto motionless quickly became active (less than 5 minutes) after *Simulium* larvae or pupae were introduced in the rearing systems seeking them.

The observation that most (83%) of the leaves with *Simulium* eggs from Boti falls and Huhunya were infested with egg of *C. oliffi* and *C. kisantuensis* is very revealing, indicating the possibility that the female chironomids intentionally lay their eggs among those of black flies in the river.

For any organism to be useful as biological control agent, it should be easy to rear it in large numbers in the laboratory. The chironomids are reared easily from egg masses to adults in the rearing system developed for *S. damnosum* by Boakye *et al.* (2000). Of 358 *C. oliffi* larvae reared in the study, 87.94% completed successfully their development to adults.

The mandate of OCP never included the control of biting nuisance. The disease onchocerciasis is no more of public health importance in West Africa. OCP is therefore coming to an end in the year 2002. The monitoring activities will be transferred to the respective countries. These countries do not have the financial resources, the skills and technical support to take over the vector control activities using chemical larvicides. The need for control measures that are cheap in the long range, self-sustaining and self-propagating is acute. It is not surprising that *C. oliffi* was found by the study to prey on *S. damnosum* in Ghana. The genus *Cardiocladius* has a long-standing reputation as *Simulium* predators (Zwick, 1978). In the 1931s, an attempt was made to import a species of this genus, *Cardiocladius australiensis* from Australia to New Zealand to control the pest *Austrosimulium* (Anonymous, 1932). The operation was not successful because as Crosskey (1990) suggested the attempt was half-hearted and was not vigorously pursued.

This work is a first step towards the ultimate use of *C. oliffi* larvae as a biological control agent of the *S. damnosum*. The study set out to incriminate the predator and to establish its potential as a biological control agent in the laboratory, which has been achieved. However, further studies need to be carried out before it can be used in the field. This study has already shown that *C. oliffi* can be reared easily in the laboratory. However,

mating, autogeny and oviposition in captivity by this species need to investigated. Further field studies are also needed. These include;

The investigation of the role of the other chironomid species (*Cricotopus kisantuensis*), as its eggs are also laid attached to those of *Simulium*.

- The detailed study of the ecological relationship between *S. damnosum* and *C. oliffi* in the river Pawnpawn.
- The study of the whole network of ecological relationships between all the living organisms that breed in the River Pawmpawm might contribute to the relative relief of the populations of the surroundings.

The studies on the seasonality and population dynamics/demographics of *C. oliffi* vis a vis *S. damnosum*.

The mapping of the geographic distribution of *C. oliffi* in similar river systems in other areas of Ghana in order to document the habitat and the micro-distribution of this organism with respect to the micro-distribution of the black flies.

- The investigation of the food chain in the other areas where *C. oliffi* might occur.

Further steps ahead will be to study the biology of the predator to find out how feasible it will be to breed them, as we know that they can be reared successfully in the rearing system developed by Boakye *et al.* (2000) for *S. damnosum*

The sequencing of *C. oliffi* PCR products to use the sequence data to develop *S. damnosum* specific primers, as the primers used also amplify chironomid DNA.

This approach combined with other methods available could contribute to bring the populations of *Simulium damnosum* at various breeding sites below biting nuisance levels, maintain the advantages of having brought onchocerciasis below public health concern and ensure the sustainable economic development of oncho-freed areas.

The predatory larvae of *Cardiocladius oliffi* observed in the laboratory may have a restraining influence on *Simulium damnosum* populations in the river Pawmpawm in the study area, if they behave in nature as much as they did in the laboratory.

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Appendices

APPENDIX I: PREPARATION OF STANDARD SOLUTIONS

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lb/sq in. for 15 minutes in an Eyela Autoclave (Rikikkaki, Tokyo).

Solutions for DNA Extraction

Bender buffer	0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS. Stored at 4°C
0.5 M EDTA (pH 8.0)	186.1 g/l in water, pH adjusted with NaOH pellets and stored at room temperature.
EtBr (10 mg/ml)	1g of EtBr was completely dissolved in 100 ml ddw and stored in the dark at room temperature.
KAc (5M K, 8M Acetate)	60 ml of 5M KAc and 11.5 ml glacial acetic acid in 28.5 ml distilled water.
RNase	10 mg/ml in water. Heated at 65°C to inactivate any DNAse and sterilised by filtration. Stored at -20°C.
TE (pH 8.0)	10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0). Stored at room temperature.

Solutions for Electrophoresis

For Agarose Gels

10x TAE buffer,	242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with ddw
0.5M EDTA (pH 8.0)	186g of EDTA, dissolved in 800 ml ddw, pH adjusted with NaOH pellets and stored at room temperature.

Urea polyacrylamide gel

10x TBE electrophoresis buffer:

108g/l Tris base, 55g/l boric acid, 9.3g/l Na_2EDTA. Use distilled water added to make up volume to 1l. Stored at room temperature on the bench. Diluted to 1x working solution for electrophoresis.

30% Acrylamide	29g acrylamide, 1g bis-acrylamide in 100 ml sddw. Filtered and stored in the dark at 4°C.

The Table below gives the required amount of ingredients necessary to prepare the solution for the 5% polyacrylamide running (separation) gel and the plug.

Ingredients	**5% polyacrylamide gel solution**
Water (ml)	30.00
Urea (g)	8.25
30% Acrylamide/0.8% Bis-acrylamide (ml)	8.50
10 x TBE Buffer (ml)	5.50

Ten microlitres ammonium persulfate (APS) and 5 µl of TEMED were added to 2.4 ml of the gel to plug the plates to avoid leakage.

Sixty microlitres of APS and 30 µl of TEMED were added to the rest of the solution (48 ml) to form the separation gel, before pouring.

The table below gives the required amount of ingredients necessary to prepare the stacking gel for a 5% Polyacrylamide gel.

Ingredients	**4.5% polyacrylamide gel solution**
Water (ml)	31.00
Urea (g)	8.25
30% Acrylamide/0.8% Bis-acrylamide (ml)	7.50
10 x TBE Buffer (ml)	5.50
Total	50.4 ml (filter, degas and store at 4 C)

3.5 ml of this solution were taken per gel, 30 µl of APS and 10 µl of TEMED were added and used as stacking gel.

The gel was stained in EtBr.

Gel Loading Buffers

5x Orange G	20% (w/v) Ficoll, 25 mM EDTA, 2.5% (w/v) orange G. stored at room temperature.
6x Bromophenol blue	0.25% bromophenol blue, 40% sucrose in water. Stored at 4°C
Bromophenol blue xylene cyanol	1 volume of Bromophenol blue xylene cyanol and 4 volumes of formamide.

DNA molecular weight size markers

The following DNA molecular weight size markers were used at various stages of the study: those that were obtained from the manufacturers e.g. 123 bp maker, 100 bp ladder marker and 1 kb marker were diluted according to their recommendations.

-λ/Hind III molecular weight marker

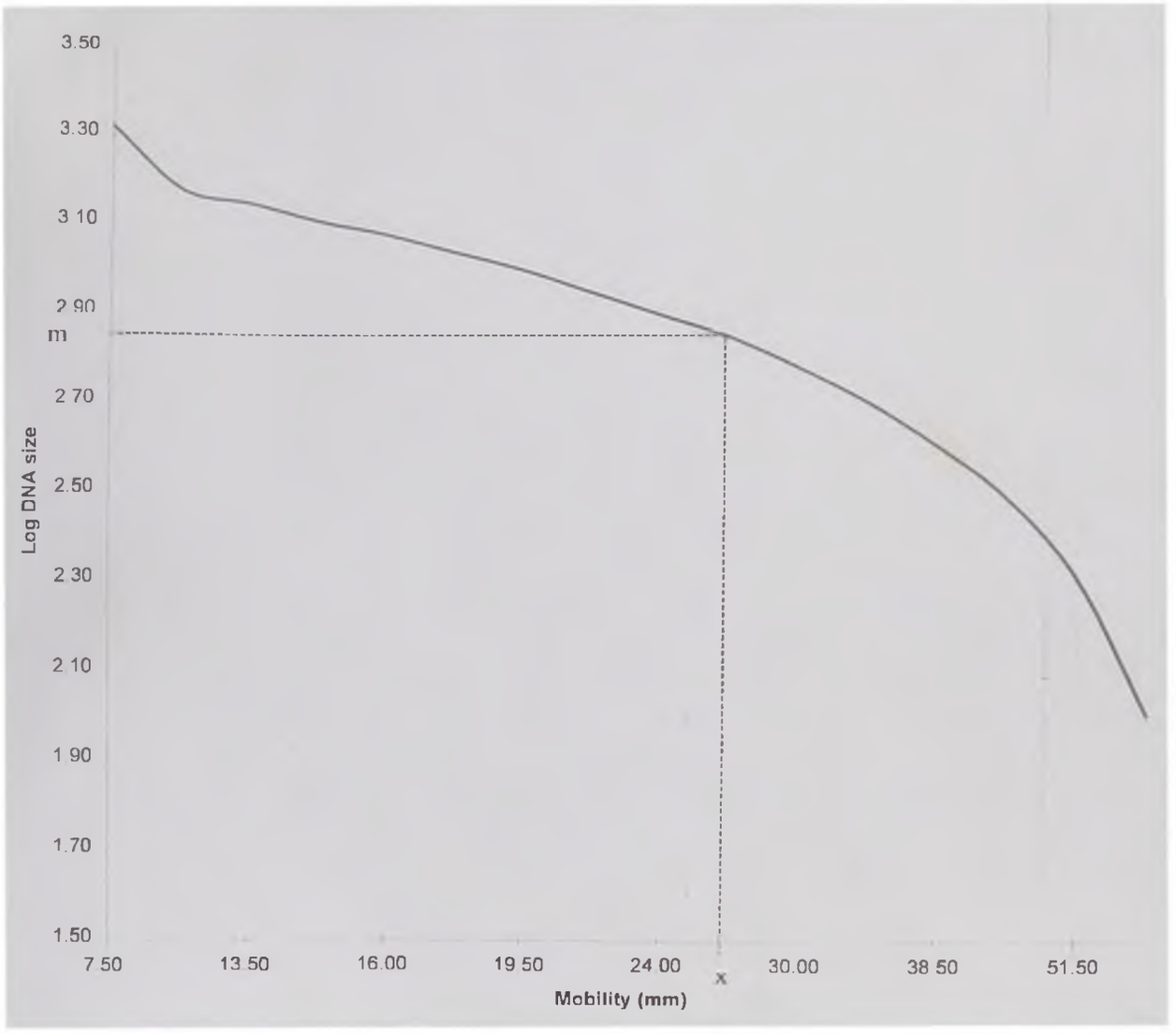
Digestion of λ/DNA with Hind III generates 8 fragments of the following lengths: 23130 bp, 9416 bp, 6682 bp, 4361 bp, 2322 bp, 2027 bp, 564 bp, 125 bp.

-123 marker: The first band size is 123 bp, the second 2x 123 bp ... and the n^{th} band nx 123 bp.

-100 bp ladder molecular weight marker: The first band size is 100 bp, the subsequent ones measure 200, 300 .... 1000 bp.

-1 kb marker: This marker has 11 bands that are of the following weights: 0.5 kb, 1.0 kb, 1.5 kb, 2.0 kb, 2.5 kb, 3.0 kb, 4.0 kb, 5.0 kb, 6.0 kb, 8.0 kb and 10 kb.

Appendix II: An example plot of log Molecular weight against mobility to determine sizes of PCR products



The size of a DNA fragment that has a mobility x; the band size is estimated by intrapolating on to the Y-axis. The anti Log of the figure where it transects the Y-axis is the molecular weight of the fragment.