

CHEMOATTRACTION BETWEEN ADULT *SCHISTOSOMA MANSONI* WORMS

by

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(Under the Direction of Raymond T. Damian)

ABSTRACT

My doctoral research addressed questions about the nature of the attraction between adult *Schistosoma mansoni* worms, which are parasitic blood flukes trematodes of man. This research represented one of the sincere attempts to identify and to purify a mate attractant factor from these organisms. My main objectives were (a) to develop an improved bioassay to assess the behavior of *S. mansoni* male and female worms towards each other, (b) to look further into the nature of the mechanism by which male and female schistosome worms find each other in the definitive vertebrate host, (c) to explore the nature of the chemotactic factor(s) released by either or both sexes to attract the opposite sex and the dynamics of the attraction, and (d) to characterize, isolate and sequence one of the factors. Most of this work focused on the female-released male-attractant factor(s). Using a combination of bioassays, size fractionations, chromatographic and chemical and enzymatic treatments, I have shown that there is mutual attraction between the opposite sexes of adult *S. mansoni* worms and absence of same sex attraction. The females were attracted to possibly more than one male-released factors; a <30kDa and a >30kDa factors. Males, were attracted to a <10kDa female released factor(s). The attractant factors were heat-labile and dead worms from both

sexes were still attractive. Male-attractant factor(s), was not in the female vomitus, and TLC showed the absence of lipids and carbohydrates. Male-attractant factor(s) were found to be sensitive to proteases and to be hydrophilic in nature. The results are consistent with the hypothesis that the female-released male-attracting factor(s) has a protein/peptide component of a hydrophilic nature. Using RP-HPLC and specific chemical treatments I have identified, purified and characterized for the first time a 3260 Da peptide released by *S. mansoni* adult females that plays a role in attracting the males. I also succeeded, using MALDI peptide-MS and MALDI-TOF/TOF, in getting a partial amino acid sequence <QVHHQK> of that peptide. Although I did not identify a homologous sequence in the available schistosomiasis database, completion of the *S. mansoni* genome will allow eventual identification of the complete peptide.

INDEX WORDS: Chemoattraction, *Schistosoma mansoni*, male worms, female worms, chemotactic factors, attractant, male released factors, female released factors, MALDI, peptide-MS, MALDI-TOF/TOF

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To Enas, Kareem, Farrah, and my Mother

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

HISTORICAL BACKGROUND

Human schistosomiasis is a world-wide health problem affecting more than 200 million people in many countries and is one of the most prevalent life threatening parasitic disease in the world, (Brillet, 2000; Gryseels, 2000a) The parasite is one of the most complex parasites of humans (Gryseels, 2000a). It is caused by certain parasitic flukes in the family *Schistosomatidae*. The *Schistosomatidae* are dioecious digenean parasites of the blood vascular system of vertebrates. Of the 13 genera in the family, only *Schistosoma* is associated with humans. Traditionally, a combination of egg morphology, intermediate host and geographical distribution has been used to assign the 19 species of the genus to 3 groups, centered on important parasites of humans: *Schistosoma mansoni* (lateral spined egg group) present mainly in Africa and South America, *S. haematobium* (terminal spined egg group) found in Africa and Middle East, and *S. japonicum* (rounded, minutely spined or spineless eggs) which is endemic in Asia (Johnston *et al.*, 1993; Lardans and Dissous, 1998; Southgate *et al.*, 1998). *S. haematobium* inhabits the veins of the genitourinary system, and *S. mansoni* and *S. japonicum* inhabit the veins of the large and small intestines respectively (Southgate *et al.*, 1998).

There are three other species of schistosomes that infect humans, but are of lesser prevalence. *S. mekongi* is restricted to the Mekong river basin between Laos, Thailand and Cambodia, and its intermediate snail host is *Neotricula aperta* (Neva and Brown,

1998; Urbani *et al.*, 2002). It was first identified by Voge *et al.*, (1978). The second is a relative of *S. japonicum*, called *S. malayensis*, and is endemic to the mountainous region of Malaysia (Greer *et al.*, 1988; Neva and Brown, 1998). The third is *S. intercalatum*, found in some regions of central and western Africa, and is considered to have arisen as a hybrid between *S. haematobium* and animal schistosomes (Zwingenberger *et al.*, 1990; Neva and Brown, 1998).

Schistosomiasis was first recognized by the Egyptians 4000 years ago (Ruffer, 1910), and its presence in Egypt as early as the 16th century B.C. has been proven by the demonstration of calcified eggs of *S. haematobium* in the kidneys of two Egyptian mummies (Adamson, 1976).

Theodore Bilharz in Cairo (1852) discovered the *S. haematobium* worm that secreted eggs with the terminal spine in the urine of patients and provided the first description of many of the pathological sequelae of acute infection, whereas, a case from the Caribbean with schistosomiasis had many eggs with a lateral spine in the feces but not found in the urine (Katz *et al.*, 1989; Sturrock, 2001). Sambon (1907) was the first to differentiate these two *Schistosoma* species on the basis of morphology and origin of the eggs in the stool and urine and the new organism, *S. mansoni* was named by him (Katz *et al.*, 1989). Katsurada (1904) was the first to identify *S. japonicum* in a wild infected cat (Katz *et al.*, 1989).

Schistosomiasis has a great global impact, with over 200 million people (of which 150 million live in Africa) who are currently infected world wide in the tropics and the subtropics, 600 million people in 74 countries who are at risk, 120 million who are symptomatic, and 20 million who suffer severe consequences of this disease (WHO,

1996; Capron, 1998; Vennervald *et al.*, 1998; Brillet, 2000; Chitsulo *et al.*, 2000; Engels *et al.*, 2002).

LIFE CYCLE, INFECTION AND ILLNESS

The schistosomes are non-replicating organisms in their vertebrate hosts, unlike protozoan parasites such as *Plasmodium* (Capron and Dessaint, 1992). They are genetically programmed to survive sequentially within two widely different specific hosts, a snail and a vertebrate, and are among the very few platyhelminths that display sexual dimorphism (Capron, 1998).

The genus *Schistosoma*: *S. mansoni*, *S. haematobium* and *S. japonicum* each have their snail intermediate hosts which belong to the genera *Biomphalaria*, *Bulinus*, and *Onchomelania*, respectively. The intermediate host of *S. mansoni* is found in Africa, the Middle East, South America and the Caribbean. The intermediate host of *S. haematobium* is found in Africa and the Near and Middle East, the intermediate host of *S. japonicum* is located in the Far East (Manson-Bahr and Bell, 1987). Human and animal excreta containing schistosome eggs contaminate fresh water harboring these snail intermediate hosts. The miracidia emerging from hatched eggs infect the snails and replicate asexually forming numerous germinal sporocysts that develop into thousands of mature cercariae. Under the influence of photic stimulation the mature cercariae escape the snail host in the water and swim searching for their vertebrate host, detected by thermal and chemical senses. When identified, the cercariae adhere to, and penetrate the skin of the host exposed to the contaminated water. Upon penetration, each cercaria becomes an immature worm, a schistosomulum, that migrates through the tissues of the skin, to the

venous system, to the heart, through the lungs, and then to the liver where they mature (Phillips and Colley, 1978; Dean and Mangold, 1983). Some cercariae may die in the skin and are rapidly cleared from there and some may fail to reach the lungs and eventually the liver and get lost (Dean and Mangold, 1983).

Adult schistosomes live *in copulo* within their definitive intravascular locations. *S. mansoni* and *S. japonicum* inhabit the inferior and superior mesenteric veins and the portal venous system, respectively, and thus the eggs they lay affect the gut and liver. *S. haematobium* schistosomula migrate to the liver for growth and maturation into adult worms after which they migrate to the venous plexus of the pelvic organs and the disease affects mainly the genito-urinary tract. Eggs laid by adult worms cause urinogenital obstructive disease, haematuria, inflammation and malignant squamous carcinoma. The lungs and the central nervous system may also be affected (Warren, 1973; Jordan and Webbe, 1982; Scrimgeour and Gajdusek, 1985; Smith and Christie, 1986;). Egg production is totally dependent upon the physical development and sexual maturation of the female pairing with a male (Moore *et al.*, 1954; Clough, 1981).

An estimated 300,00-500,000 deaths occur each year from the complications of schistosomiasis, which include liver fibrosis, urinary bladder cancer and damage to the central nervous system (Capron *et al.*, 1994). These complications arise mainly from the host's immune system which attacks the parasites' eggs trapped in the tissues and organs; e.g. the liver, of the host (Warren, 1973; Phillips and Colley, 1978; Edungbola and Schiller, 1979; Newport and Colley, 1992;).

The host response to schistosomiasis is often divided into general aspects. First is the response to the invading, developing and mature worms, which is concerned with

resistance to infection. Second is the response to the biological products of that organism, primarily the eggs, which is concerned with pathologic processes and morbidity.

Dermatitis, which results from cercarial penetration of the skin, is due to hypersensitivity reactions of both immediate and delayed immune types (Webbe, 1981).

Katayama fever (acute schistosomiasis) occurs in intense initial infection (Ister *et al.*, 1984) or after overwhelming superinfection (Ottessen *et al.*, 1978). It usually coincides with the onset of egg laying by the worms (Clarke *et al.*, 1979; Hiatt *et al.*, 1979; Zuidema, 1981) as experimentally confirmed in the baboon model (Damian *et al.*, 1992). Acute schistosomiasis is associated with malaise, weight loss, gastro-intestinal signs and systematic manifestations such as fever, skin rash, eosinophilia and hepatosplenomegaly (Hiatt *et al.*, 1979).

The clinical consequences of the disease are chronic in nature (Vennervald, 1998). Chronic schistosomiasis is characterized by a wide variety of lesions in different organs (Warren, 1973, 1977; Pope *et al.*, 1980). Entrapped eggs in the liver release soluble antigens eliciting T-cell dependent granulomas that accumulate around each egg (Davis *et al.*, 1974).

The severity of the disease schistosomiasis is often directly proportional to the infection load: the number of adult worms present, the number of eggs produced, and the sites of the lesions they provoke (Goldsmith, 1983; Chan *et al.*, 1996; Lima *et al.*, 1996; Andrade *et al.*, 1997; Brillet 2000).

HUMAN IMMUNITY TO SCHISTOSOMIASIS

The concept of concomitant immunity was first introduced by Smithers and Terry (1969). This type of immunity describes the condition when an actively infected host resists, or partially resists, a subsequent challenge infection by the same organism (Arnon, 1991). Support for the concept has developed from studies of *S. mansoni* in baboons (Taylor *et al.*, 1973; Damian *et al.*, 1976), *S. mansoni* in mice (Sher *et al.*, 1974), and *S. haematobium* in the baboons (Webbe and James, 1973). Bradley and McCullough, 1973, proved the existence of this concomitant immunity to *S. haematobium* in man as well.

Gother (1963) exposed himself 16 times to *S. haematobium* cercariae which had been attenuated by DDT, then infected himself again using active unattenuated cercariae. Neither eggs nor other symptoms of the disease were observed.

Clinical infection data give evidence for acquired immunity in humans. Man lives under constant re-exposure as in the case of Egyptian peasants and the rice farmers of the Orient, who are in almost daily contact with cercariae-containing water since childhood. Such individuals do not manifest overwhelming infection, which surely indicates an acquired resistance to re-infection (Stirewalt, 1963).

Many studies have been done examining the resistance to reinfection in humans. Wilkins and colleagues worked on *S. haematobium* in Gambia (Wilkins *et al.*, 1984). They found that the acquisition of new infections by adults over 25 years old was 1000-fold less than that of 5-8 years old children, a difference that could not be attributed to a comparable 1000-fold reduction in water contact levels among the adults. When they studied the intensities of reinfection following treatment for *S. haematobium* infections

they found that for each level of exposure there was marked age-dependent reinfection, with heavily-exposed 2-9 year old children showing intensities of infection 100-fold greater than comparably-exposed individuals of more than 15 years of age.

Hagan *et al.*, (1985) studied the role of eosinophils in resistance to *S. haematobium* in man. They confirmed that human eosinophils could mediate antibody dependent damage to schistosomula of *S. haematobium in vitro* and demonstrated a significant association between high eosinophil counts and subsequent lack of re-infection in 8-13 year old children.

Butterworth and Hagan (1987) proposed a hypothesis correlating resistance to reinfection with age and immune response. The hypothesis states that during early infections of young children, the major immunogenic stimuli are antigens released from eggs (which are present in much greater mass than either larvae or adult worms). These antigens, including polysaccharides or heavily glycosylated glycoproteins, elicit blocking antibody responses, which include immunoglobulin M or possibly certain isotypes of immunoglobulin G, IgG2 and IgG4. Blocking antibodies would then cross-react with major glycoproteins on the schistosomulum surface, thereby blocking the binding of antibodies that mediate effector functions, such as eosinophil-dependent killing of schistosomula. During early infections, the balance of the response is towards the blocking antibodies and, although potentially protective antibodies can be detected, the child is not immune. Subsequently, as the child ages, the balance may switch from a predominantly blocking to a predominantly protective type of response, and the child may then express the capacity to resist reinfection.

Much work has been done that support the above hypothesis (Chandiwana *et al.*, 1991; Hagan, *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993; Ndhlovu *et al.*, 1996; Webster *et al.*, 1997; Gomes *et al.*, 1998; Gomes *et al.*, 2002) and shows that the acquired immune responses of humans that are associated with resistance to re-infection are all of the Th2 type including IL-4 (Medhat *et al.*, 1998), IL-5 (Roberts *et al.*, 1993; Medhat *et al.*, 1998), Ig E (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992) and eosinophils (Hagan *et al.*, 1985).

Karanja *et al.*, (2002) have shown some evidence for the existence or the development of immunological age-independent resistance to reinfection in adults.

Also it has been argued that physiological factors like skin thickness, lipid content (Hagan *et al.*, 1998b), and hormone levels also vary with age, and might modify innate or acquired resistance to infection (Daynes *et al.*, 1990; Shurrs and Verheul, 1990; Suzuki *et al.*, 1991; Daynes *et al.*, 1993; Besser and Thorner, 1994; Herbert, 1995; Parker, 1995; Shealy, 1995; Agnew, *et al.*, 1996; Fulford *et al.*, 1998; Hagan *et al.*, 1998b)

There is some evidence regarding specific genetic elements in humans that regulate the capacity to mount protective immune responses leading to the development of resistance to schistosomiasis (Abel *et al.*, 1991; Marquet *et al.*, 1996)

Yet, and in spite of all of the above, there is great debate on the ability of human beings to develop resistance (Gryseels, 2000; Hagan *et al.*, 2000), and whether or not it is age-related (Gryseels, 1994).

PREVENTION AND CONTROL

Schistosomiasis is a behavioral disease in that people expose themselves to infection with cercariae often through a lack of choice and availability of safe water supplies for recreational and domestic purposes (Hagan *et al.*, 1998a). The main causes of infection were determined among three target populations: swimming in infested water in youth, clothes-washing in infested water in adult women, and exposure to infested water in production activities in adult men, with the latter being difficult to avoid as long as the old production style continues (Xiaonong *et al.*, 2002)

Approximately 80% of those infected live in sub-Saharan Africa (WHO, 1997; Chitsulo *et al.*, 2000) where the rapid development of irrigation and hydroelectric projects created fresh water habitats for the snail, and this has given rise to the spread of the disease (McManus, 1992). In Africa, the construction of the Aswan dam on the Nile, the Volta dam in Ghana and the Bafing dam on a major tributary of the Senegal river in Mali have given rise to schistosomiasis outbreaks (El-Katsha and Watts, 1995; Lardans and Dissous, 1998). Land reclamation has extended transmission of schistosomiasis to new areas in Egypt (El-Sayed *et al.*, 1995) such that the number of infected people in Egypt has not reduced significantly (Savioli *et al.*, 1997).

In spite of the fact that there have been important local successes in the control of the disease, for example in China, Brazil and Tanzania, globally the infection rate has increased during the last decade. This has been primarily due to population increases in endemic countries and partly due to migration, which resulted both in increases in endemic areas and the introduction of the parasite into uninfected areas (Bergquist, 1993).

Control of snails, whether chemically, biologically or by modification of the snail habitat, can achieve success, although limited, only by a carefully selected combination of measures. The use of molluscicides has contributed its share of benefits but has raised problems as regards to their effects on humans, animals and plant resources (WHO, 1985). For assessing the success of mollusciciding, the reduced number or absence of snails, the determination of the infection rate in snails and measurement of cercarial numbers by animal exposures or filtration techniques have been used (Muller, 1975).

In China, the anti-flood policy of 'returning the cultured lands into lakes, and breaking levees for storage of flood water' will be implemented with the consequence of snail habitats re-appearing through the construction of embankments in 90% of formerly snail eliminated areas (Xianyi, 2002).

Snails genetically resistant to infection with schistosomes were advocated as potential tools for the control of schistosomiasis (Rupple *et al.*, 2002)

Chemotherapy is one of the most valuable tools as a primary means of controlling schistosomiasis (Jordan, 1977; Lucas, 1980; Webbe, 1981). It is a practical strategy in the reduction of prevalence, intensity, and associated morbidity due to schistosomiasis, thus leading to the reduction of transmission (Stephenson *et al.*, 1985; El-Tayeb *et al.*, 1988; King *et al.*, 1988). For a long time, only harsh antimonial drugs were available for the chemotherapy of schistosomiasis (Cioli *et al.*, 1995; Cioli, 1998; Harder, 2002). The three drugs metrifonate (active against *S. haematobium*), oxamniquine (active against *S. mansoni*) and praziquantel (PZQ) are now used for mass treatment in different parts of the world, with PZQ being the drug of choice as it is effective against all relevant

schistosomes (Harder, 2002). Other drugs approved by the WHO are also available such as artesunate and artemether (Cioli, 1998; De Clercq *et al.*, 2002; Shuhua *et al.*, 2002).

Treatment with PZQ results in egg clearance in 60-90 % of infected people, and in a reduction in egg loads of more than 95% among those who remain with patent infection after treatment. Furthermore it results in a significant regression of schistosome-induced lesions, especially those in their early stages (Day *et al.*, 1992). A combination of chemotherapy, using PZQ, and mollusciciding program in the Fayoum area of Egypt led to a decreased prevalence of *S. haematobium* from 46% to 7% in about 12 years (Abdel Salam *et al.*, 1986). In Beheira, Egypt, a schistosomiasis control program that began in 1983 succeeded in reducing the prevalence of *S. mansoni* infection from 60.3% to 24.8% and that of *S. haematobium* from 37.6% to 5.5% (Spencer *et al.*, 1990).

The most obvious effects of the drug on the schistosome are tegumental disruption, the induction of paralytic muscle contraction, and the induction of Ca²⁺ influx into the tegument, of which the exact mechanism for the latter is not yet clear (Day *et al.*, 1992; Redman *et al.*, 1996). It also induces changes in the antigenicity of the parasite causing the exposure or release of previously concealed parasite surface antigens on the tegument, and consequently the formation of antibodies of other immune mechanisms that work in synergy with direct actions of the drug, allowing the elimination of the parasite (Sabah *et al.*, 1985; Xiao *et al.*, 1985; Brindley and Sher, 1987; Modha *et al.*, 1990; Day *et al.*, 1992). The main limitation on the use of PZQ for control is the high reinfection rate in endemic areas even after mass treatment (Bergquist, 1993). The impact of chemotherapy in an endemic community is of limited duration even if combined with

snail control (Bergquist, 1990). Repeated treatment is therefore necessary but it is not yet clear at what intervals such treatment should be undertaken (Bergquist, 1993).

The main concern about chemotherapy is the emergence of drug resistance, although the exact definition of 'resistance' and how it should be measured are still arguable (Fallon *et al.*, 1996; Coles and Kinoti, 1997; Giboda and Smith, 1997; Picquet *et al.*, 1998; Van Wyk, 2001; Coles, 2002). Resistance has developed under both laboratory and field conditions (Butterworth, 1988; Anonymous, 1992; Stelma *et al.*, 1995; Bennett *et al.*, 1997). In Brazil, field resistance to schistosomicides was first reported with hycanthone and oxamniquine (Katz *et al.*, 1973; Campos *et al.*, 1976; Hussain *et al.*, 1983). Coles *et al.*, 1987, showed that in Kenya worms resistant to oxamniquine were probably present even before extensive chemotherapy had been used. Evidence was presented for a *S. mansoni* PZQ-resistance phenotype in an infected population in Egypt (Ismail *et al.*, 1996; Hagan *et al.*, 1998a) and in Senegal (Anonymous, 1993; Fallon *et al.*, 1995). In Senegal, alternative use of oxamniquine gave the expected high cure rate in the same population that showed PZQ resistance (Stelma *et al.*, 1997). Some reports exist for PZQ resistance in *S. haematobium* (Herwaldt *et al.*, 1995; Murray-Smith *et al.*, 1996) and in *S. mekongi* (Ajana, 1986). There are laboratory data that support the existence of *S. mansoni* resistance to PZQ (Fallon and Doenhoff, 1994; Fallon *et al.*, 1995; Liang *et al.*, 2001) and to oxamniquine (Fallon and Doenhoff, 1994). The above suggests that schistosomiasis is no exception to the general rule for infectious diseases, that in circumstances of heavy drug pressure, selection of drug-resistant strains will eventually occur (Butterworth, 1988). Therefore, there is further need to look for new synthetic or natural antischistosomal drugs (Harder, 2002). It can also be seen that the control of

schistosomiasis cannot be achieved by chemotherapy alone, and potent, cheap and safe vaccines are in great need (Butterworth *et al.*, 1987; Wilkins, 1989). A vaccine against schistosome infections would be of great value by strongly reducing the establishment of adult worms and hence a reduction both in pathology and in egg excretion (Butterworth, 1988; McManus, 1992), though some have argued about its real worthiness (Gryseels, 2000 a and b).

The first step towards the production of anti-schistosomal vaccines is the identification of target antigens presented by the schistosomula or adult worms (Dissous *et al.*, 1981; Smith and Clegg, 1985), or more generally, an antigen, whether surface or non-surface, capable of evoking a host immune response which results in elimination of the parasite (Norden *et al.*, 1982; James *et al.*, 1987). Vaccination in animal models against human schistosomes using defined schistosome antigens has been reported, with the level of immune protection in these immunogens varying from 20-80% as measured by worm burden recoveries (James and Sher, 1990).

HOST-PARASITE INTERACTIONS AND VACCINE DEVELOPMENT

The host immune responses are absolutely central to the strategies for controlling both infection and pathology (Capron, 1992; Capron, 1998). Parasite development, egg production, granuloma formation, disease severity, resistance to reinfection and drug efficacy - all have been shown to depend, sometimes essentially, on immunological factors (Capron, 1992; Dunne *et al.*, 1995; McCarthy and Nutman, 1996; Brunet *et al.*, 1998; Capron, 1998; Davies *et al.*, 2001; Viney, 2002).

One of the major research efforts in schistosomiasis is the development of an effective vaccine despite the fact that vaccine-induced immunity is never complete. Other limitations towards a human vaccine are due both to differences observed among animal models and the danger of direct extrapolation from experimental systems to humans (Capron, 1998). Another hurdle faced is the basic physiological differences between hosts (Gryseels, 2000a). Also to be considered is that the results from independent testing are disappointing (Bergquist and Colley, 1998; Gryseels, 2000b). These difficulties lead some to raise the question of “Do we really need a vaccine?” and to consider the area of schistosome population genetic structure to be more important than that of vaccine development (Bennett, 2000).

Still, progress has been made and several experimental vaccines have been developed that have reached human trial level. These human vaccine candidates have been reviewed by Bergquist *et al.*, (2002). The importance of vaccine development lies in the realm that they hold the promise of long-term prevention, while drugs provide short-term reduction of morbidity (Hagan *et al.*, 2000).

The main arguments in the field of schistosome vaccine development are focused on the efficacy of the vaccine candidates and the type of immune response needed to be elicited for vaccine effectiveness (Bennett, 2000; Gryseels, 2000a & b; Hagan *et al.*, 2000; Wynn and Hoffmann, 2000).

SENSORY SYSTEMS IN *S. MANSONI*

S. mansoni, like other platyhelminths, has a relatively complex nervous system, that employs peptidergic messengers, that likely play an integrative neuroendocrine-like

role in the regulation and the coordination of processes such as growth and reproductive development, and also is likely to control other bodily and behavioral activities that are essential to their complex life cycle (Halton *et al.*, 1992). Their tegument, in general, is structurally adapted, among other things, for communication with the neuromuscular system via gap junctions (Thompson and Geary, 1995; Halton and Gustafsson, 1996).

In the Digenea, there is a wide array of sensory organs in the tegument, in particular in miracidia and cercariae. Adults have less variety. Unciliated bulbous endings are found on the anterior portion of the cercariae of *S. mansoni*, similar to but smaller than those on the adult (Schmidt and Roberts, 2000).

In their review, Halton *et al.*, (1994), presented that in *S. mansoni*, an extensive plexus of peptidergic fibers and associated cell bodies has been observed in the lining of the gynacophoric canal that could indicate a sensory function in the orientation of male and female pairing (Sekuce *et al.*, 1990a). Also, a neuropeptide involvement in contact communications has been suggested by the peptide-rich innervation of the dorsal tubercles in the male, and by the peptide-immunoreactivities in the ciliated, putative sense-receptors that are scattered throughout the general body tegument of both sexes (Gustafsson, 1987; Basch and Gupta, 1988; Skuce *et al.*, 1990a). In addition to the adult worms, peptidergic substances have been detected in the miracidia and the cercariae stages of *S. mansoni* (Skuce *et al.*, 1990b). The mode of action of these regulatory peptides is suggested to be through specific receptors linked to ion channels or through G-protein-linked 7-transmembrane receptors (Halton *et al.*, 1994).

GENERAL OUTLINE AND OBJECTIVES

A main concept that has emerged from the war against schistosomiasis is that the potential control of the disease will rely on multiple and integrated strategies (Capron, 1998). Going on with this view, Modha *et al.*, (1998), put forward some unanswered questions in schistosome biology that if answered would be of great value in the control effort. Among them is “What are the chemical stimuli required for pairing of male and female schistosomes?” in the vertebrate host. This question is the focus of this study. There were two specific aims set forth for the project: (a) to develop an improved bio-assay to assess the behavior of *Schistosoma mansoni* male and female worms towards each other, and (b) to look further into the nature of the mechanism by which male and female schistosome worms find each other in the definitive vertebrate host. Both (a) and (b) are covered in chapter 2. The hypothesis that is to be tested here states that if a worm of one sex can detect the presence and move towards another worm of the opposite sex placed behind a permissive barrier then there must be some factor(s) released by the imprisoned worm to attract the free moving one. *S. mansoni* was used for these experiments because of its availability and ease of maintaining its life cycle in the laboratory.

In Chapter 3 are explored the nature of the chemotactic factor(s) released by either or both sexes to attract the opposite sex and the dynamics of the attraction. Chapter 4 reports the characterization, isolation and sequence of one of the factors, using *in vitro* assays and biochemical and mass spectrometric techniques.

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CHAPTER 2
DYNAMICS OF ADULT *SCHISTOSOMA MANSONI* MUTUAL SEXUAL
ATTRACTION *IN VITRO*.

In vitro experiments on adult *Schistosoma mansoni* worms have shown the existence of mutual attraction between males and females, carried out by factor(s) released by both sexes. Incubation media of male or female worms were pooled and tested against worms of the opposite sex placed individually in single chambers, 4 cm x 1 cm each. Males were attracted to female-released factor(s), which was found in <30 kDa fractions. The female worms were attracted to possibly more than one factor; a short range factor(s), <30 kDa, and a long-range factor(s), >30 kDa. Boiling reduced the attraction both of males towards female incubate and vice versa.

INTRODUCTION

It has been suggested that the sexes of *S. mansoni* find each other by trial and error and that pairing is mediated by thigmotaxis (Armstrong, 1965). On the other hand, evidence exists from *in vitro* studies for the operation of chemotaxis by factors released by either or both sexes to attract and find their opposites (Eveland *et al.*, 1982; Shirazian and Schiller, 1982; Eveland *et al.*, 1983; Eveland and Haseeb, 1989; Haseeb and Eveland, 1991). This is no surprise since chemoattraction between the opposite sexes of various species of nematodes and trematodes has been shown to exist *in vitro* (Salm and Fried, 1973; Bone and Shorey, 1977; Bone *et al.*, 1977; Roberts and Thorson, 1977; Belosovic and Dick, 1980; Fried *et al.*, 1980; Fried and Robinson, 1981; Glassburg *et al.*, 1981; Glassburg *et al.*, 1983).

The importance of understanding how the opposite sexes in the schistosome species find each other in general comes from the fact that several studies have shown

that in some species the pairing of females with males is necessary for female maturation (Vogel, 1942; Moore *et al.*, 1954), the migration of worm pairs to their definitive intravascular location in their host (Standen, 1953) and for fertilization and oviposition to occur (Armstrong, 1965; Michaels, 1969; Shaw, 1977). However, single females of the genus *Schistosomatium* (*S. douthitti*) can regularly reproduce by parthenogenesis and lay viable eggs (Short *et al.*, 1952 a, b; Jourdane *et al.*, 1995). Incomplete parthenogenesis in the genus *Schistosoma*, where non-viable eggs are laid by females in single sex infections, has been shown to occur in *S. mattheei* (Taylor *et al.*, 1969) and *S. hematobium* (Sahba and Malek, 1977). Using chromosomal evidence, when *S. mansoni* females were paired with *S. douthitti* males, parthenogenesis occurred in a way in which the females laid viable eggs without being inseminated (Basch and Basch, 1984)

In the schistosome species, the importance of finding a male by the female is further emphasized by the fact that pairing is important for the maintenance of the female mature state. On separating female schistosomes from their mates, they stop egg laying and begin regressing back to the juvenile stage (Clough, 1981; Popiel *et al.*, 1981; Popiel *et al.*, 1984). On pairing again they regain maturity and resume their reproductive activity (Clough *et al.*, 1981). Pairing has been shown to be neither species specific (Vogel, 1941, 1942) nor genus specific (Armstrong, 1965; Short, 1948).

Since reproduction is the key to the existence of trematode parasites, and since the egg is the basis of transmission and distribution and in schistosomes is also the agent of pathogenesis (Basch, 1986) and since, as mentioned above, reproduction and egg laying is dependent on mate finding in the majority of the schistosome species, the importance

of knowing the chemical basis of mate finding become very clear as a tool that has the potential of being utilized to break the life cycle of the schistosome and to reduce or prevent pathogenesis.

The chemical nature of the chemoattractants between male and female adult worms of *S. mansoni* is still unclear. These factors have been suggested to be lipidic in nature (Shaw, 1977) on the basis of their partition in *n*-hexane (Haseeb *et al.*, 1989; Haseeb and Eveland, 1991) and in *n*-pentane and ether (Gloer *et al.*, 1986).

In this chapter, we tested media in which live adult worms had been incubated (“the incubates”) for chemoattractive properties *in vitro*. Factors so revealed are hypothesized to be released in the bloodstream of the vertebrate host by either or both sexes to attract the opposite sex. Here we confirm chemoattraction between adult male and female *S. mansoni* worms *in vitro*, report on the characteristics and dynamics of this attraction in our bioassay system, and compare chemoattraction of adult male and female worms to opposite sex incubates and defined fractions thereof. The latter experiments were done to provide information on the approximate molecular weight of the factor(s), their resistance to boiling, and whether each sex responds to one or more factors of the approximate molecular weights.

MATERIALS AND METHODS

Parasite collection

C57BL/Ca6 or CBA/J6 mice of both sexes were each infected by subcutaneous injection of 150–200 *S. mansoni* cercariae (KEB strain, Damian *et al.*, 1972) per mouse.

Before the mice were killed at 6–8 weeks post infection to harvest adult worms, each was injected with 0.1 ml PBS solution containing 30 μ g/ml Na heparin (Shirazian *et al.*, 1986). A non-anesthetic method, carbon dioxide inhalation, was used to kill the mice to avoid any effects that anesthetics, *e.g.* pentobarbital, might have on the worms. The worms were collected by perfusion from the hepatic portal veins (Duvall and DeWitt, 1967), then washed in PBS 3–4X before being used either in preparing incubates or in the bioassays.

Pilot experiments

Based on a previous assembly by Imperia *et al.*, (1980), the *in vitro* assay was carried out in a plexiglass chamber containing linear channels. The dimensions of the channels were 4 cm long, 1 cm wide, and 1.5 cm high, being 1 cm longer than what was previously used by Imperia *et al.* (1980). Each channel was marked into 0.5 cm zones along its length. For the assay individual worms were placed either at one end or in the middle of a channel. The channel floors were overlaid by 1 ml of 2% agar to provide a suitable substrate for the worms migration and each channel was filled with migration medium (0.1% glucose and 0.5% lactalbumin hydrolysate in 1X Earl's balanced salt solution) (Clegg, 1965), for performance of the assays, which were carried out in the dark at 5% CO₂ and 37°C in a humidified incubator chamber.

To confirm the attraction between the sexes, bioassays using live adult worms were carried out as pilot experiments. In these experiments live worms of either sex were used as bait for worms of the opposite sex. A single worm, behind a Whatman No. 4 filter paper barrier, 0.8 cm wide and 1.5 cm long that had been perforated with common pins, was positioned one end of each of the assay channels while a single worm of opposite sex

was put in the middle of the same channel. In other experiments, multiple worms served as bait. The filter paper barrier served to confine the bait worm(s) and allow any chemoattractants released by the bait worm(s) to diffuse freely into the rest of the medium in the channel. Movement of the worms towards the bait was checked at 0.5, 1, 2 and 3 hours. Stretching of a worm into the first 0.5 cm zone was not scored as movement, because worms were usually curled or twisted on themselves when placed in the migration channels. Only if they were in the second 0.5 cm zone or beyond was it scored as movement. Also, a worm had to be at least half its length into a zone for it to be scored as movement. Two criteria for zone scoring were used: the first was direction of movement, positive movement (attraction) if 50% or more of the worms moved towards the bait and negative movement (no attraction) if more than 50% remained at the zero zone or/and moved away from the bait. The second criterion was to distinguish between different baits that scored either positive or negative movement. This was based on the percentage of worms that moved all the way to the bait, the higher the better. In all cases, the progress of the worms' movement in general was considered when comparing between groups, but with more emphasis on their distribution at the end of the experiment, after 3 hours. Worm viability was checked microscopically after each bioassay. The complete loss of motility of the anterior regions, and loss of gut contractions were taken as indicators of death which invalidated the experiment. This occurred only rarely and then only in the initial steps of this investigation. In each trial a minimum of 32 worms was used.

Worm incubate preparation

Worm-released products (“incubates”) were prepared by incubating individual worms in 200 ul of migration medium, described above, in a microtiter plate (Corning Costar Corporation, Cambridge, MA) for one hour (5% CO₂, 37°C). The incubates for each sex of worms were then pooled and later used in the assay. Each well was checked under the microscope, before collecting media from it, for the overt presence of hematin material regurgitated by the worms. Worm incubate containing hematin was not collected. The incubates were either not subjected to any treatments before testing or were treated. The treatments were either boiling or fractionation by centrifugation using Centricons (Millipore Corporation, Bedford, MA) of defined cut-off value, 30kDa. The filtrate and the retentate were then tested. The retained fraction, the >30kDa, was washed 3X, by re-suspending in PBS and then centrifuging, before being collected from the Centricon assembly and used.

Assays using worm incubate

Cylindrical agar blocks, approximately 0.5 cm in diameter and 0.5 cm long, in which the worm incubate had been incorporated, were then used as bait in lieu of living worms to test for the presence or absence of attracting factor(s) in the incubates. The agar blocks were prepared by mixing 30 ul of the pooled incubate with 150 ul of 2 % molten agar at 45-50 °C in PBS, in round-bottom Linbro micro-titer wells (ICN Biomedical, Aurora, OH) and left to solidify. The bioassay was performed by placing an agar block containing incubate from one sex at one end of each channel and a worm of the opposite sex at the other end or in the middle. Again, distances covered by migrating worms in either

direction and their viability was recorded. Criteria for zone scoring were as described above.

Statistical analysis

Experiments were performed in triplicate. Analysis of the results of the bioassays was carried out by one-way analysis of variance (ANOVA). The location of each worm at every time point was used as the parameter for analysis. The results for each group of experimental set of worms were then compared to the other groups for statistically significant differences between them. Differences were considered significant when $P < 0.05$. The P values, at 3hrs, for experiments done on one sex are shown in relation to the random movement of the same sex, unless otherwise stated. The results of the experiments were expressed in the form of the average percentage of worms present at each marked 0.5 cm zone at each time point.

The one-tailed T test was used to test the statistical significance of data when the % total movement of worms towards the bait was less than 50% at 3hrs. For that purpose, the following formula was adapted from Anderson *et al*, (1994):

$$z = \frac{\bar{x} - \hat{\mu}}{\hat{\sigma}_x}$$

where \bar{x} is the sample mean, $\hat{\mu}$ is the population mean set here to be the 50%, $\hat{\sigma}_x$ is the standard deviation of the sample mean and z is the test statistic used to detect whether \bar{x} deviates enough from $\hat{\mu}$. The difference of the sample mean when its value was less than

50% was not considered significant when z was less than the critical value 1.645. The source of the critical value of $t_{0.05} = 1.645$ at df is Anderson *et al*, (1994).

RESULTS

Pilot experiments

These experiments showed that adult female worms are clearly attracted (70%) to single adult male worms, $P = 0.001$ (Fig. 2.1) as compared to the random movement of the females (49%, $z = 2.19$), in which the majority of the females did not advance beyond zone 1 (Fig. 2.2). They also showed the clear attraction of adult male worms (73%) to single adult females, $P = 0.001$ (Fig. 2.3) when compared to the random movement of the males (15%, $z = 3.8$) in which almost none went beyond zone 1 (Fig. 2.4). No female-female attraction was detected (24%, $P = 0.296$, $z = 4.03$) (Fig. 2.5) as compared to the random movement of the females. On the contrary, there was detectable (but rare and not statistically significant) male-male attraction (24%, $P = 1.00$, $z = 7.85$) (Fig. 2.6) as compared to the random movement of the males. The experiments showed little or no enhancement of migration of either sex by using multiple (59% and 76% for Figs. 2.7 and 2.8 respectively) versus single (Figs. 2.1 and 2.3) bait worms. The P value was 0.001 for both figures 2.7 and 2.8. The P values for Fig. 2.1 in relation to Fig 2.7 was 1.00, and for Fig. 2.3 in relation to Fig. 2.8 was 1.00.

Behavior of adult female worms in the presence of boiled and non-boiled male worms incubate

Adult female worms were attracted (55%) to the untreated male incubate, $P = 0.001$. Up to 38% of them moved all the way toward the bait after 3 hrs (Fig. 2.9). When male worm incubate was boiled before testing, a great reduction in attraction was

observed, (20%, $P = 1.00$ and $z = 4.23$) (Fig. 2.10). To test the possibility that reduction of attraction was due to alteration of migration medium by boiling (i.e. had repellent effect), the medium was boiled, then mixed with non-boiled male incubate in 1:1 ratio and tested. It had no reduction effect on attraction, (53%, $P = 0.001$) (Fig. 2.11).

Behavior of adult male worms in the presence of boiled and non-boiled female worms incubate

Reciprocally with adult female worms, adult males were attracted to the untreated female worm incubate, (69%, $P=0.001$). Up to 39% of males had moved all the way toward the bait after 3 hrs (Fig. 2.12). Again, on boiling the female worm incubate, reduction of attraction behavior was noticed (21%, $P=1.00$, $z=13.14$) (Fig. 2.13).

Behavior of female worms in presence of male incubate fractions

More female worms (72%) moved towards the <30kDa fraction, $P = 0.001$, when they were placed at the middle of the chambers (Fig. 2.14, and 2.15). For the female response to >30kDa (14%), $P = 1.00$ and $z = 3.5$. When they were placed at the other end, opposite to the agar block and farther from the bait, more moved towards the >30kDa fraction (Fig. 2.16 and 2.17). Fig 2.18 shows the random movement of females when placed at one end. The P values for the female response, when placed at one end, toward both >30kDa and <30kDa male fractions, are 0.05 and 1.00 respectively, in relation to the female random movement when the females were placed at one end of the chamber.

Behavior of male worms in presence of female incubate fractions

Male worms were attracted to the <30kDa fraction, (56%, $P = 0.001$), when they were placed at middle of the chambers opposite to the agar block (Fig. 2.19 and 2.20 respectively). For the male response to >30kDa (26%), $P = 1.00$ and $z = 9.68$. Contrary to

the observation with the female worms, when they were placed at the opposite end of the chamber and farther from the bait they still moved more towards the <30kDa fraction (2.21, 2.22 & 2.23). The P values for the male response, when placed at one end, toward both <30kDa and >30kDa female fractions, are 0.001 and 0.572 respectively, in relation to the male random movement when the males were placed at one end of the chamber.

Figure 2.1: Percentage of adult female *Schistosoma mansoni* attracted to single live adult males *S. mansoni*. $P = 0.001$ with respect to adult female random movement at 3 hrs. In all the figures each row on the x-axis represents the distribution of the female worms at each time point indicated on the y-axis and the columns represent the average of determinations at each time point at each zone.

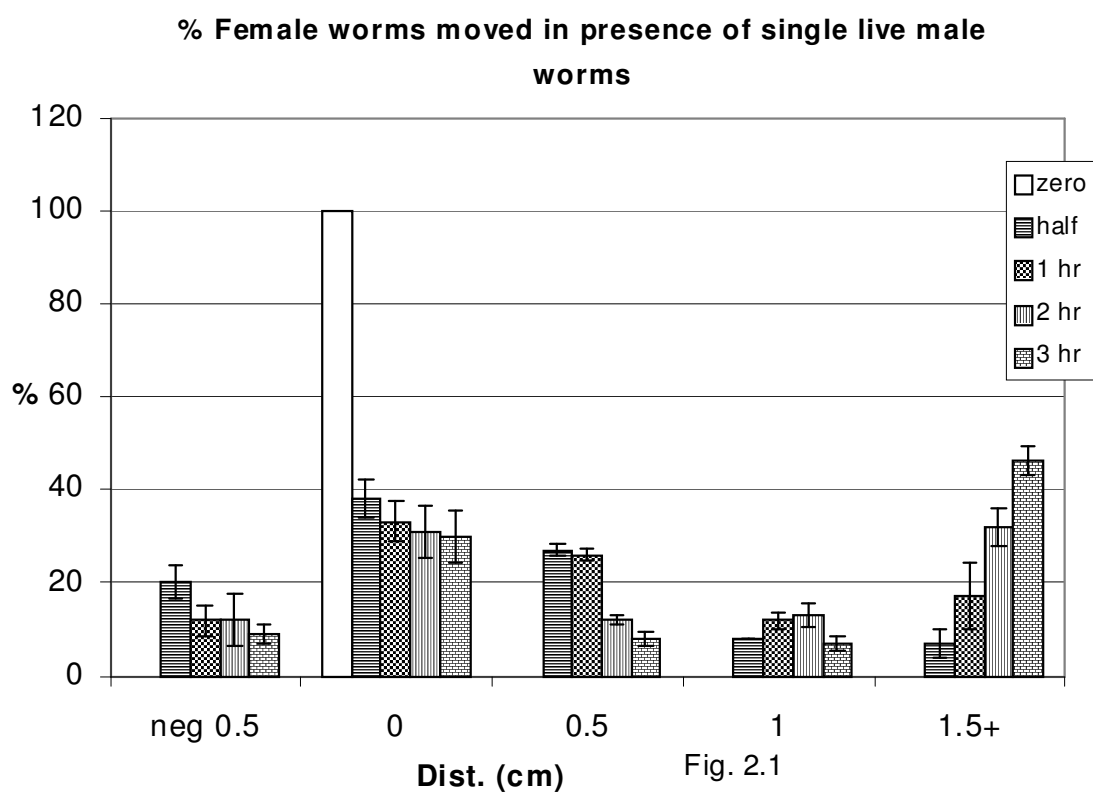


Figure 2.2: Percentage of adult female *S. mansoni* distributed on random movement ($z = 2.19$). $P = 0.358$ in relation to the male random movement.

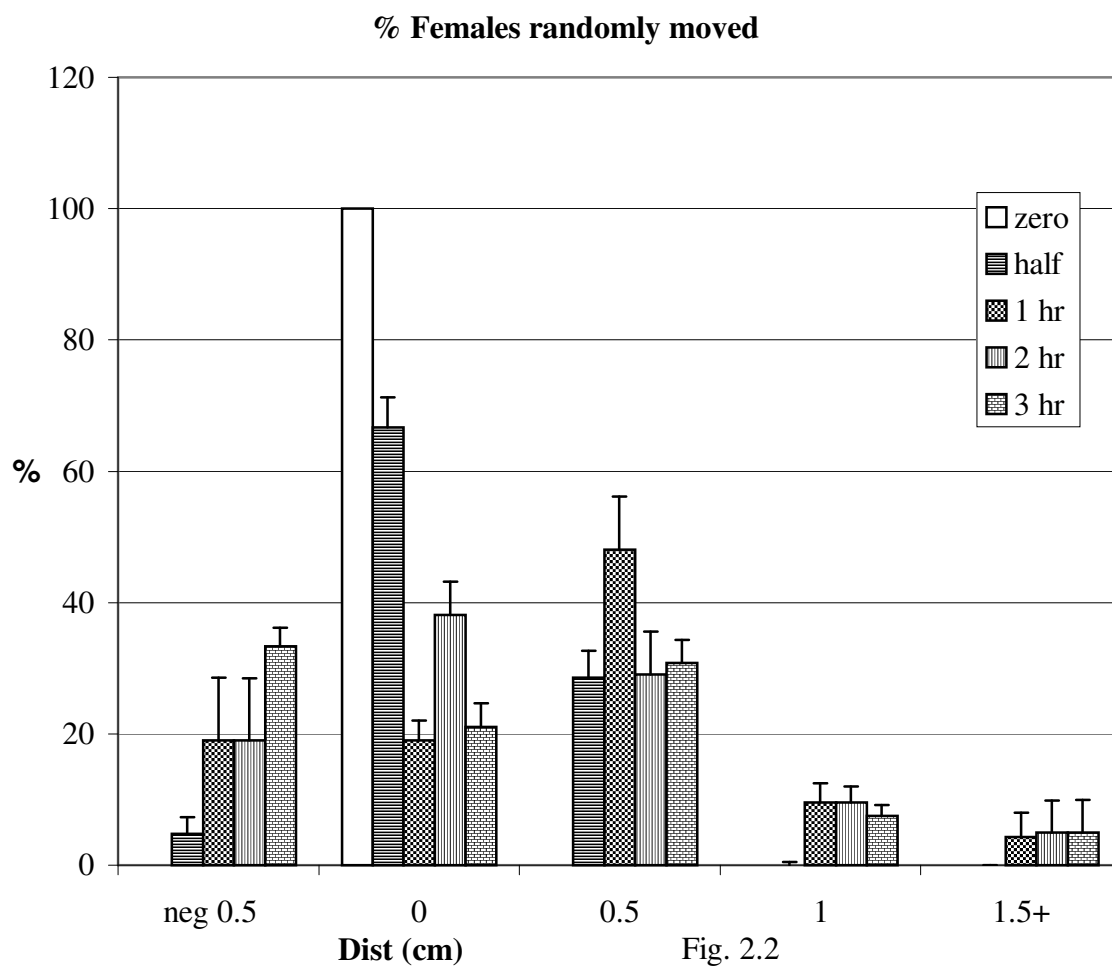


Figure 2.3. Percentage of adult male *S. mansoni* attracted to single adult live female *S. mansoni*. $P = 0.001$ with respect to adult female random movement at 3 hrs.

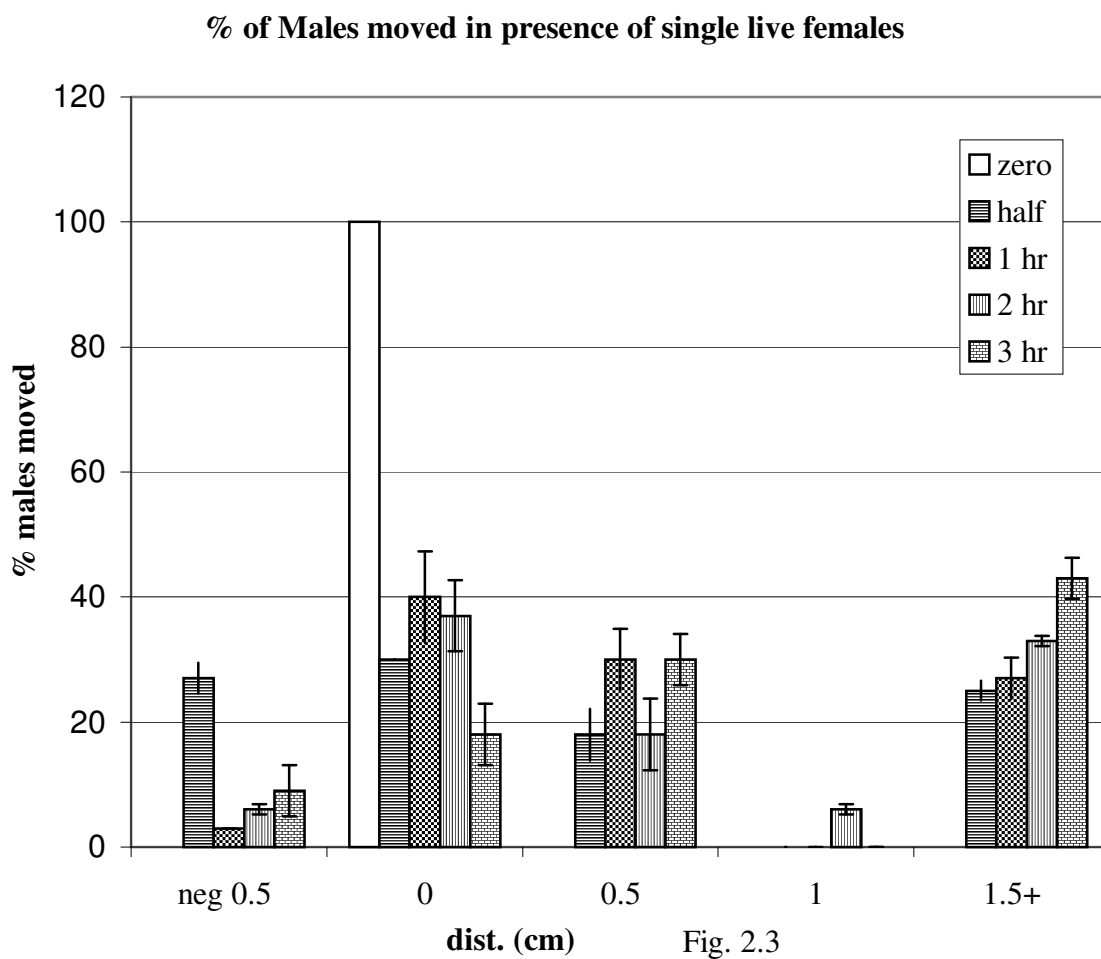


Figure 2.4: Percentage of adult male *S. mansoni* distributed on random movement
($z = 3.8$).

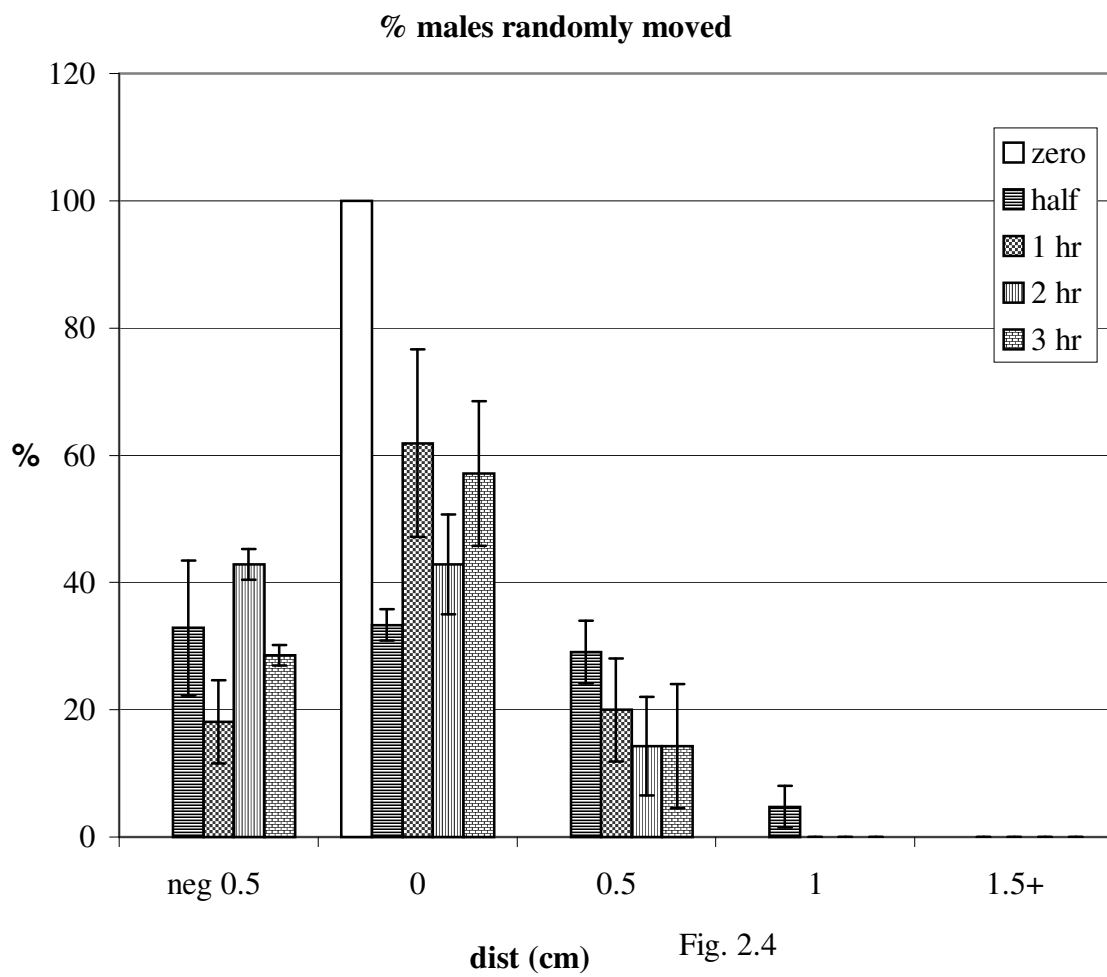


Figure 2.5: Percentage of adult female *S. mansoni* attracted to single live adult female worms ($P = 0.296$, $z = 4.03$).

% Female worms moved in presence of single live females

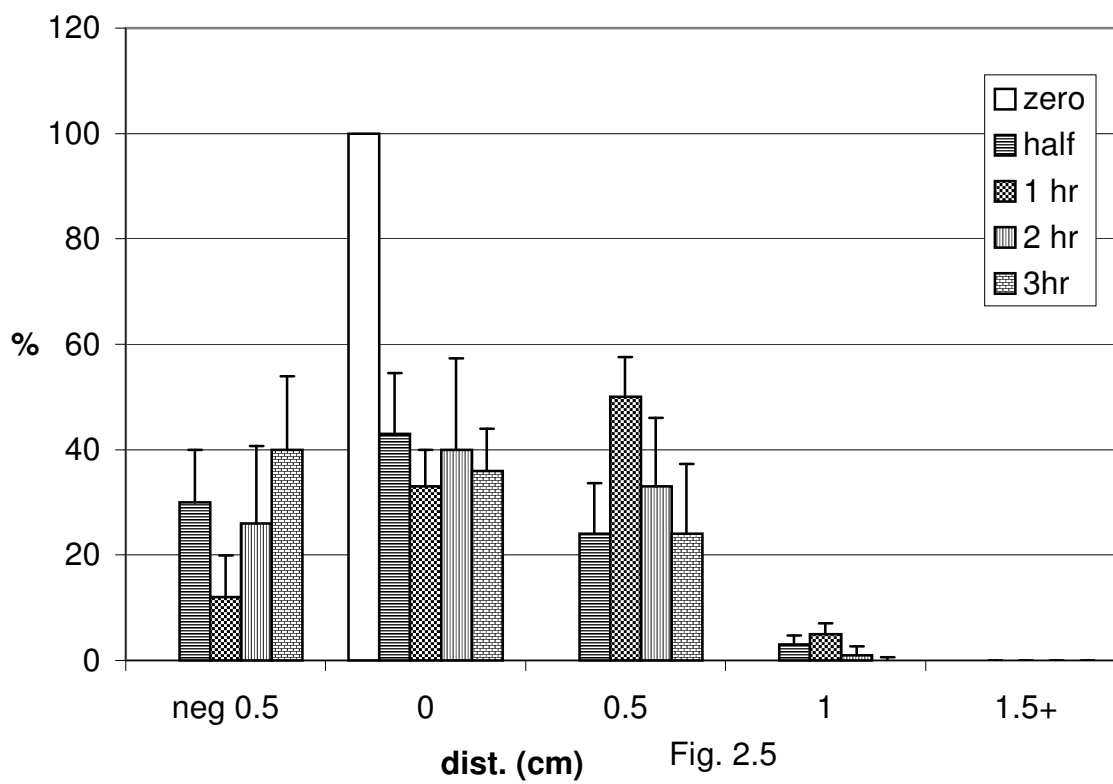


Figure 2.6: Percentage of adult male *S. mansoni* distributed in the presence of single adult male *S. mansoni* ($P = 1.00$, $z = 7.85$).

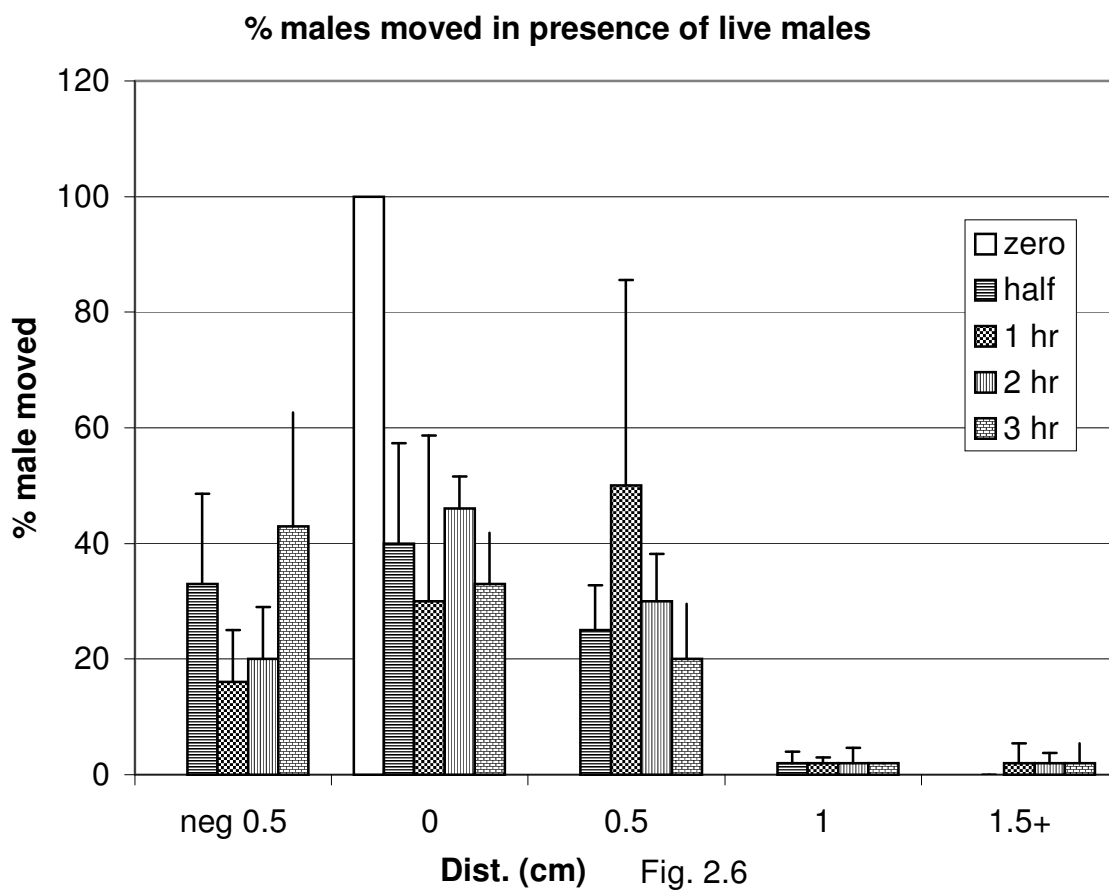


Figure 2.7: Percentage of adult female *S. mansoni* attracted to multiple males put together. $P = 0.001$ with respect to adult female random movement at 3 hrs.

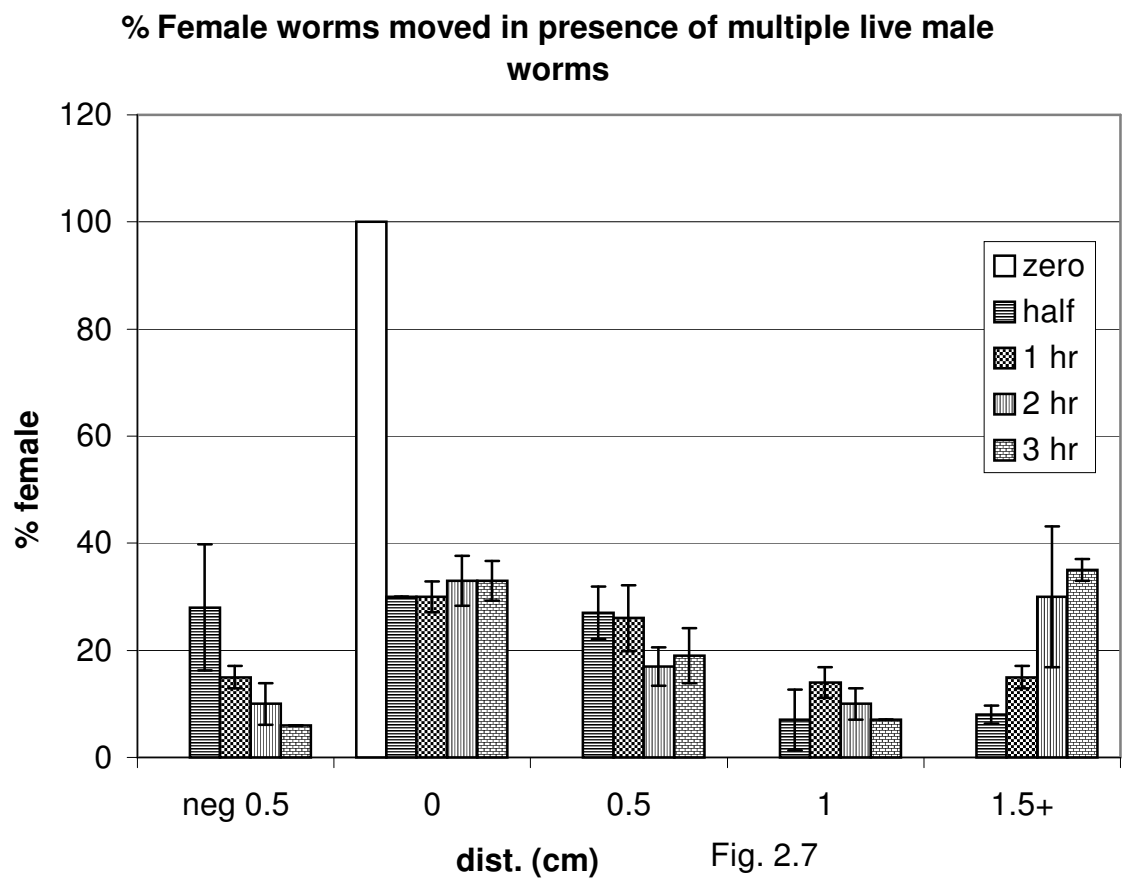


Figure 2.8: Percentage of adult male *S. mansoni* attracted to multiple females put together. $P = 0.001$ with respect to adult male random movement at 3 hrs.

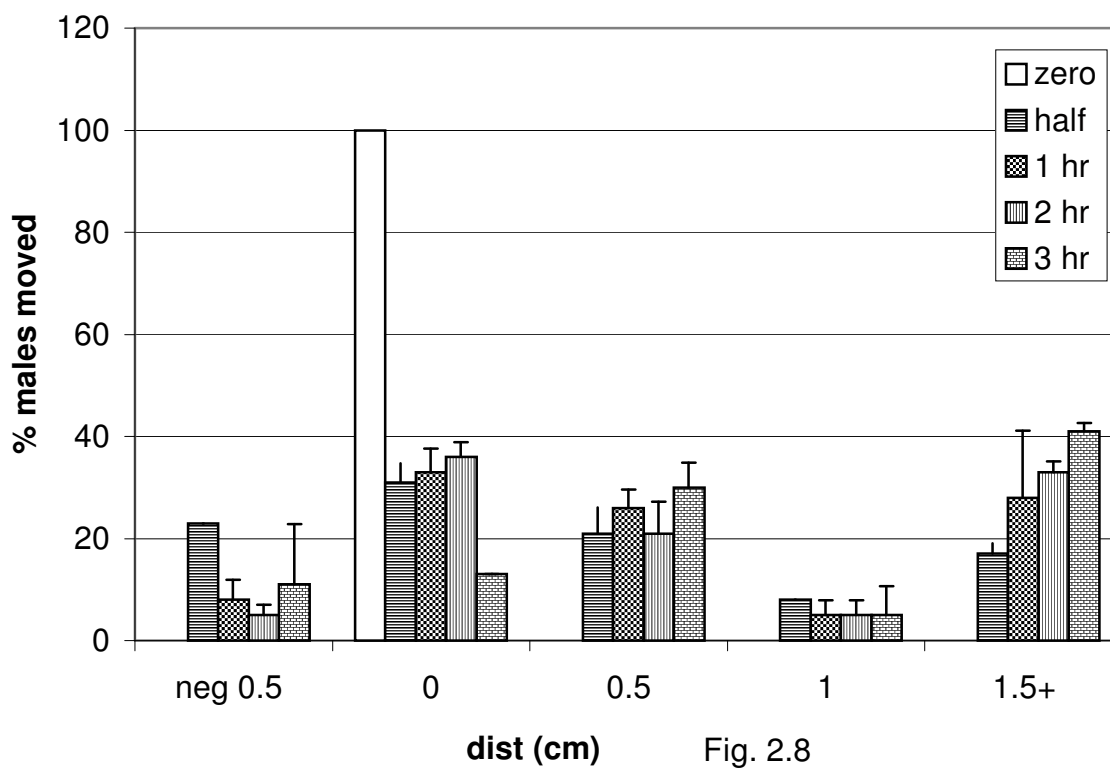
% Males moved in presence of multiple live females

Fig. 2.8

Figure 2.9: Percentage of adult female *S. mansoni* attracted to untreated male incubate. $P = 0.001$ with respect to adult female random movement at 3 hrs.

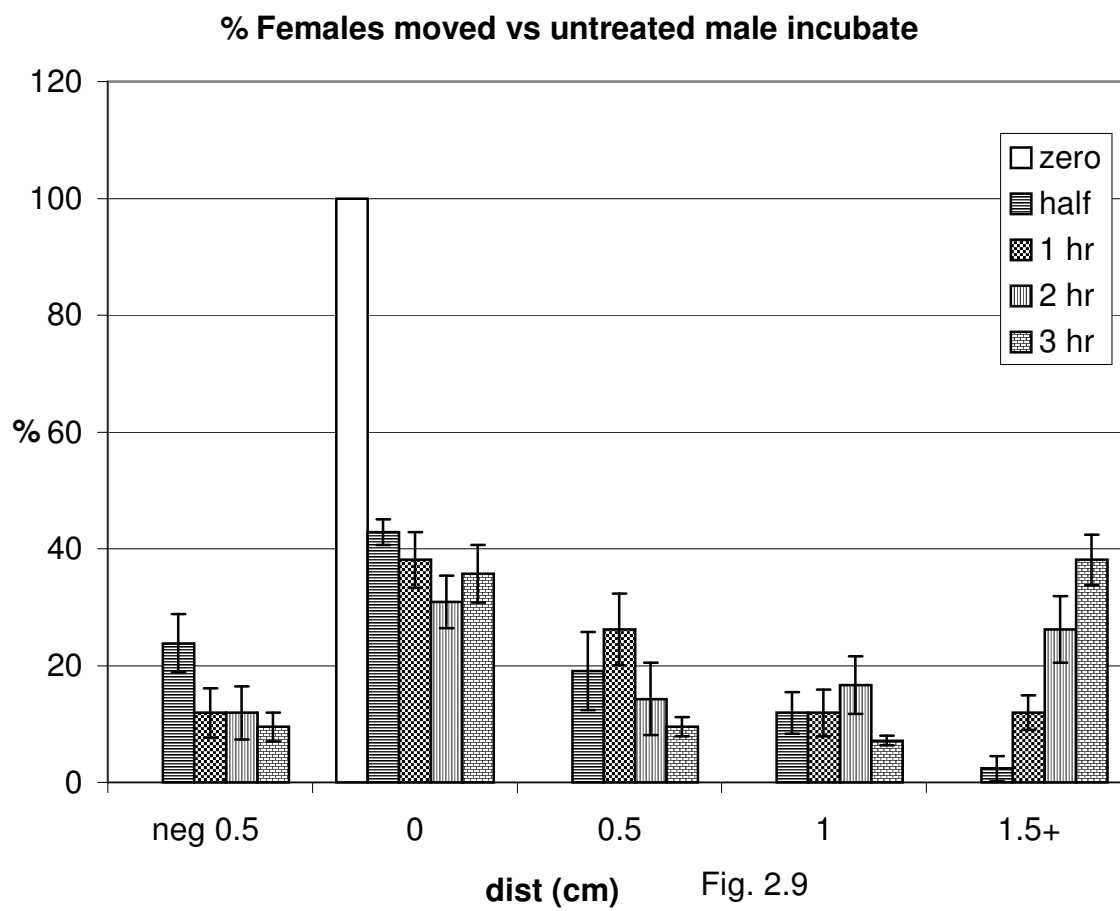


Figure 2.10: Percentage of adult female *S. mansoni* attracted to boiled adult male worm incubate (P = 1.00, z = 4.23).

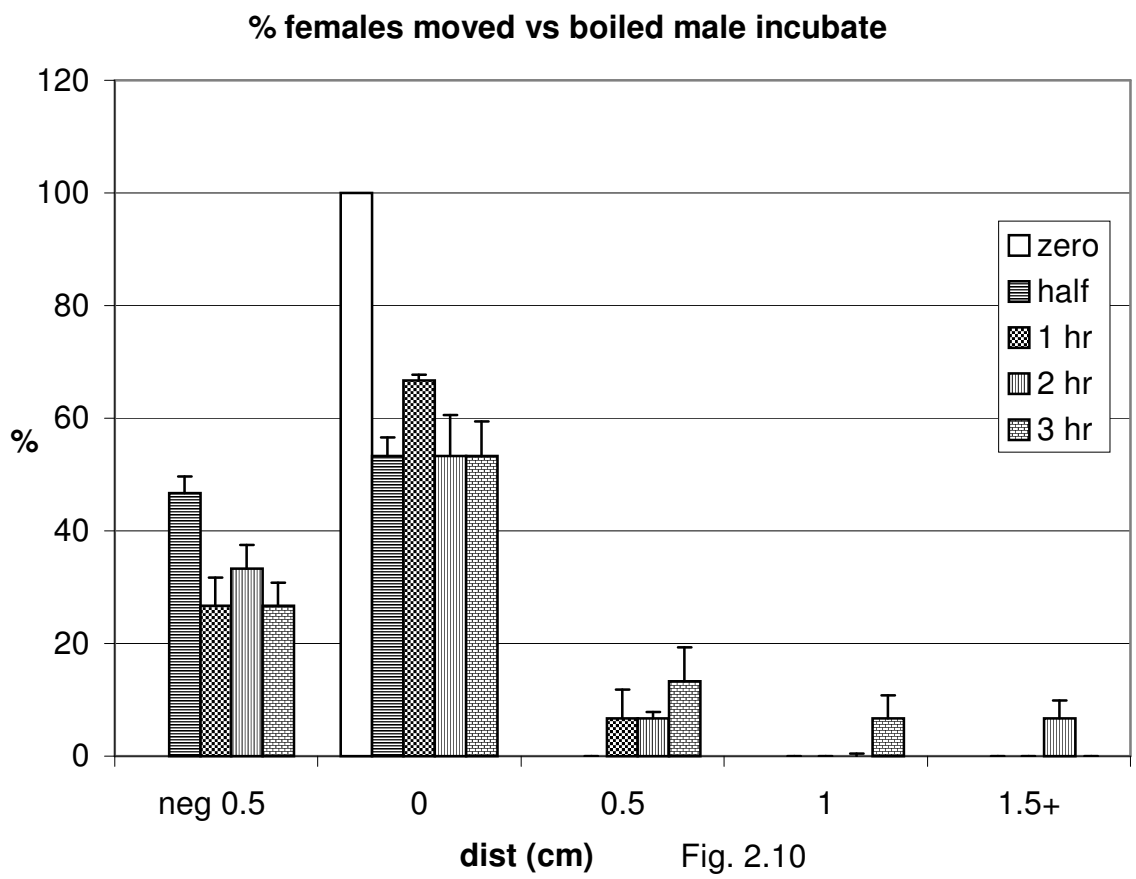


Figure 2.11: Percentage of total positive movement of adult female *S. mansoni* attracted to untreated, boiled and 1:1 boiled: untreated adult male worm incubate. For untreated and for boiled: untreated mix, $P = 0.001$ with respect to adult female random movement at 3 hrs.

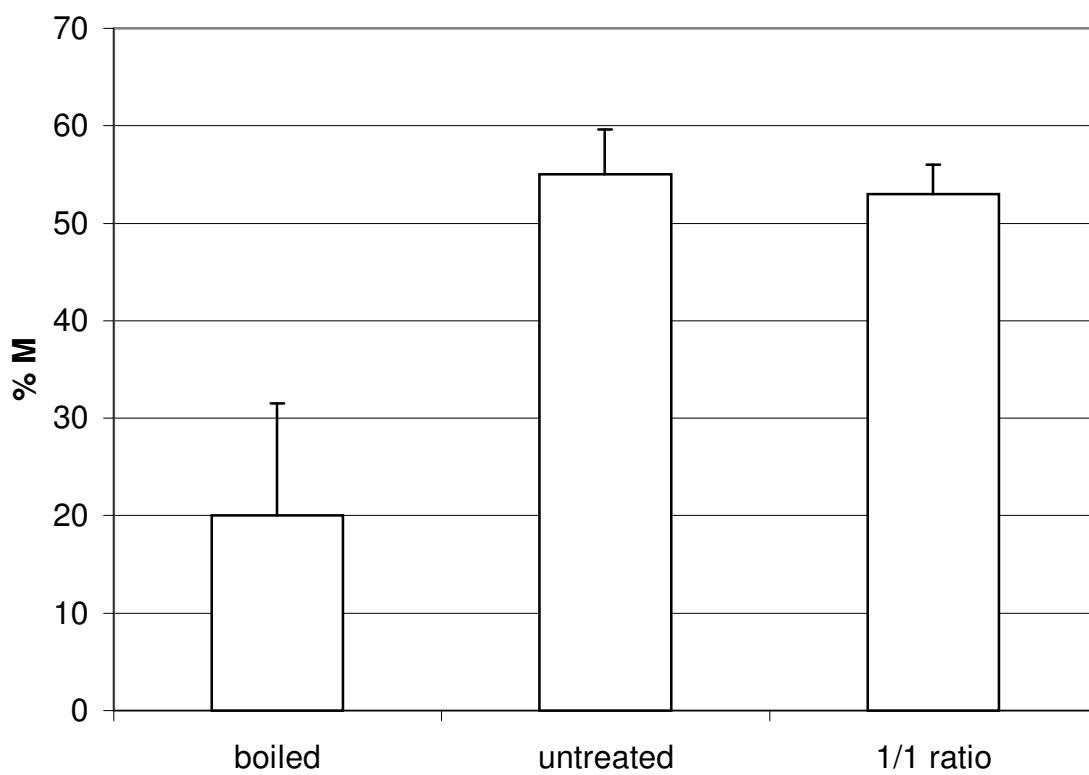
F vs untreated, boiled & 1:1 boiled:non boiled M incubate

Fig 2.11

Figure 2.12: Percentage of adult male *S. mansoni* attracted to untreated adult female worm incubate. $P = 0.001$ with respect to adult male random movement at 3 hrs.

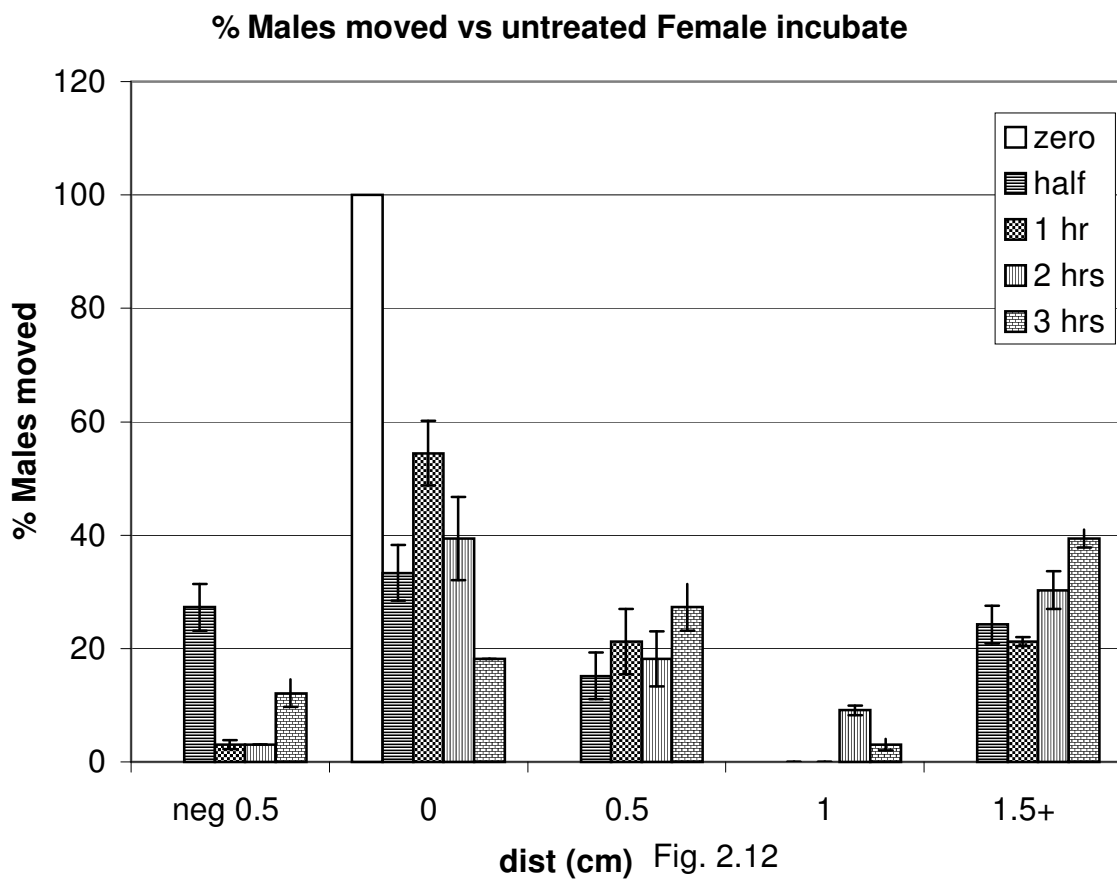


Figure 2.13: Percentage of adult male *S. mansoni* attracted to boiled adult female worm incubate (P = 1.00, z = 13.14).

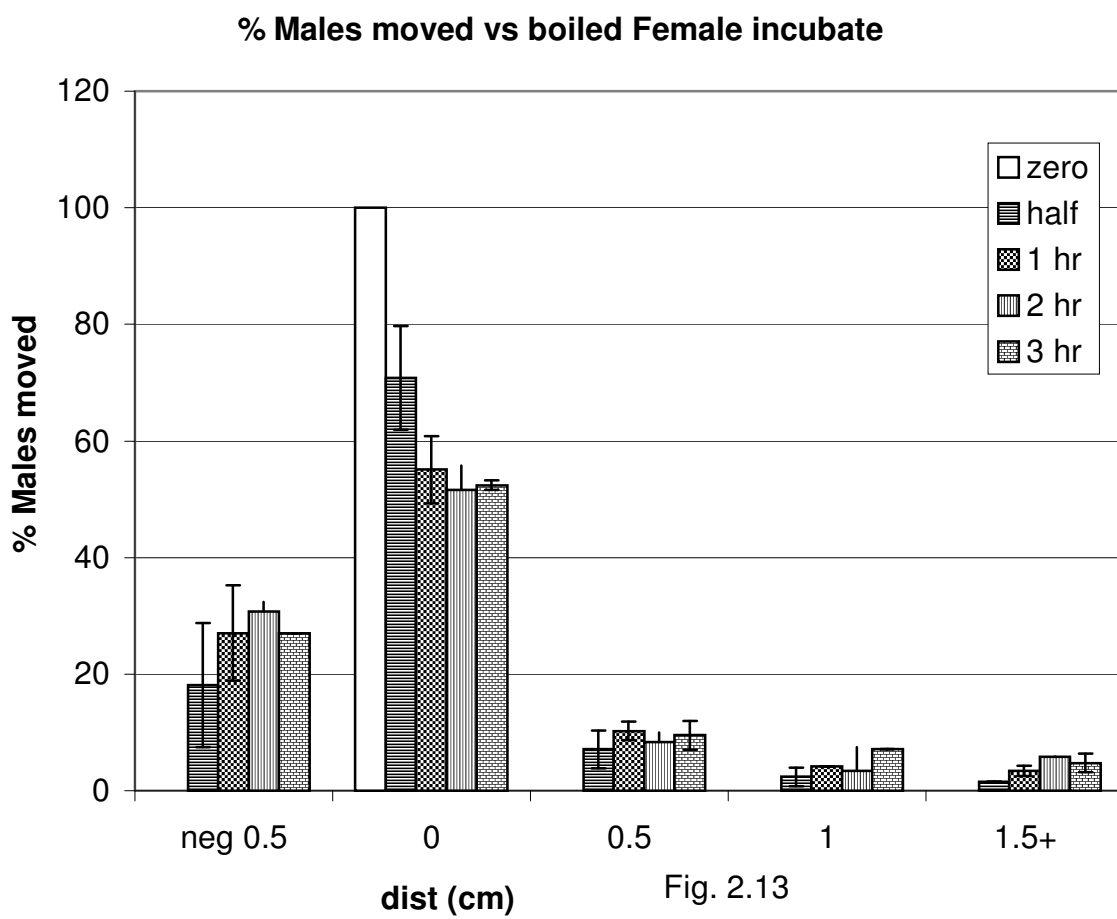


Figure 2.14: Percentage of adult female *S. mansoni* distributed in presence of adult male *S. mansoni* >30kDa incubate fraction. Female worms were placed at the middle of the wells ($P = 1.00$, $z = 3.5$).

% F moved in presence of >30kDa M fraction. F started at the middle.

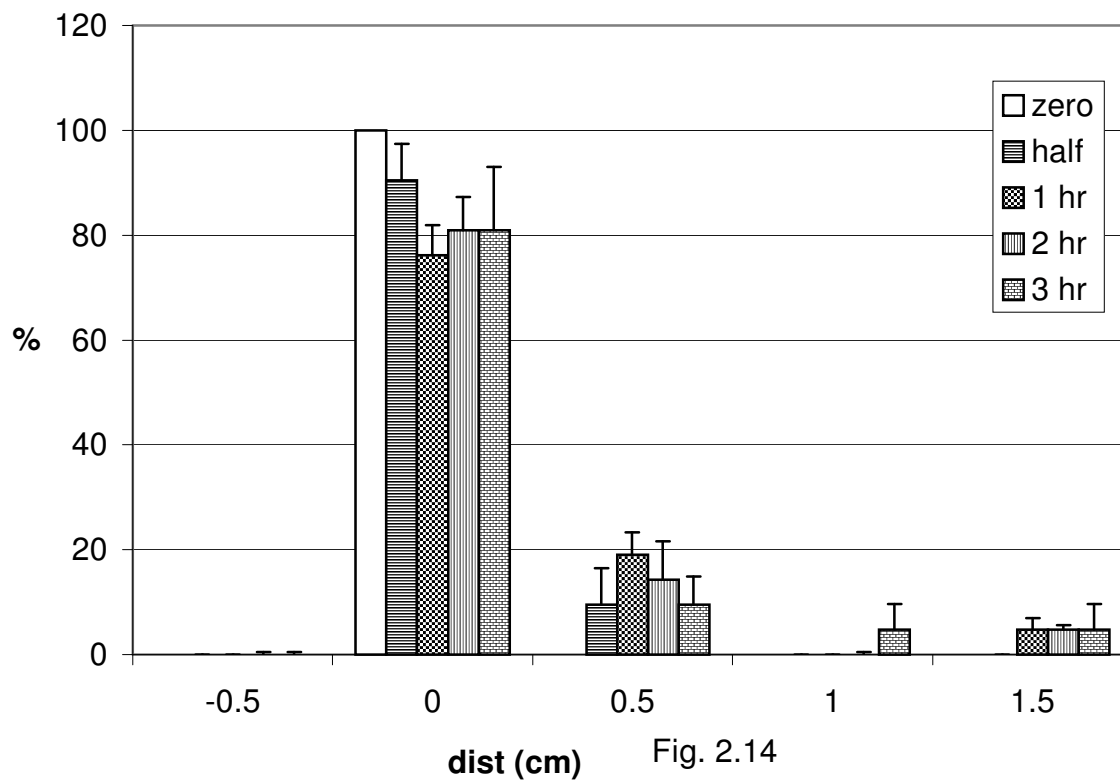


Figure 2.15: Percentage of adult female *S. mansoni* distributed in presence of adult male *S. mansoni* <30kDa incubate fraction. Female worms were placed at the middle of the wells (P = 0.001).

% F moved in presence of <30kDa M fraction. F started at the middle.

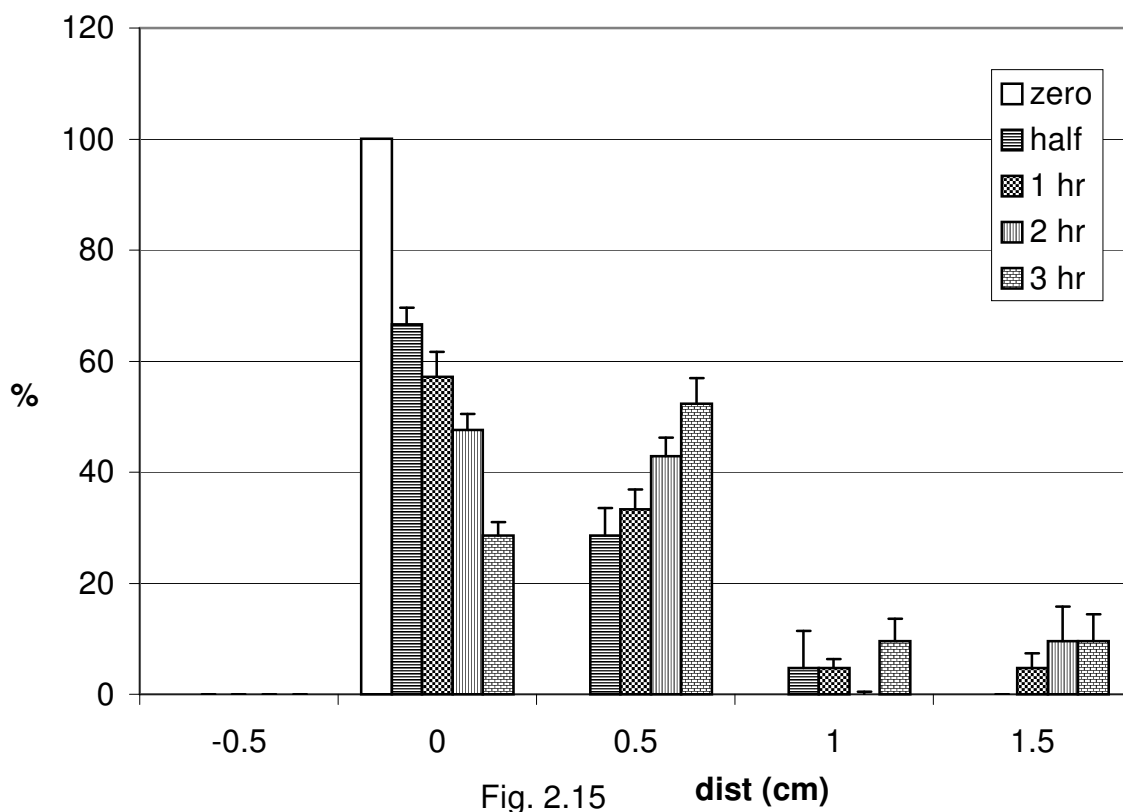


Figure 2.16: Percentage of adult female *S. mansoni* distributed in presence of adult male *S. mansoni* >30kDa incubate fraction. Female worms were placed at the opposite end of the agar blocks containing the male fraction. $P = 0.05$ with respect to adult female random movement at 3 hrs starting at one end, Fig 2.18.

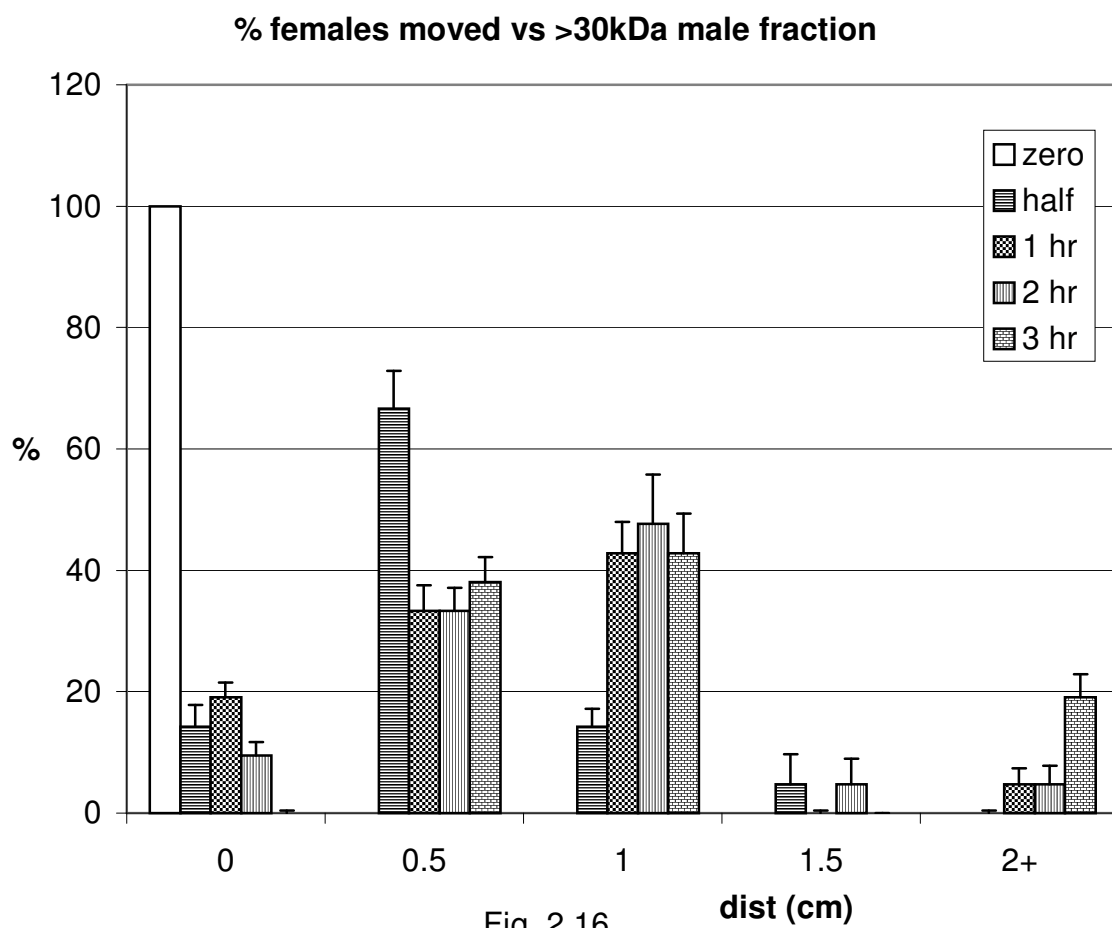


Figure 2.17: Percentage of adult female *S. mansoni* distributed in presence of adult male *S. mansoni* <30kDa incubate fraction. Female worms were placed at the opposite end of the agar blocks containing the male fraction. $P = 1.00$ with respect to adult female random movement at 3 hrs starting at one end, Fig 2.18.

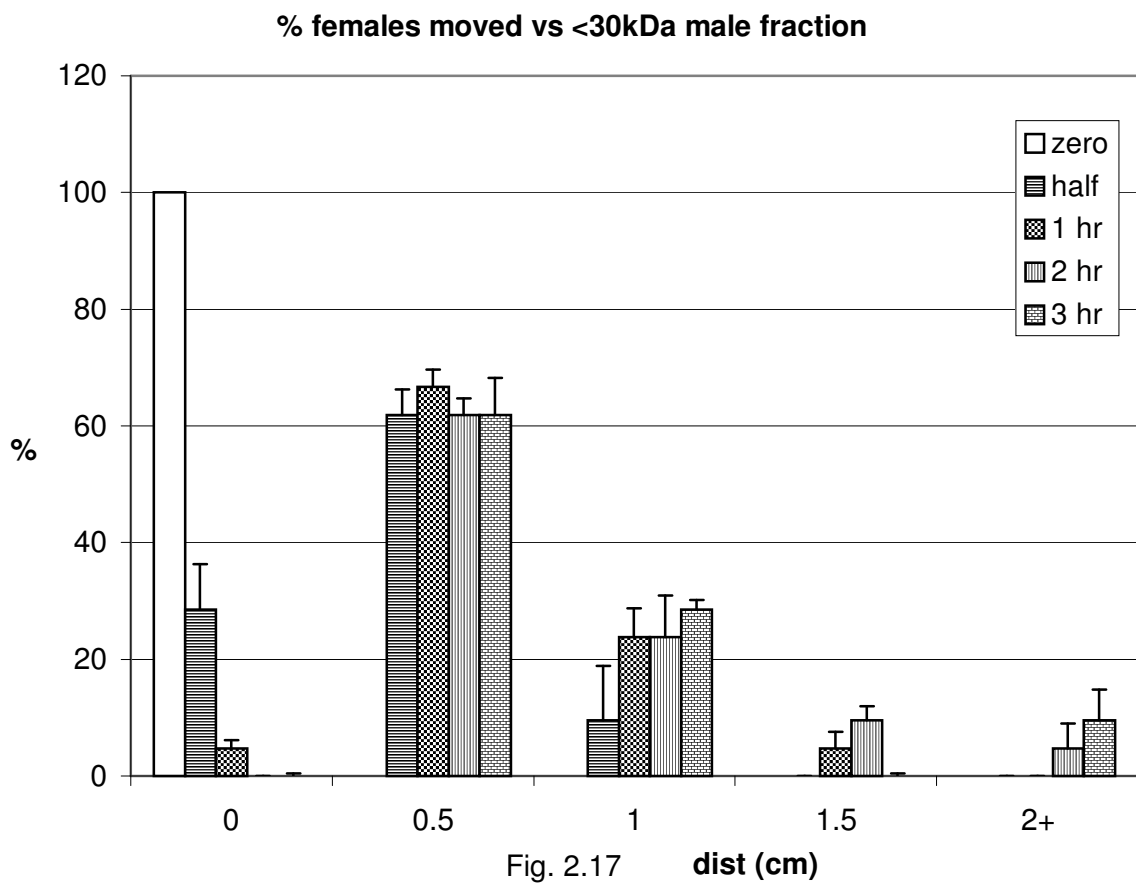


Figure 2.18: Percentage of adult female *S. mansoni* distributed on random movement when placed at one end.

random movement of Females starting at one end

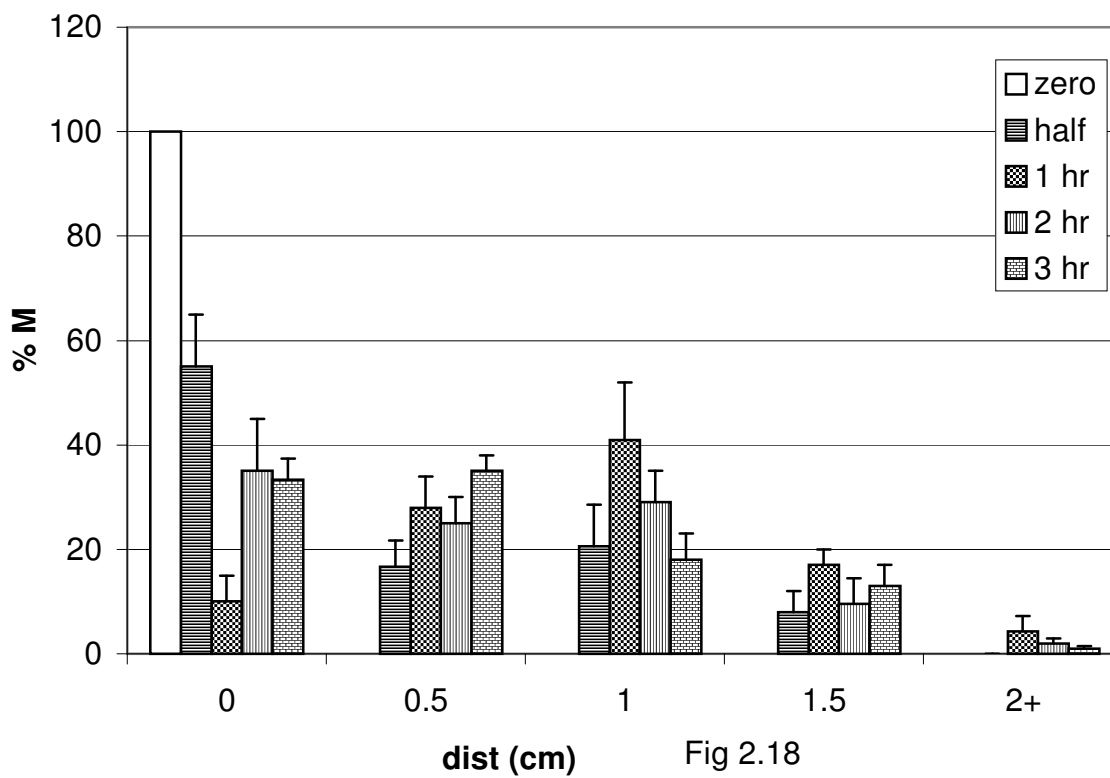


Fig 2.18

Figure 2.19: Percentage of adult male *S. mansoni* distributed in presence of adult female *S. mansoni* <30kDa incubate fraction. Male worms were placed in the middle. $P = 0.001$ with respect to adult male random movement at 3 hrs.

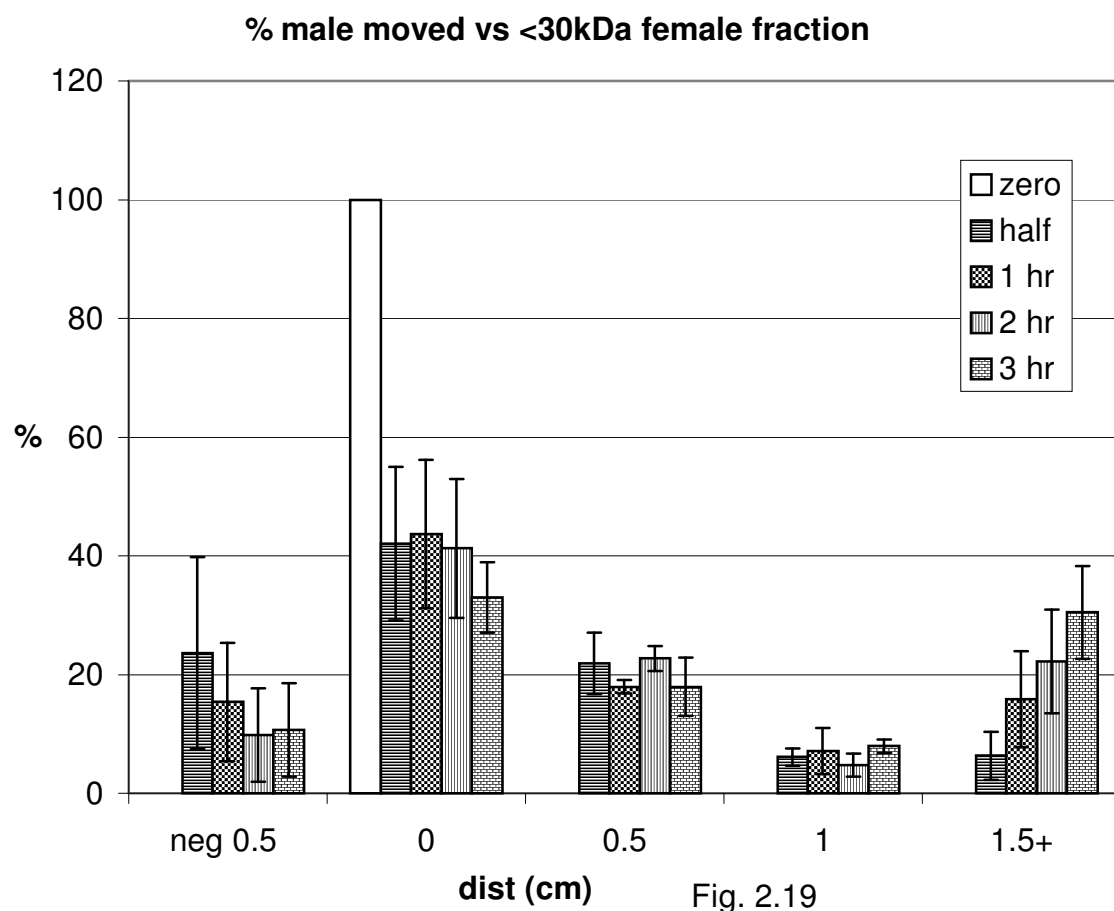


Figure 2.20: Percentage of adult male *S. mansoni* distributed in presence of adult female *S. mansoni* >30kDa incubate fraction. Male worms were in the middle (P = 1.00, z = 9.68).

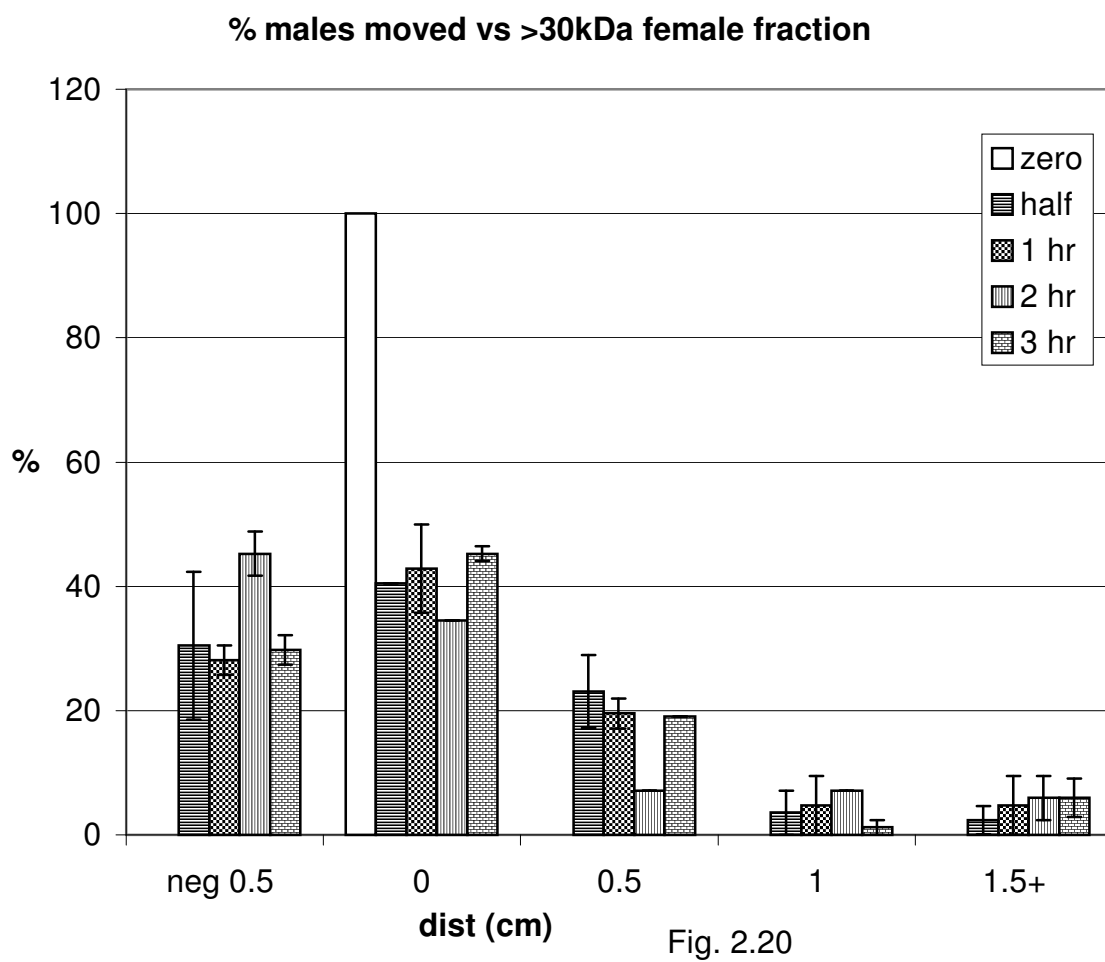


Figure 2.21: Percentage of adult male *S. mansoni* distributed on random movement when placed at one end.

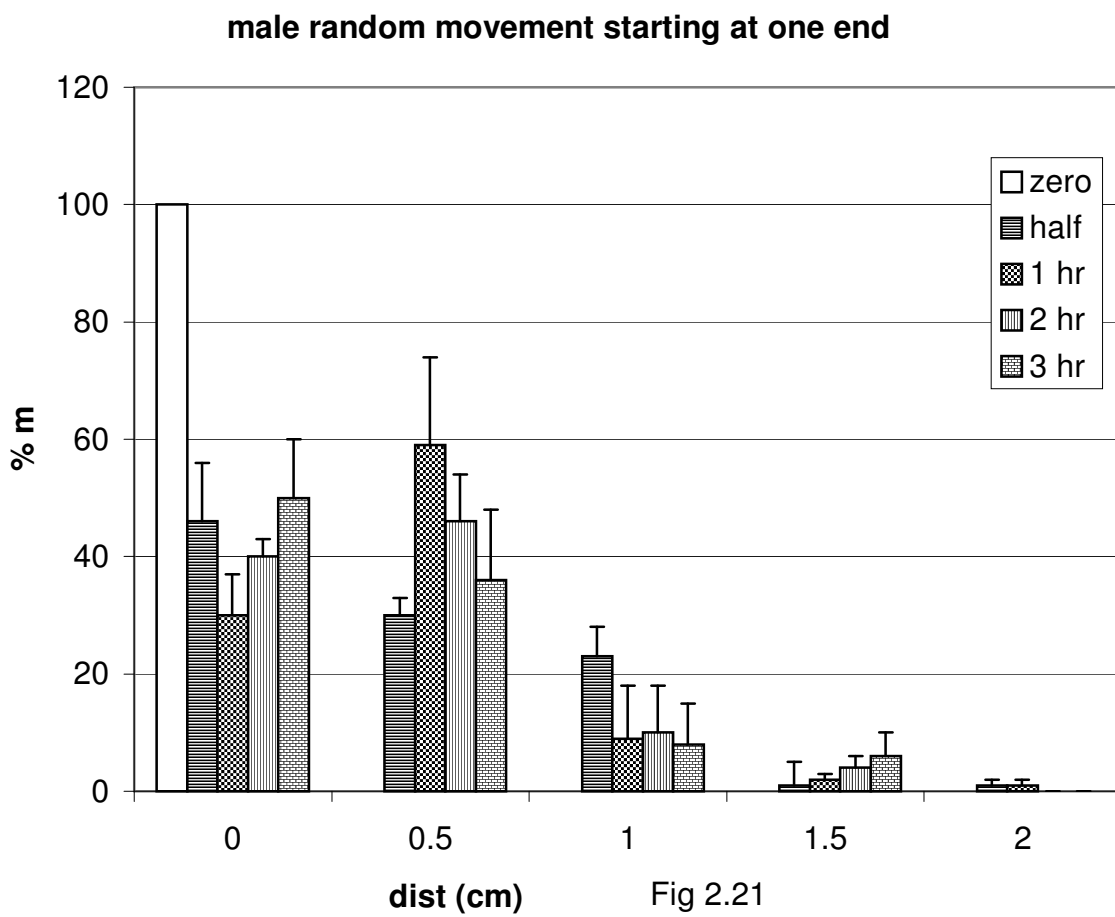


Figure 2.22: Percentage of adult male *S. mansoni* distributed in presence of adult female *S. mansoni* <30kDa incubate fraction. Male worms were placed at the opposite end. $P = 0.001$ with respect to adult male random movement at 3 hrs.

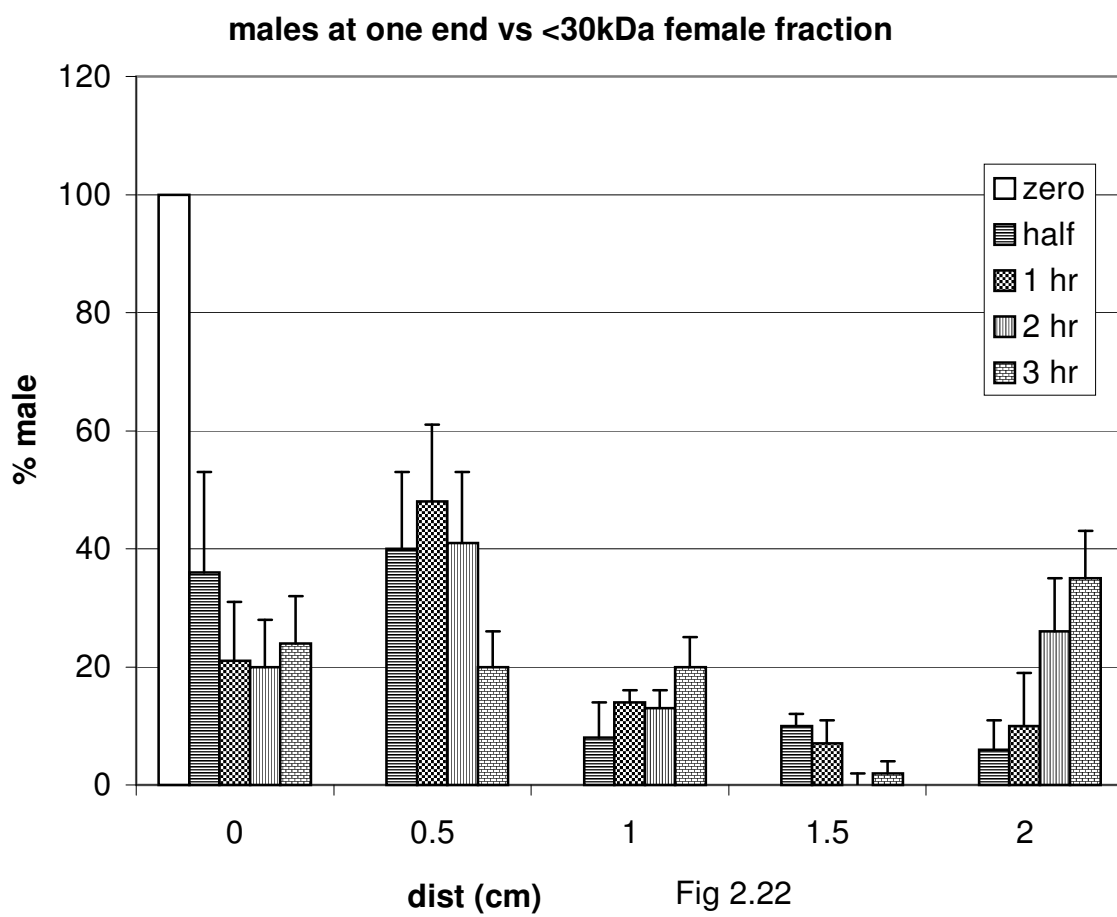
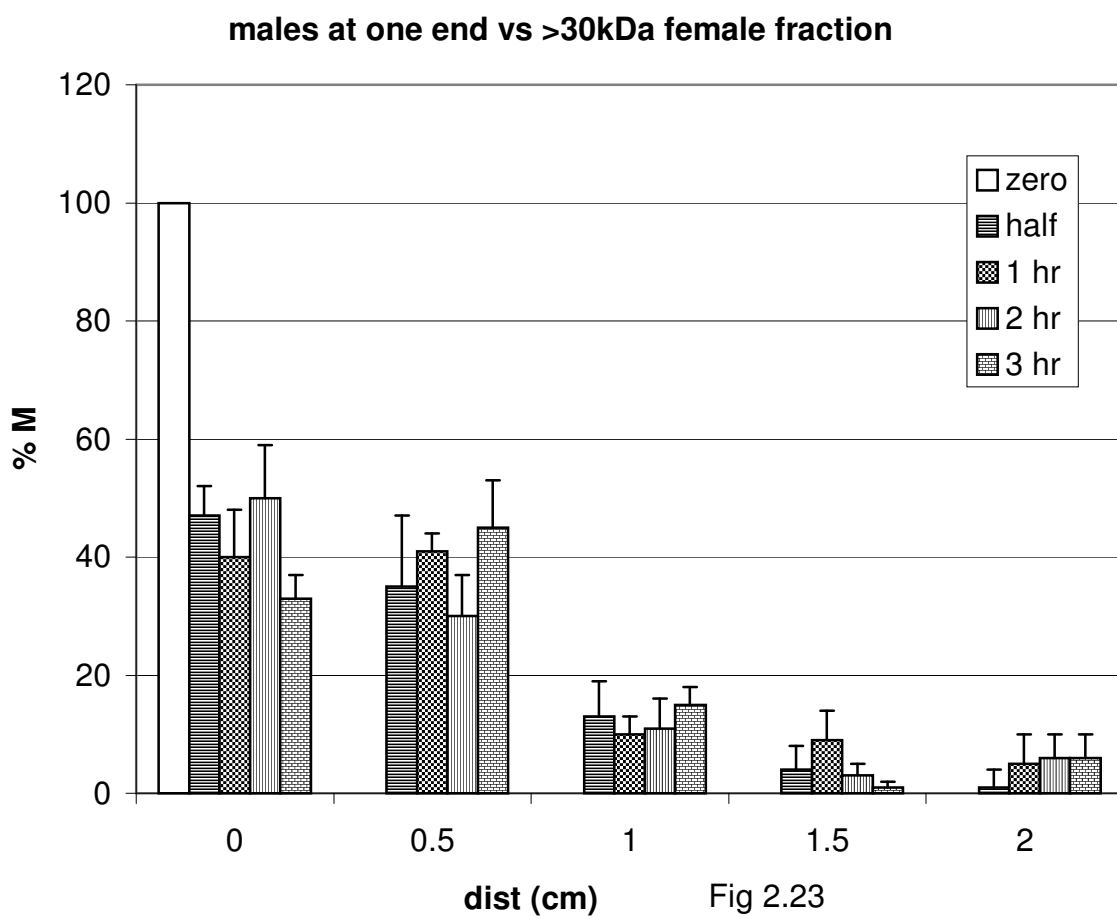


Figure 2.23: Percentage of adult male *S. mansoni* distributed in presence of adult female *S. mansoni* >30kDa incubate fraction. Male worms were placed in the middle (P = 0.572).



DISCUSSION

Behavior of adult worms in the presence of live worms of the same or opposite sex and the presence of untreated incubate of the opposite sex

The behavior of both genders in the presence of a live opposite sex worm (Figs. 2.1 & 2.3), and in the presence of untreated incubates thereof (Figs. 2.9 & 2.12), showed clearly that there is no quantitative preference as to which sex is attracted to the other. This is in contrast to the findings of Eveland *et al.* (1982) in their system in which females were preferentially attracted to males. Mutual attraction could be a way of doubling the chances for pairing to occur in a flowing environment of blood in the vertebrate host. It is an advantage to female worms in particular and to their ability to propagate their progeny outside the vertebrate host, since the females of *S. mansoni* fail to reach maturity and lay eggs if not coupled with a male, in contrast to some other species of schistosomes in which numerous viable eggs can be produced by parthenogenesis in unisexual infections (Short, 1952a & b, 1983). It is also noteworthy that the results of random movement experiments showed that the females are innately more active than the males, and that the difference between the two groups is statistically significant ($P < 0.05$) at 1, 2 and 3 hrs (but not at 0.5 hr). This observation seems to be fitting with the hypothesis mentioned above.

The experiments done on the random movement of both male and female worms showed that the majority of the worms failed to migrate beyond the 0.5 cm zone.

Homosexual attraction was rarely detectable between males but never seen between females. This is in contrast to the findings of Eveland *et al.* (1982), when they used perforated barriers. Male attraction, if real, is in agreement with the finding of males

in copula occasionally seen *in vivo* when adult worms are harvested from animals infected unisexually or bisexually.

In disagreement with the work of Eveland *et al.* (1983), we found that male worms showed no preference for attraction to single versus multiple female worms. Also, contrary to the finding of Imperia *et al.* (1980), there was no statistically significant tendency for the female worms not to be attracted to multiple males as compared to single males, though the percent of females at 3hrs that were attracted to multiple males, was 11% less than those attracted to single males. These results could be viewed at two levels: the social level and the chemical attractant/ pheromone characteristic level.

At the social level, the lack of preference of males for multiple or single females could be viewed as a way to increase the chances of female mating, fertilization, the survival and maturation of the females. It has been established that sperm is not the stimulus for female maturation (Erasmus, 1973 and Shaw, 1977) and so the frequent observation of a single male enveloping more than one female could be looked at in the frame of the above explanation since it is unclear whether the male with more than one female in possession is actually fertilizing only one or all of them at the same time.

Looking at the level of the chemical attractant/pheromone characteristic, the failure of multiple worms of one sex to either increase the number of attracted worms of the opposite sex or to decrease the attraction peak time shows that one worm is as good as many in issuing attraction of the opposite sex *in vitro*. This could be due to limited number of receptors. The less significant female attraction to multiple males, as compared to single males, observed in the system used by Imperia *et al.* (1980), can be viewed as a side effect of using barbiturates to kill the infected mice before collecting the

worms: a byproduct of metabolic breakdown of the barbiturates by the male worms could be having some repellent effect on the females. This metabolite's concentration is increased in the test chamber and, as a consequence, its effect on the females is augmented when multiple males are present. If true, then this was most likely avoided in our system when we used CO₂ to kill the mice, thus leading to the differences observed in the female behavior.

The very close similarity between Figures 2.1 and 2.9 and that between Figures 2.3 and 2.12 suggest that the worms are not attracted to byproducts found in the worms' blood meal, mainly hematin. This can be drawn from the fact that in our preparation of worm incubate we excluded any that overtly contained hematin.

Time seems to be a factor in attraction. By looking at zone 1.5+ the number of worms at this zone almost always increased in time and reached its peak at 3 hrs. This is in contrast to the findings of Eveland *et al.* (1982) in which the peak was within 30 min in the presence of barriers. This could be due to differences in the length of the chambers between the system used by us and that used by Eveland *et al.* (1982).

Behavior of adult worms in the presence of boiled incubate of the opposite sex

The purpose of these experiments was to give us an idea about the stability and the nature of the released factor(s). Since boiling affected the factor(s) in a way that clearly reduced attraction of worms of the opposite sex the possibility that the factor(s) is protein/peptide in nature is suggested.

Fractionation of adult worms incubate

Filtration was used to divide worm incubates into <30kDa and >30kDa fractions. Both the <30kDa and the >30kDa male fractions attracted the female worms, although

the dynamics of the attraction for the 2 fractions tended to differ. Female worms moved better towards the <30kDa fraction when they were placed at one end of the chambers opposite to the agar block (Figs. 2.16 and 2.17), while when they were placed in the middle of the chamber (closer to the bait) they moved better towards the >30kDa fraction (Figs. 2.14 and 2.15). This result suggests the possible existence of 2 types of factors released by the male worms. One type acts as a short range attracting factor, the <30kDa, and the other acts as a long-range attracting-factor, the >30kDa.

Contrary to the observation with the female worms, the position of the male worms relative to the agar bait did not affect their behavior towards it, they being clearly better attracted towards the <30kDa fraction in all cases. A conclusion that could be drawn is that the female factor(s) that attracts the male worms is present only in the <30kDa fraction and absent in the >30kDa fraction.

Another noticeable phenomenon with attractive fractions (with the exception of the <30kDa and the >30kDa male fractions) is the existence of what we termed the “dip” at the 1.0 cm zone. The “dip” zone contained the least percentage of worms at all time points. This phenomenon could be interpreted that the worms are in active “search mode” for the factor(s) within a certain range. Once they find it they move straight towards the source, thus creating this “dip”.

Also, one of the differences between our system and that used by others is the fact that we used a non-anesthetic method, carbon dioxide inhalation, while others have used anesthetics such as Nembutal (Eveland *et al.*, 1982; Eveland *et al.* 1983; Eveland and Haseeb, 1989; Haseeb *et al.*, 1989; Haseeb and Eveland, 1991). The new findings and the differences regarding the movement dynamics of the worms could, at least, be partially

explained by possible anesthetic effects that barbiturates could have on the worms. This is drawn from the practical fact of the ease of worm collection from mice first injected with barbiturates versus the relative difficulty of the same procedure when using un-anesthetized dead mice.

In conclusion we can say that both sexes of the *S. mansoni* species appear to attract each other equally. We hypothesize that the worms are in active search of the opposite sex. Once a signal from the opposite sex is detected the worms migrate toward the source giving what has been shown *in vitro* as the aforementioned “dip”. The factors released by each sex to attract their opposites are probably of different sizes but are likely to be of the same general chemical nature. The disagreement of some of our results with those done before is most probably due to our use of a non-anesthetic method to harvest worms, a factor that needs to be considered when studying the behavior of a living organism.

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CHAPTER 3**EFFECT OF ENZYMATIC AND CHEMICAL TREATMENTS OF
SCHISTOSOMA MANSONI FEMALE INCUBATE ON MALE ATTRACTION.
PARTIAL CHARACTERIZATION OF FEMALE-RELEASED MALE
ATTRACTANT FACTOR.**

In vitro experiments on *Schistosoma mansoni* worms have shown the existence of mutual attraction between males and females, carried out by factor(s) released by both sexes. Female worms were incubated individually in single wells in media and the incubate was collected after 1 hour. A <30 kDa fraction of the incubate was collected and subjected to Thin Layer Chromatography (TLC) analysis. It was also treated with different proteases and different concentrations of acetonitril, then was tested for its ability to attract adult male worms. TLC showed the absence of lipids and carbohydrates. Males showed attraction behavior towards methanol-insoluble female fraction and towards female incubate treated with either Proteinase K enzyme, or 5% acetonitril. No attraction was shown towards methanol-soluble female fraction or towards female incubate treated either with Pronase E, Trypsin or 80% acetonitril. The results emphasize our hypothesis that the female-released male-attracting factor(s) have a protein/peptide component of a hydrophilic nature.

INTRODUCTION

For a long time, *in vitro* chemoattraction between the opposite sexes of various species of nematodes and trematodes has been known to exist (Bone and Shorey, 1977; Bone *et al.*, 1977; Roberts and Thorson, 1977; Belosevic and Dick, 1980; Fried *et al.*, 1980; Imperia *et al.*, 1980; Fried and Robinson, 1981; Glassburg *et al.* 1981; Eveland *et al.*, 1982; Shirazian and Schiller, 1982; Eveland *et al.*, 1983; Glassburg *et al.*, 1983; Eveland and Haseeb, 1989; Haseeb and Eveland, 1991).

Ward and Bone (1983) have shown that in the nematode *Nippostrongylus brasiliensis*, the male-attracting female-released pheromone has two components. One was suggested to be a peptide, based on its sensitivity to pronase digestion, and the other to be a hydrocarbon.

The nature of the chemoattraction between male and female adult worms of the *Schistosoma mansoni* species is still unclear. These factors have been suggested to be lipidic in nature, on the basis of their partition in *n*-hexane (Haseeb, *et al.* 1989; Haseeb and Eveland, 1991) and *n*-pentane and ether (Gloer *et al.* 1986).

In the present study we partially characterized the chemoattractants released by one sex with focus on female-released male-attracting factor(s). We tested the effects of vomitus-rich incubates and worms of one sex killed by different methods on their ability to attract the opposite sex. We utilized size-exclusion gel filtration chromatography and molecular weight cut-off filters to estimate the size(s) of the chemo-attractants. Also, we tested the effects of dilution and the treatment by acetonitril and different proteases on the chemoattraction property of the adult *S. mansoni* female worm incubate. These experiments were done to provide us with information on the possible nature of the chemoattractant factor(s) that are released by the females to attract the males.

MATERIALS AND METHODS

Parasite collection, worm-incubate preparation, experiment set up and statistical analysis were carried out as shown in Chapter Two. The one-tailed T test was used to test the statistical significance of data showing % total movement of worms towards the bait

was less than 50%. For that purpose, the following formula was adapted from Anderson *et al*, (1994):

$$z = \frac{\bar{x} - \hat{\mu}}{\hat{\sigma}_x}$$

where \bar{x} is the sample mean, $\hat{\mu}$ is the population mean set here to be the 50%, $\hat{\sigma}_x$ is the standard deviation of the sample mean and z is the test statistic used to detect whether \bar{x} deviates enough from $\hat{\mu}$. The difference of the sample mean when its value was less than 50% was not considered significant when z was less than the critical value 1.645. The source of the critical value of $t_{0.05} = 1.645$ at df is Anderson *et al*, (1994).

The effect of both vomitus-rich incubate, and dead worms of one sex on the attraction behavior of the opposite sex worms

To confirm that the attraction of one sex to the incubate of the opposite sex was not towards any material in the vomitus released by the worms into the medium, we collected vomitus-rich female incubate and tested it against both worm sexes. We could not collect vomitus-rich male incubate since we could not detect any overtly released vomitus from males. We also tested the effect of the presence of dead worms on the behavior of the opposite sex primarily to confirm the reality of the attraction behavior to the opposite sex, and secondarily to see whether attractants could possibly leach out of dead worms. The worms were killed either by ethanol for 1 min at room temperature (RT) or by incubation in PBS overnight at 4C. The dead worms were washed briefly 3x in PBS before use.

Size estimation of female-released male-attracting factor(s)

Spin filters of specific cut-off sizes and gel filtration were used to estimate the size of the female-released male-attracting factor(s). Centricon spin filters of 10kDa and 3kDa cut-off were used to fractionate the female incubate. After fractionation both the filtrate and the retentate were tested for male attraction activity. For gel filtration, <30kDa female incubate fraction was run over Biol-Sil 2000 TSK column (Bio Rad, Hercules, CA) (separation range 250k – 1k). Elution rate was at 0.5 ml/min using 10 mM HEPES, pH 7 and 150 mM NaCl for elution, and 500µl fractions were collected. Aliquots from the fraction were taken and pooled in several different small batches that were then tested for activity. Caution was taken so the final dilution of any aliquot in a pool did not exceed 1:50 when being tested.

Titering the female incubate

To examine whether the attracting factor(s) are present in abundance and to estimate the maximum dilution that can be used, the female incubate was diluted 1:10, 1:25, 1:50, 1:75 and 1:100 in PBS, then tested for attraction.

High performance thin layer chromatography (HPTLC)

A <30kDa female incubate fraction, prepared by passing the media through Centricon filters (Millipore Corp., Bedford, MA) of defined cut-off value, 30kDa, was analyzed by HPTLC for the presence of lipids and carbohydrates. Thirty-five ml of the fraction was lyophilized and about 1 mg of it was used. Analytical HPTLC was performed on silica gel 60 plates (10 cm running length; 200 µM layer thickness; E. Merck, Darmstadt, Germany), using chloroform/methanol/water (50:40:10 v/v/v, containing 0.003% w/v CaCl₂) as the mobile phase. Samples were dissolved in a

minimum amount of isopropanol/hexane/water (55:20:25, v/v/v), and applied to the origin of the plate by streaking from 5 μ l Micro-caps (Drummond, Broomall, PA). Three separate plates were run, with detection made by (1) primulin dye (Sigma-Aldrich, St. Louis, MO; 0.01% in 80% aqueous acetone; after spraying, the plate is dried briefly and viewed under UV light) (Leverly *et al.*, 1989); (2) Bial's orcinol reagent (orcinol 0.55% [w/v] and H₂SO₄ 5.5% [v/v] in ethanol/water 9:1 [v/v]); the plate is sprayed and heated briefly to ~200-250°C) (Bohm *et al.*, 1954); and (3) molybdenum blue reagent (Sigma-Aldrich, M3389) (Zinzadze, 1935). The first procedure is used to detect lipids by their yellow fluorescence, the second is used to detect glycoconjugates such as glycolipids by their purple staining, and the third to detect phosphate groups of phospholipids, staining dark blue against a light blue background.

Enzymatic treatments of the female incubate

The purpose of these experiments was to test the hypothesis that the female released factor(s) are protein/peptide in nature.

Pronase-E digestion: Nonspecific complete degradation of protein was carried out using a mixture of proteolytic enzymes, Pronase-E (Sigma, P5147) to test generically the nature of the attracting factors. Pronase-E was added to the female <30kDa fraction to a final concentration of 1% (w/w pronase to lactalbumin) in a 37°C water bath for 4 hrs. The female fraction digest was then collected and tested for attraction property. Two controls were done, one in which Pronase-E was first incubated alone in phosphate buffered saline (PBS) for 4 hrs under the conditions mentioned before, then it was added as before to the female worms' incubate immediately before using it in the experiment. In

the other control both Pronase and the worm incubate were incubated separately for 4 hrs, then mixed as before.

Proteinase K digestion: Pro-K acrylic beads (Sigma, P0803), 1.2 units, were used to treat the female incubate. The coated beads were added to the female worm incubate in a tube and incubated for 4 hrs in a water bath at 37°C. The tubes were then centrifuged to pellet the coated beads and the supernatant was collected for testing.

Trypsin digestion: Trypsin coated beads (Sigma, T1763), 2 units, were used under conditions mentioned above. After incubation the coated beads were removed as described above. The supernatant was then dialyzed against PBS in a 500-pore dialysis tube to remove the excess calcium chloride added for the trypsin activity. After dialysis the fraction was tested for attraction property. As a control, untreated female fraction was also dialyzed before testing.

For each of the above enzymatic treatments a control was performed to ensure that the enzymes were active. Two ug bovine serum albumin (BSA)/ml reaction buffer was used with each enzyme. The degradation of the BSA was then ascertained by an SDS-PAGE gel.

Chemical treatments of the female incubate

To test the effect of organic solvents on the female incubate, trifluoroacetic acid (TFA) and acetonitrile (ACN), the two chemicals commonly used in reversed phase chromatography, were used at the following concentrations: 0.1% TFA acid and 5% or 80% ACN. The TFA is used a proton donor and the ACN is the organic solvent used in a gradient in the mobile phase for elution. To purify TFA from impurities, Sep-Pack columns (Bio Rad) were washed first with ACN 5x volumes, then with ddH₂O, 5x

volumes. The column was then washed 2x with 5% TFA solution and equilibrated before passing the remainder of the 5% TFA solution through it. The cleaned TFA was then used in preparing two working buffers, the first contained 5% ACN, 95% incubate, 0.1% TFA and the second 80% ACN, 20% incubate, 0.1% TFA. The buffers were incubated for 1hr at RT, then dried in speed vacuum. The dried pellet of each was resuspended in 1 ml ddH₂O or in 2 mg BSA/ ml ddH₂O and tested for activity.

The effect of methanol was tested by adding it to female incubate at a 5:1 (methanol: incubate, v/v) ratio, left at RT for 2 hrs and then centrifuged. Both the precipitate (1° precipitate) and the supernatant were collected and saved. The supernatant was dried twice in a speed vacuum, resuspended in 25 ml methanol (100% methanol) and dried again. The final dried pellet (2° precipitate) was saved. Both the 1° and the 2° precipitates were re-suspended in PBS + BSA (2 mg/ml), vortexed or sonicated for 5 min, then tested.

RESULTS

The results for the vomitus-rich incubate and dead worms were compared at 3-hrs-time-point for the total number of worms that moved in the direction of the tested material (bait). Figs. 3.1 and 3.2 show that vomitus-rich female incubate significantly reduced male attraction (10%), $z = 12.54$ (Fig. 3.1). They also show a significant difference in the behavior of males in the presence of females killed by refrigeration ($z = 13.38$ for Fig. 3.1) and in those killed by ethanol. Ethanol-killed females showed no

reduction (69%) on male attraction while those killed by refrigeration showed significant reduction (34%). For males random movement, $z = 3.8$ (Fig. 3.1).

Fig. 3.3 & 3.4 show significant female attraction to males killed by refrigeration (53%), or by ethanol (42%) ($z = 1.28$ for Fig. 3.3) while no significant attraction (41%) was shown with vomitus-rich female incubate ($z = 7.76$ for Fig. 3.3). For the female random movement $z = 2.19$ (Fig. 3.3).

Size estimation:

Fractionation using spin filters showed that the factor(s) is most probably less than 10kDa (Fig.3.5). The ‘Z’ values for >30kDa, >10kDa, and <3kDa fractions were 9.68, 11.95 and 1.4 respectively, The % of males attracted to <30kDa, >30kDa, >10kDa, <10kDa, <3kDa, and >3kDa female incubate fractions were 57%, 26%, 9%, 50%, 42%, and 51% respectively. For gel filtration, Fig 3.6 shows two pools, 25-28 min and 35-38 min that showed weak to good attraction individually and best when combined together. The estimated approximate size ranges of these two pools were between 3kDa and 8kDa and between 15kDa and 20kDa respectively. Pools 1 & 2 are representatives of all other different pools that did not show activity. The ‘z’ values for pools 1, 2, and 3 were 3.55, 2.22 and 0.22 respectively.

Serial dilution:

The serial dilution results (Fig. 3.7) have shown that 1:50 is the critical dilution beyond which attraction was greatly reduced. Attraction was relatively greatly maintained with 1:10 (66%) and 1:25 (65%) when compared to the 1:50 (52%). Significant reduction in male attraction was seen with both 1:75 (39%, $z = 1.83$) and 1:100 (24%, $z = 3.58$).

HPTLC

Neither carbohydrates nor lipids nor phosphate groups were detected in the sample.

Enzymatic treatments

Both Pronase E and trypsin treatments (Fig. 3.8 and 3.9 respectively) greatly reduced attraction (18% and 29% respectively) while Pro-K treatment (Fig. 3.10) did not show reduction in attraction (62%). The z values for Pronase E and trypsin were 65.96 and 12.35 respectively.

ACN and methanol treatments

0.1% TFA/5% ACN treatment (Fig. 3.11) had no effect on attraction (53%) while 0.1% TFA/80% ACN (Fig. 3.12) reduced attraction (37%, $z = 18.31$).

As for methanol treatment, the methanol insoluble fraction (the primary precipitate), showed high attraction activity, 77% (Fig. 3.13), whereas, the methanol soluble fraction (the secondary precipitate) yielded much reduced attraction, 38%, $z = 4.8$, (Fig. 3.14).

Figure 3.1: Comparison of total positive movement of adult male *S. mansoni* at 3 hrs in presence of vomitus-rich adult female incubate and of dead females. The z values for male worms response to vomitus-rich female incubate, females killed at 4C and their random movement, are 12.54, 13.38 and 3.8 respectively.

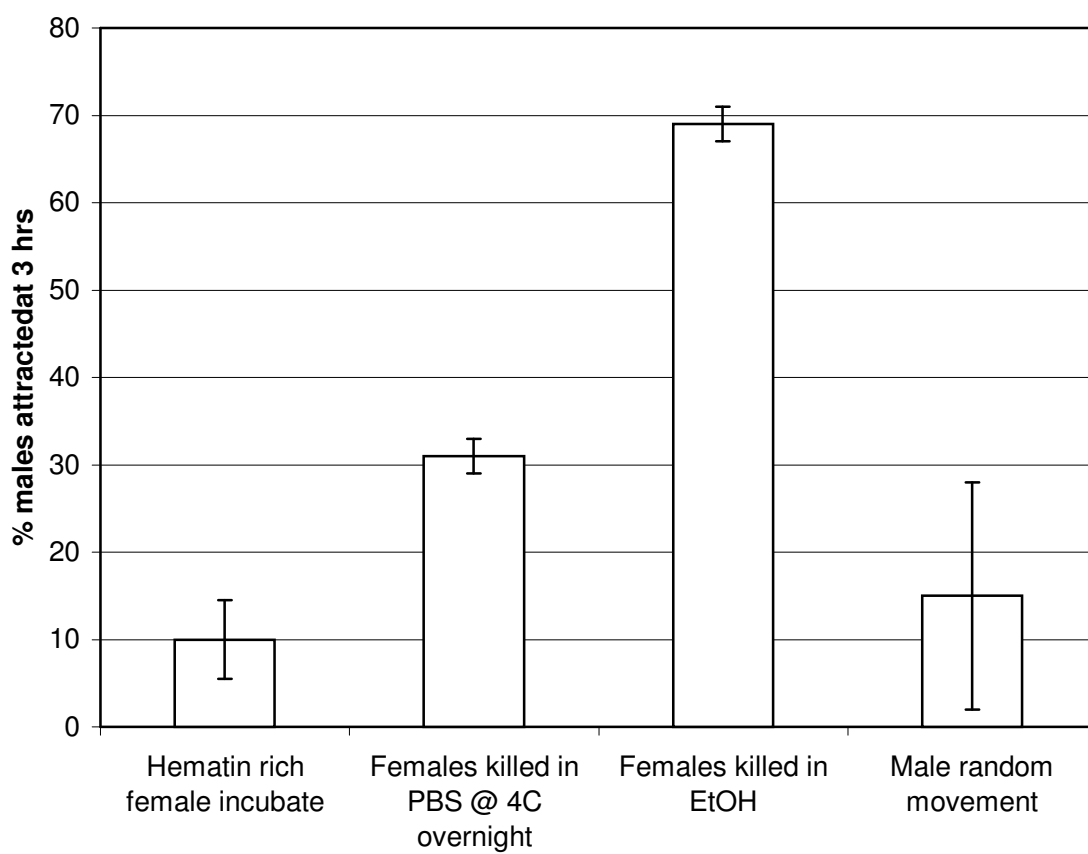


Fig 3.1

Figure 3.2: Comparison of adult male *S. mansoni* that moved the full length to the bait at 3 hrs in presence of vomitus-rich adult female incubate and of dead females.

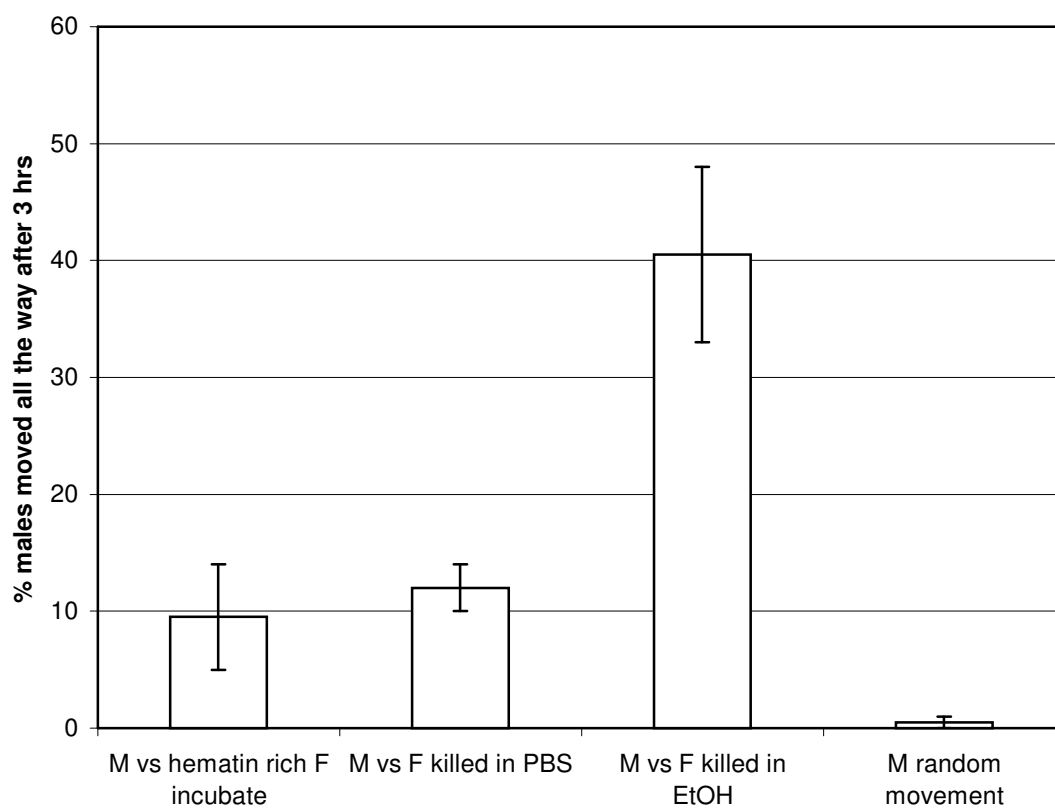


Fig 3.2

Figure 3.3: Comparison of total positive movement of adult female *S. mansoni* at 3 hrs in presence of vomitus-rich adult female incubate and dead males. The z values for the female response to vomitus-rich female incubate, males killed by ethanol and their random movement, are 7.76, 1.28 and 2.19 respectively.

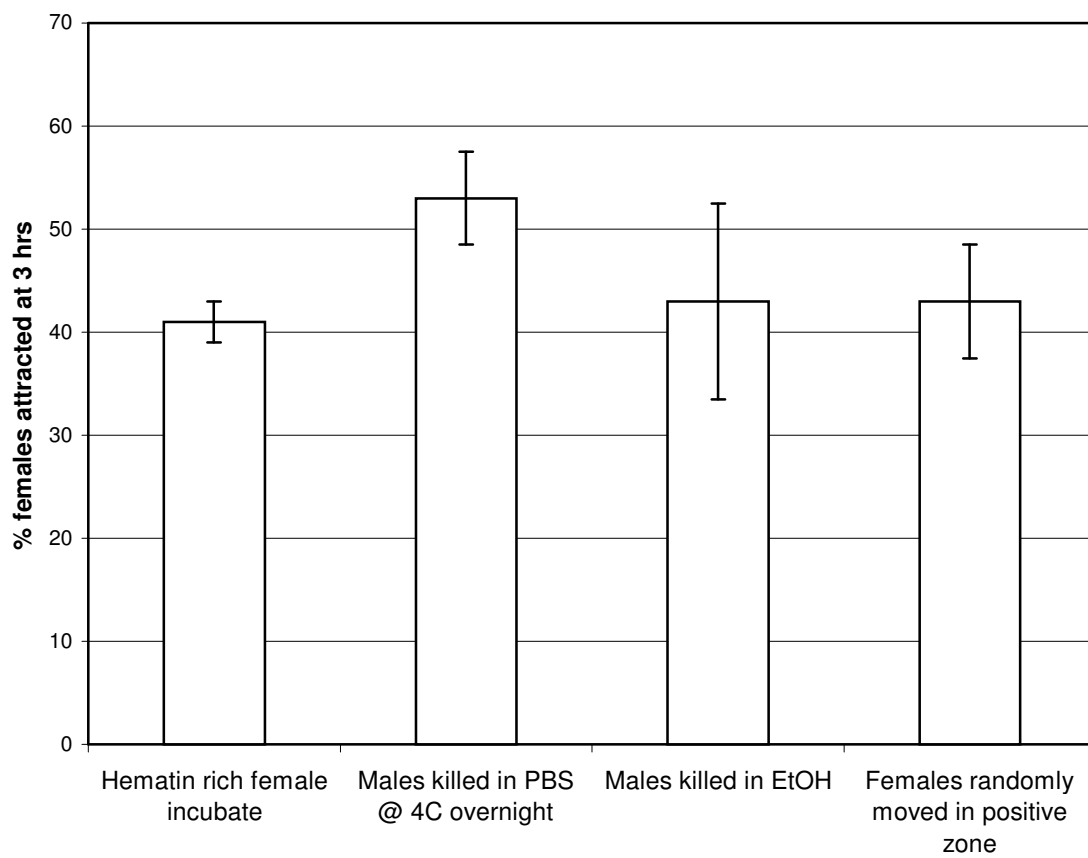


Fig 3.3

Figure 3.4: Comparison of adult female *S. mansoni* that moved the full length to the bait at 3 hrs in presence of vomitus-rich adult female incubate and dead males.

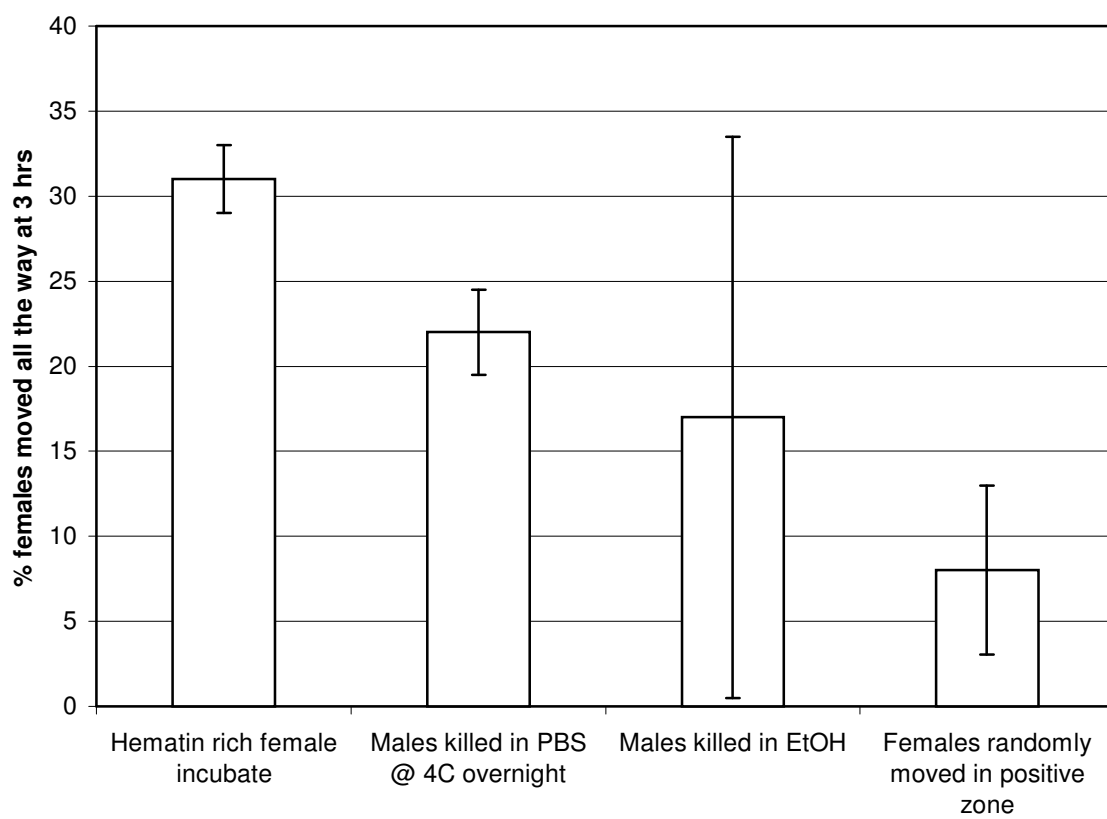


Fig 3.4

Figure 3.5: Comparison of total male positive movement at 3 hrs to different filter-fractionated female incubate fractions. The z values for >30kDa, 10 kDa, and <3kDa are 9.68, 11.95 and 1.4 respectively.

Total positive movement after 3 hrs for all female incubate fractions by cut-off filter

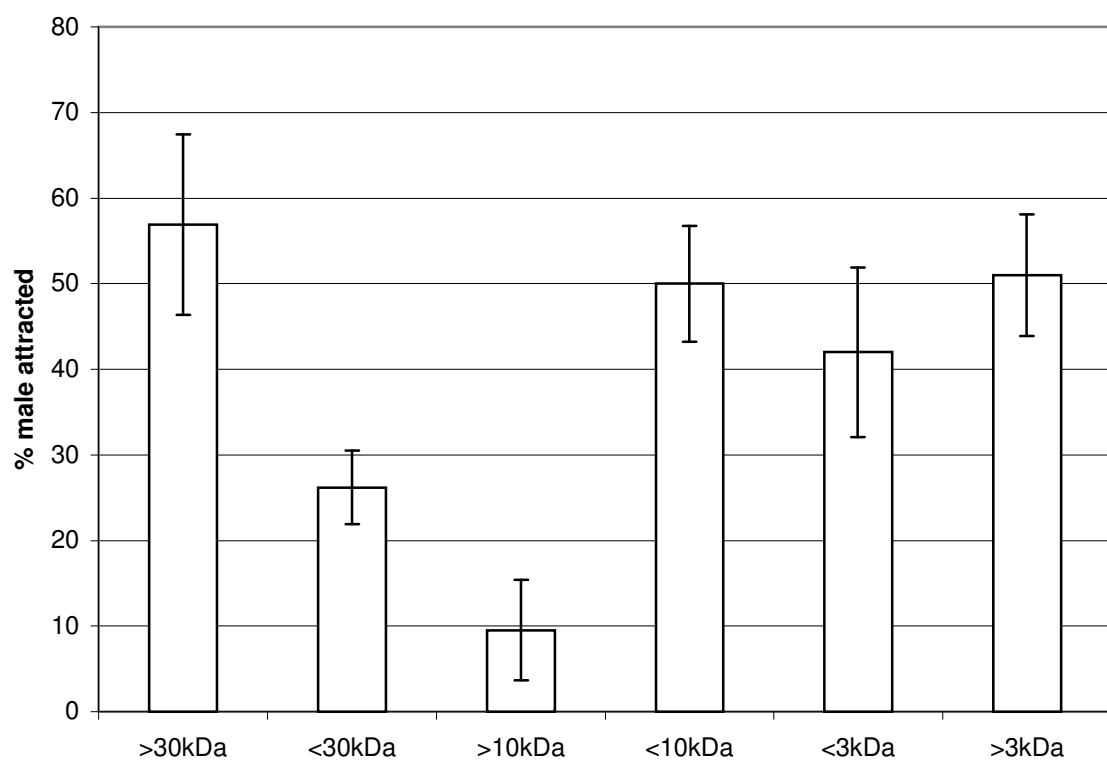


Fig 3.5

Figure 3.6: Comparison of total male positive movement at 3 hrs to different gel filtration pools of female incubate fractions. The z values for pool 1, pool 2 and fraction 35-38 min are 3.55, 2.22, and 0.22 respectively.

comparison of total attraction for gel filtration

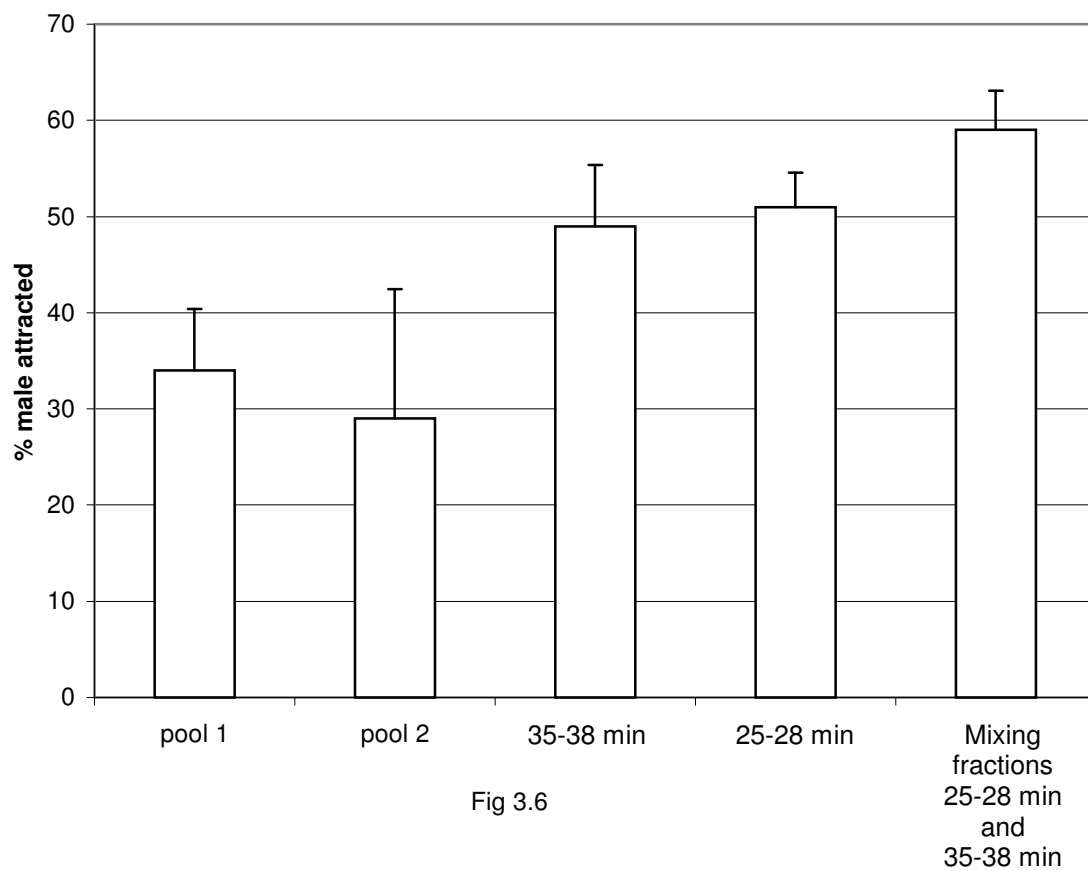
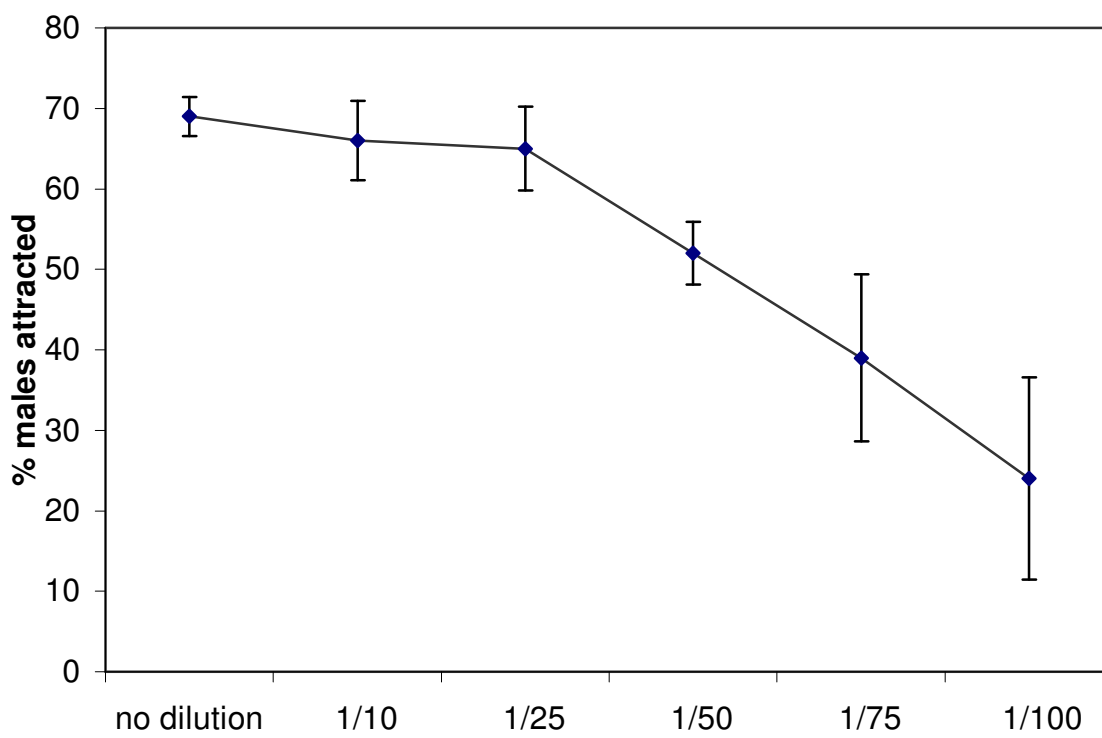


Fig 3.6

Fig. 3.7: Percent of males attracted to a series dilution of female worm incubate at 3 hrs. The z values for 1:75 and 1:100 are 1.83 and 3.58 respectively.

% Males attracted to a series dilution of female

Dilution Fig 3.7

Figure 3.8: Percentage of males attracted to PronaseE-treated female incubate ($z = 65.96$).

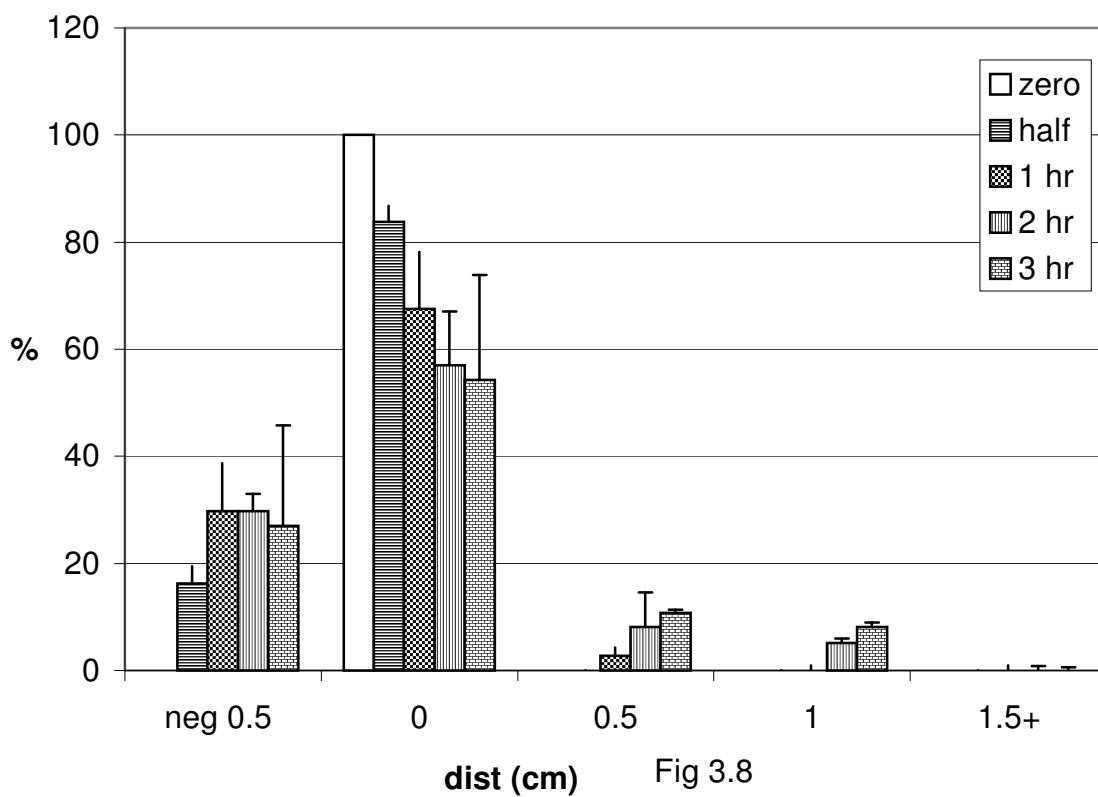
% males moved vs Pronase treated female incubate

Figure 3.9: Percentage of male attracted to trypsin-treated female incubate ($z = 12.35$).

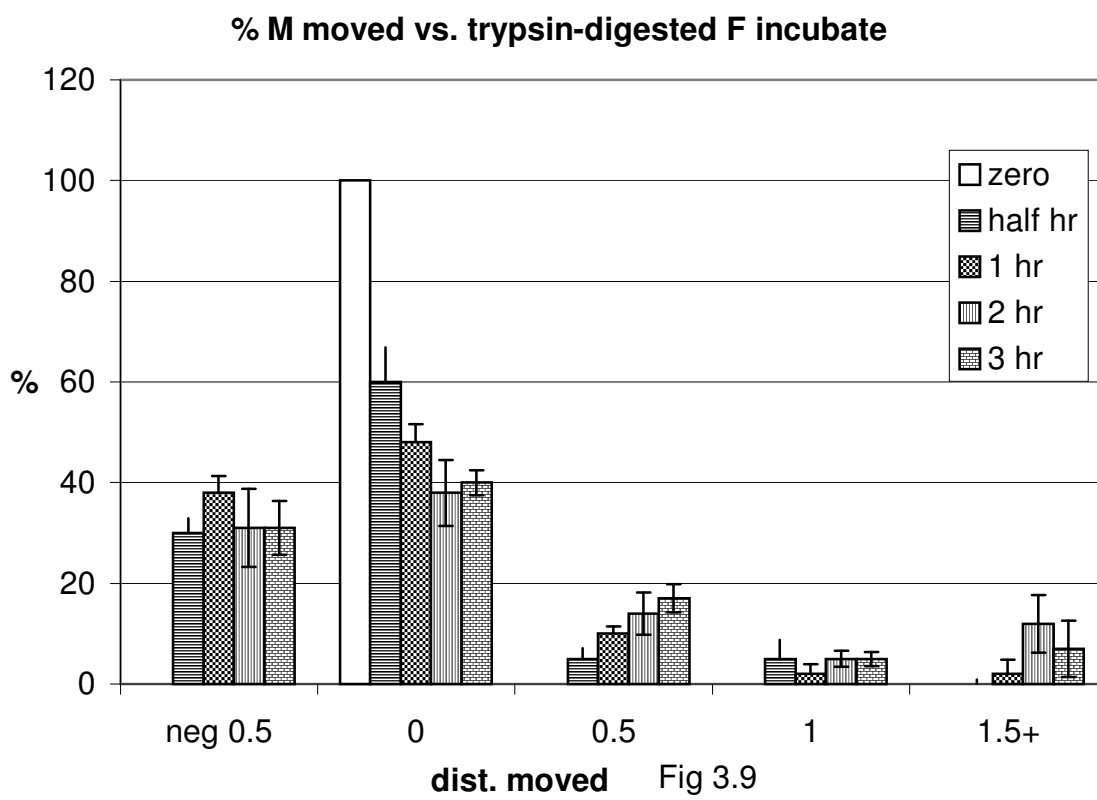


Figure 3.10: Percentage of males attracted to ProK-treated female incubate.

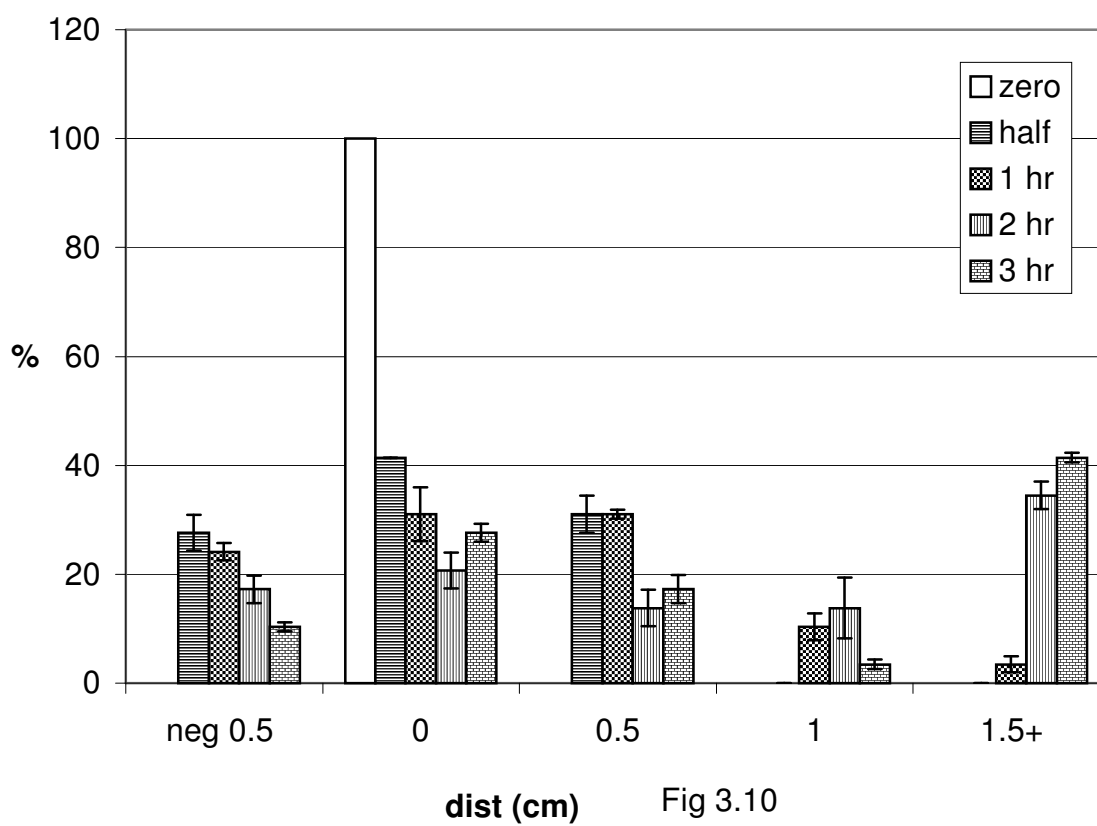
% males moved vs ProK treated female incubate

Fig 3.10

Figure 3.11: Percentage of males attracted to 0.1% TFA/5% ACN-treated female incubate.

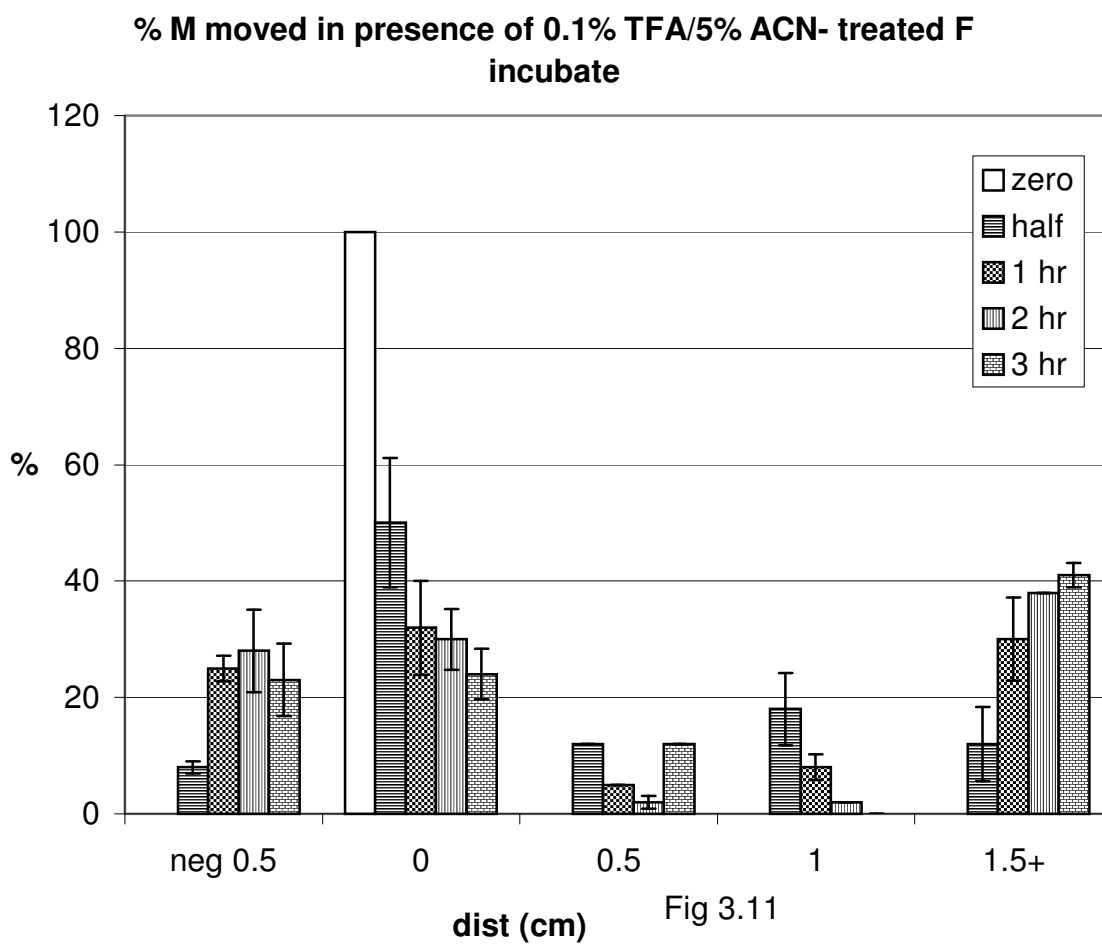


Figure 3.12: Percentage of males attracted to 0.1% TFA/80% ACN-treated female incubate ($z = 18.31$).

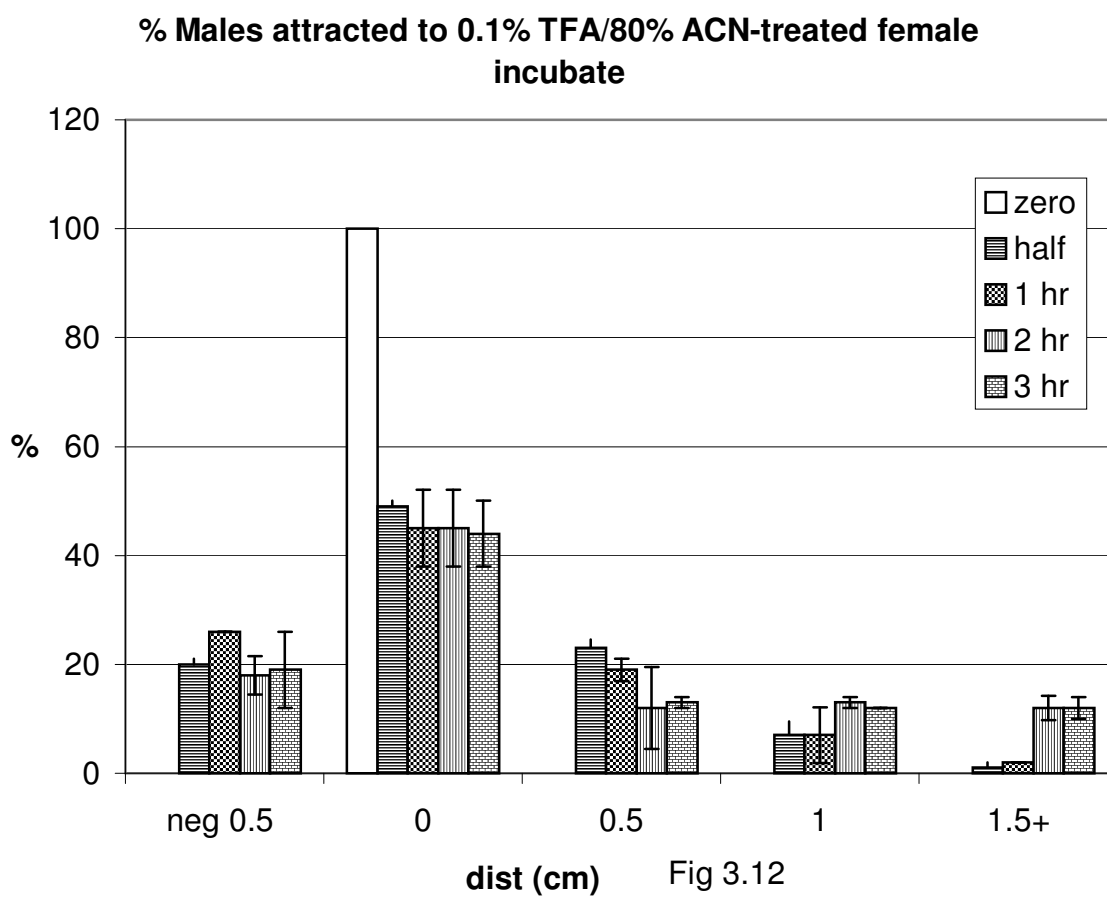


Figure 3.13: Percentage of males attracted to methanol insoluble female incubate fraction.

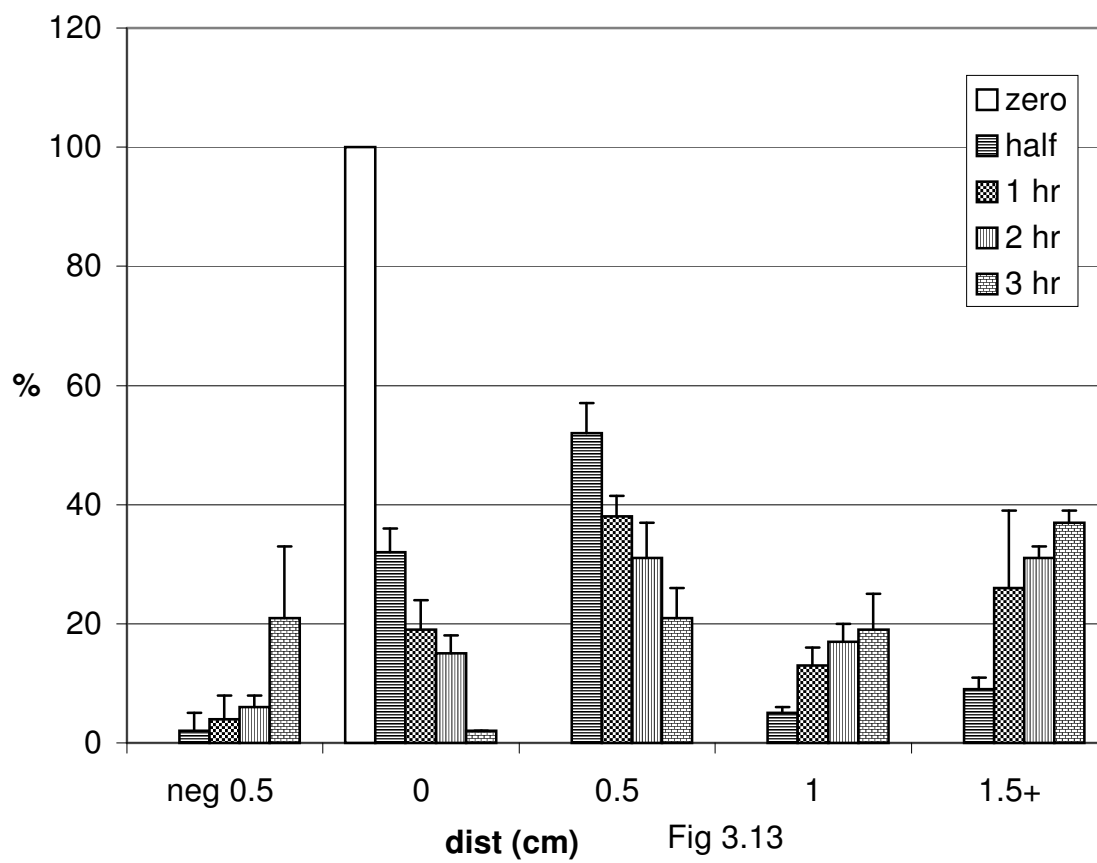
Males in presence of methanol insoluble female fraction

Figure 3.14: Percentage of males attracted to methanol soluble female fraction ($z = 4.8$).

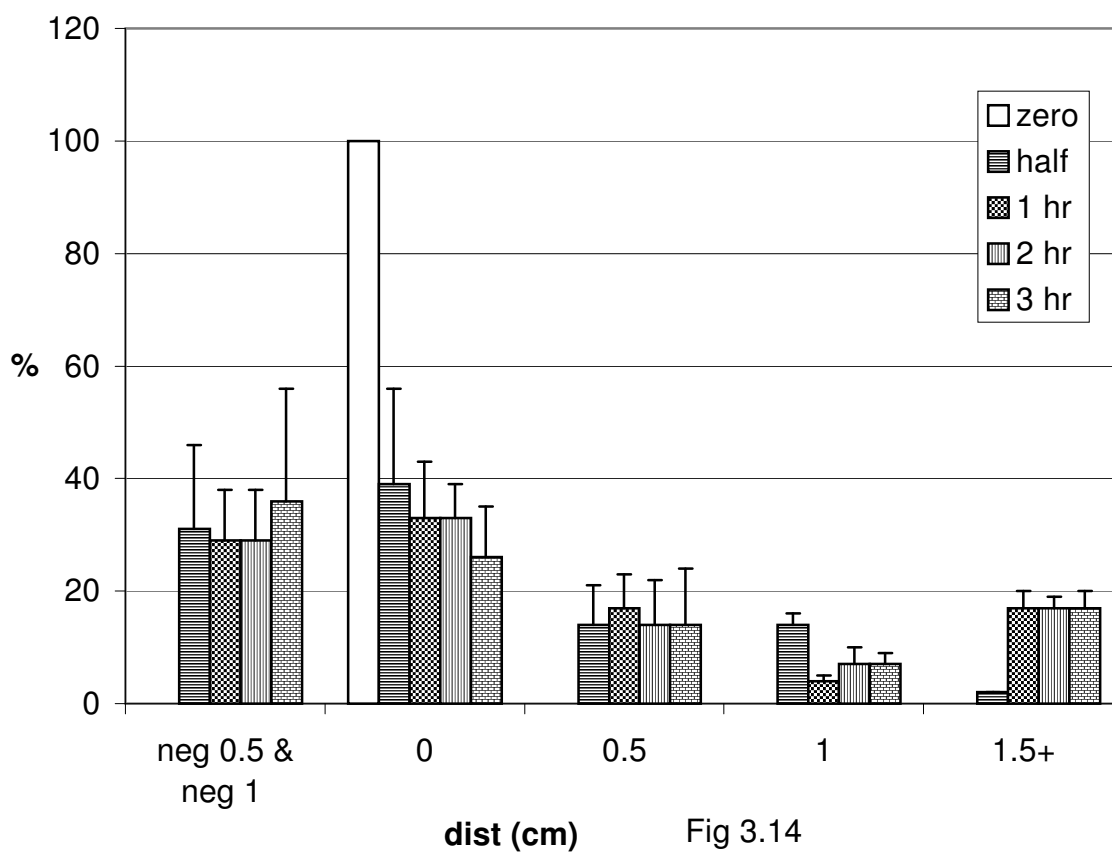
Males in presence of methanol soluble female fraction

Fig 3.14

DISCUSSION

Chappell and Dresden (1986) have considered that the digestive tract components of *S. mansoni* are represented by their vomitus. It has been shown that it contains large quantities of digestive enzymes as well as other degraded materials (Timms and Bueding, 1959; Chappell and Dresden, 1986; McKerrow and Doenhoff, 1988; McKerrow, 1989; Dalton *et al.*, 1995; Caffrey *et al.*, 1997). The reduction in attraction of males to vomitus-rich female incubate may have adaptive significance since young males would not waste energy by migrating toward mature females *in copulo*, that produce copious quantities of vomitus. On the other hand, young virginal females produce little vomitus, so they would be highly attractive to males. The results also indicate that at least the male attractants are not food by-products released by the females, and suggest that these attractants are susceptible to the digestive enzymes found in the female vomitus. The persistence of male attraction to ethanol-killed females but not refrigerated females suggests that the female attractants are insoluble in organic solvents. Also ethanol would have denatured the digestive enzymes in the vomitus thus leaving the attractants intact. Refrigeration would only slow the process of degradation.

The reduction of female attraction to ethanol-killed males, though not statistically significant, suggests that the male released factors might be soluble in organic solvents. The attraction of females to refrigerated males suggests that either the male attractants are not easily degraded, or it could be due to the fact that males do not produce much vomitus and so the male released factors are not subjected to proteolysis.

The reduction of attraction behavior of males after trypsin digestion indicates that the female released factor(s) has arginine and/or lysine (Craik *et al.*, 1985; Perona and

Craik, 1995) and that cleaving the factor(s) at these sites inactivates it. On the other hand the insensitivity of the attraction to ProK treatment indicates that the active portion of the factor(s) does not contain amino acids that either are hydrophobic aliphatic, *e.g.* valine, leucine and isoleucine, hydrophobic aromatic, *e.g.* tyrosine, phenylalanine and tryptophan, or hydrophobic, *e.g.* proline, methionine and alanine (Kraus *et al.*, 1976). Arginine and lysine are polar hydrophilic with charged side chains. Also, it can be deduced that at least the active portion of the factor(s) does not contain esters since both enzymes hydrolyze esters (Hedstrom *et al.*, 1994; Borhan *et al.*, 1996; Barrett *et al.*, 1998).

The results of ACN and methanol treatments indicate that the factor(s) are probably hydrophilic in nature due to their sensitivity to high ACN concentration and their apparent insolubility in high concentrations of methanol. The proposed hydrophilic nature of the factor(s) seems to be fitting the nature of the environment, the blood, in which the worms live.

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CHAPTER 4

IDENTIFICATION, PURIFICATION AND PARTIAL AMINO ACID

SEQUENCE OF A *SCHISTOSOMA. MANSONI* FEMALE-RELEASED MALE-

ATTRACTANT PEPTIDE.

Mass spectrometry was used to determine the amino acid sequence of a *S. mansoni* male-attractant peptide that is released by the females. A <10 kDa methanol insoluble fraction (MIF) of the female incubate was fractionated by reversed phase-high performance liquid chromatography (RP-HPLC). We found a fraction containing one peak that gave the best attraction. The purity of the peak was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). At x1000 concentration, multiple bands were seen on the gel after silver staining. The M_r of the bands were approximately the multiples of the M_r of the smallest band detected on the gel (about 3 kDa). At a concentration of about x200 only a single band of about 3 kDa was seen on the gel. The findings were confirmed by MALDI-TOF MS. A major signal indicating the presence of a peptide of mass 3268 was obtained. Several other minor signals of masses that were the multiples of that peptide were also obtained, confirming the findings of the SDS-PAGE. The amino acid sequence of a peptide fragment was then determined using MALDI-TOF/TOF. The sequence was QVHHQK.

INTRODUCTION

In 1959 it was shown for the first time that mass spectrometry (MS) can be used to identify the amino acid sequence of peptides, and since then it has contributed to certain areas of protein structure determination such as the identification of N-terminal blocking groups, which are a severe obstacle in Edman degradation, and the identification of unusual or modified amino acids in peptides (Biemann, 1986). It has become a valuable tool for the

characterization of proteins and peptides for measuring their molecular weights while intact, for determining their identity and delineating their structure, and for detecting post-translational modifications of amino acids especially at the terminal residues (Yergey *et al.*, 2002). Many of the protein modifications, such as carbohydrates, cannot be identified by Edman degradation (Carr *et al.*, 1988). This procedure is based on selective coupling of a peptide's amino-terminal amino acid with phenylisothiocyanate in the presence of an organic base. This derivatized amino acid (a phenylthio-hydantoin form) is then cleaved from the remainder of the peptide by a strong organic acid in repetitive cycles, and the derivatized amino acids are then collected from each cycle and run on reverse phase HPLC to give a unique chromatogram for each derivatized amino acid (Hunkapiller, 1986). Some of the protein modifications are destroyed during the relatively harsh chemical derivatization steps that are employed. In addition, the amino-termini of recombinant proteins are often blocked preventing confirmation of this region by Edman degradation. Carboxy-terminal sequencing with carboxypeptidases is difficult because of the differential rate of hydrolysis of peptide bonds and, in some cases, lack of accessibility of this region to the proteases (Carr *et al.*, 1988).

The high sensitivity of modern MS and the high selectivity of protein database searches have enabled the identification of proteins in the sub-picomole level on a larger scale and have fueled the rise of proteomics as a new field of research (Wattenberg *et al.*, 2002). Protein identifications are now accomplished using several approaches, often in combination, of which Matrix Assisted Laser Desorption-Time of Flight (MALDI-TOF) is the first method employed by which exact masses of peptide resulting from endoprotease

digestions are used in conjunction with a number of data base searching routines (Yergey *et al.*, 2002).

The MALDI-TOF approach has one clear advantage, speed of analysis (Lay, 2001). Since its development and introduction by Karas and Hillenkamp (1988) and Tanaka *et al.*, (1988) it has been broadly used in the analysis of proteins (Karas *et al.*, 1989; Cordwell *et al.*, 1995; Wasinger *et al.*, 1995; Cordwell *et al.*, 1997; Dukan *et al.*, 1998; Fukuo *et al.*, 1998; McAtee *et al.*, 1998; Wolf *et al.*, 1998; Loo *et al.*, 1999; Wall *et al.*, 1999; Fournier *et al.*, 2000; Heinzle, 2000; Kaji *et al.*, 2000; Bray *et al.*, 2001; Marvin *et al.*, 2001; Kim *et al.*, 2002; Wittmann and Nam *et al.*, 2002; Zatylny *et al.*, 2002).

MALDI-TOF has been used also for the characterization of synthetic and natural polymers and for the analysis of tannins (Behrens *et al.*, 2003, and references therein). Some have used it for DNA analysis (Tang *et al.*, 1994; Chang *et al.*, 1995; Taranenko *et al.*, 1996; Berkenkamp *et al.*, 1998; Taranenko *et al.*, 2002) and in sequencing short DNAs (Koster *et al.*, 1996; Rosky *et al.*, 1996;).

Taranenko *et al.* (2002) have reported that MALDI-TOF/MS can be used to measure molecular weights of polymerase chain reaction (PCR) products of 16S rDNA and restriction fragment length polymorphism (RFLP) fragments to help identify various microbes. The use of MALDI-TOF/MS as a tool for quantitative analysis has been demonstrated by Wittmann and Heinzle (2000). In their reviews, Fenselau and Demirev (2001) and Lay (2001) have shown the applications of MALDI-TOF/MS to obtain biomarker profiles directly from unfractionated microorganisms, including viruses, bacterial and fungal vegetative cells, and spores. Taxonomic identification of bacteria based on constituent proteins using MALDI-

TOF/MS has been shown to be possible, in some cases down to the subspecies level (Lay, 2001).

The principle of MALDI-TOF/MS is based on mixing, on a metal chip, the sample with a special proton donor matrix that then interacts with proteins and forms crystalloid structures with them. These structures are targets for a laser beam causing the sample and the matrix to volatilize and ionize without the fragmentation of the sample. Such an ionization mode results in the formation of single charged protein ions, although protein ions of two, three or more charges can also be formed (Govorun and Archakov, 2002). The ions formed are accelerated by a high voltage supply and then allowed to drift down a flight tube where they separate according to mass. Arrival at the end of the flight tube is detected and recorded by a high-speed recording device (<http://www.biotech.iastate.edu/facilities/protein/maldi.html>).

Tandem MS is a technique that is a nearly ideal analytical approach for structural characterization of proteins and sequence analysis of specific peptides and has two main advantages. First, it is a nonsequential technique and not limited by the presence of posttranslational modifications, in contrast to the Edman degradation. Second, posttranslational modifications, if present, generally survive and can be analyzed. The data provided by tandem MS can then be used to establish the specific residue that is modified, confirm the mass of the modification, and determine the sequence of the peptide containing this modification (Carr *et al*, 1988). In addition, it has the ability to provide extensive sequence information over the whole length of a protein chain in a single series of experiments that involve minimal effort directed toward separation and purification of oligopeptide fragments (Hunt *et al*, 1986).

The interpretation of the spectra of unknowns requires extensive knowledge of the various fragmentation processes, the effect of specific amino acids on the relative abundance of the resulting ions, and the effect of experimental conditions (i.e., collision energy, collision gas, etc.) on the relative distribution of these ion series (Martin *et al.*, 1988). The currently used nomenclature for the resulting ions was first proposed by Roepstroff and Fohlman (1984).

The recent introduction of a MALDI ion source coupled to a mass spectrometer with a tandem time-of-flight analyzer has provided a means to obtain MS and MS/MS data from the same sample on the same instrument (Wattenberg *et al.*, 2002). Several have already exploited it for protein identification (Krutchinsky *et al.*, 2000; Loboda *et al.*, 2000; Shevchenko *et al.*, 2000 and 2001; Baldwin *et al.*, 2001).

Yergey *et al.*, (2002) have argued that the MS/MS spectra by the MALDI-TOF/TOF are of such quality that sequence determinations based strictly on these spectra are both exact and straightforward to obtain. They used it to get *de novo* sequences of a set of peptides derived from tryptic digestion of electrophoretically separated sea urchin egg membrane proteins. They detected errors in published sequences and were able to develop sequences from peptides differing in mass by one dalton. Another example was the successful identification and characterization of bovine heart proteins using MALDI-TOF/TOF by Bienvenut *et al.*, (2002). In their work, they showed two examples of the utilization of the MALDI-TOF/TOF for peptide discrimination and sequence validation. Also, using *de novo* interpretation of the MS/MS spectrum obtained from one peptide, they identified a previously unknown amino acid residue that proved to be a doubly oxidized tryptophan.

In the previous chapters we have shown that there is mutual attraction between the opposite sexes of adult *S. mansoni* worms and that there is no attraction between worms of the same sex. We have shown that the females were attracted to possibly more than one male-released factors; a <30kDa short-range factor and a >30kDa long-range factor. Males, on the other hand, were attracted to a <10kDa female released factor(s). Boiling the worm incubate of one sex reduced the attraction of the worms of the opposite sex, suggesting that the attracting factors released by both sexes are protein/peptide in nature or have a protein/peptide component. The female-released factors were shown not to be in the female vomitus, since vomitus-free female incubate did attract the males, while vomitus-rich female incubate did not. Also, we have shown that mate attractants can leach out of the dead worms, and that the strength of the attraction of dead worms of the opposite sex is probably dependent on the method used to kill the worm. Males were strongly attracted to ethanol-killed females, but not to females killed by overnight incubation in PBS at 4C. On the contrary, the females were attracted to males killed either by overnight incubation in PBS at 4C or by ethanol, though to a lesser extent to the latter. This showed that probably the male attractants are not soluble in organic solvents and that the female attractant(s) are either partially soluble in organic solvents or that some components are soluble in organic solvents and were lost in the ethanol, thus reducing the female attraction to ethanol-killed males.

To test the hypothesis that the female-released factor(s) is protein/peptide in nature, the female incubate was analyzed by thin layer chromatography. It was also treated with different proteases, with methanol, and with different concentrations of acetonitril. The thin layer chromatography showed the absence of lipids and carbohydrates. Males were attracted to female incubate treated with either Proteinase K, with 5% acetonitril, and to the methanol-

insoluble fraction. Their attraction was greatly reduced towards female incubate treated with Pronase E, trypsin, or with 80% acetonitril, or to the methanol-soluble fraction. The results are consistent with the hypothesis that the female-released male-attracting factor(s) has a protein/peptide component of a hydrophilic nature.

Here we present our results in characterizing, purifying and the partial amino acid sequencing of a polypeptidic male-attractant factor released by *S. mansoni* adult female worms. MALDI-TOF was used to get exact molecular weight size and MALDI-TOF/TOF was used as the tool for the amino acid sequence determination.

MATERIAL AND METHODS

Fractionation of female incubate using Reverse Phase-High Performance Liquid

Chromatography (RP-HPLC):

Female incubate was treated with 4 volumes of methanol and left at 4°C overnight for protein precipitation. The precipitate was collected, pooled, reconstituted in ddH₂O to 1/100 the original incubate volume, sonicated for 10 min and then centrifuged at 4000xg to remove any insoluble material. The supernatant, the methanol-insoluble female incubate fraction (MIF), was collected, while any pellet after sonication was discarded. A 10kDa cut-off spin filter (Microcon YM-10, Millipore, Bedford, MA) was used to filter 50 µl MIF + 500 µl ddH₂O/0.1% TFA to eliminate the albumin and any other high molecular weight proteins before running on the RP-HPLC to prevent over-saturation of the column with albumin in particular. RP-HPLC for the <10kDa MIF fraction was then carried out using analytical scale, 5µm particle size, C18 column (Jupiter, 150x4.6 mm, Phenomenex, Torrance, CA).

The column was injected with 500ul of the <10kDa fraction. A linear gradient of 5-90% ACN containing 0.1% TFA was used as the elution buffer, at flow rate of 0.5 ml/min for 55 min. Six fractions corresponding to the following percentages of ACN (5-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%) were collected. Absorbance was measured at 215nm. Fractions collected were frozen then dried using speed vacuum, re-suspended to the eluted volume in ddH₂O/BSA (2mg/ml), sonicated or vortexed, and then tested for activity. The fraction that gave the best attraction was chosen for further fractionation. Our target was to find a fraction with a single elution peak that gave the best attraction.

After determining that best activity was in the 30-40% fraction, 500ul of the <10,000 MIF was injected. The column was equilibrated for 10 min with 20% ACN/0.1% TFA followed by 10 min linear gradient to 0.1% TFA/28% ACN. Five fractions corresponding to the following percentages of ACN: 28-31%, 31-33%, 33-35%, 35-37% and 37-41%, were collected during a linear gradient from 0.1% TFA/28% ACN to 0.1% TFA/41% ACN over 1hr. Absorbance was measured at 215nm. Fractions collected were frozen then dried using speed vacuum, re-suspended to the eluted volume in ddH₂O/BSA (2mg/ml), sonicated or vortexed, and then tested for activity.

Statistical analysis:

The one-tailed T test was used to test the statistical significance of data showing percent total movement of male worms towards different RP-HPLC fractions, for the assays of which their value was less than 50%. For that purpose, the following formula was adapted from Anderson *et al*, (1994):

$$z = \frac{\bar{x} - \hat{\mu}}{\hat{\sigma}_x}$$

where \bar{x} is the sample mean, $\hat{\mu}$ is the population mean set here to be the 50%, $\hat{\sigma}_x$ is the standard deviation of the sample mean and z is the test statistic used to detect whether \bar{x} deviates enough from $\hat{\mu}$. The difference of the sample mean when its value was less than 50% was not considered significant when z was less than the critical value 1.645. The source of the critical value of $t_{0.05} = 1.645$ at df is Anderson *et al.*, (1994).

SDS-PAGE:

Pre-cast 10% and 16% Tris-HCl SDS-PAGE, and 16.5% Tris-Tricine SDS-PAGE mini gels (Bio Rad) were used. Samples were run under non-denaturing conditions at constant voltage 120V. After separation the gels were silver stained using Focus-Fast Silver stain kit (Geno Technology Inc., St. Louis MO).

Carboxyamidomethylation:

Carboxyamidomethylation of both the crude MIF and the peptide was done to simultaneously check the presence of cystine residues and to see the effect of the reduction and capping of the sulfide groups, if cystine was present, on the peptide multimerization. This was done using iodoacetamide, which is a reagent commonly used for that purpose. The procedure was adapted from Ferranti *et al.* (1995). For the crude MIF, 50 μ l of it was reduced in 8M urea, 0.2M Tris-HCL (pH 8.8), and 40mM DTT for 1hr at 55°C. After 1hr the reduced MIF solution was made 80mM in iodacetamide and allowed to incubate at room temperature in the dark for 30 min. After reduction and carboxyamidomethylation the MIF, salts were removed using C18-Zip Tip (Millipore, Bedford, MA) solid phase extractions into 10 μ l of 1:1 0.1% TFA:ACN. The salt-free MIF solution was brought to 550 μ l by addition of 0.1%TFA subjected to RP-HPLC under the same conditions described above. The RP-HPLC

purified peptide fraction was frozen, dried under vacuum, and resuspended in 10ul of 1:1 0.1%TFA: acetonitrile for MALDI analysis.

For carboxyamidomethylaion of the peptide, 40ul was used and treated as shown above.

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF):

Alpha-Cyano-4-Hydroxycinnamic acid (Sigma-Aldrich) was used as the proton donor matrix. One ul of the sample was added along with an equal volume of the matrix solution (saturated matrix solution in 1:1-0.1% TFA:ACN) on a gold plated metal chip. On drying, using G2024A vacuum pump (Hewlett Packard, GA), the matrix formed a crystalloid structure with the peptide. This structure was then subjected to the laser beam (40 uJ). The LD-TOF system G2025A (Hewlett Packard) was used, in the positive ion mode, as the laser source to vaporize and ionize the peptide. The MALDI-TOF was used to check the purity of the peptide and to get an exact molecular weight size of it. MW sizes of detected peaks are recorded as mass:charge ratio (m/z).

Edman Degradation for N-terminal sequence:

N-terminal sequencing of the peptide by Edaman degradation was done at the Macromolecular Structure Facility, Michigan State Univ., East Lansing, MI.

MS/MS Analysis and de Novo Sequencing

MS and MS/MS analysis was performed on an Applied Biosystems 4700 MALDI TOF/TOF Proteomics Analyzer (Applied Biosystems, CA) at the Molecular Genetics Instrumentation Facility, Univ. of Georgia, Athens, GA. The dried purified peptide was re-suspended in 10ul of 1:1 0.1%TFA: ACN for MS/MS analysis. A 0.5 μ l sample was co-crystallized with the same amount of matrix (saturated α -cyano-4-hydroxycinnamic acid).

Samples were analyzed both in MS and MS/MS mode with a 200 Hz laser repetition rate. The collision-induced dissociation (CID) was performed using air as the collision gas at a collision energy of 1kv.

Peptide Digestion

To confirm the presence of lysine residues the purified peptide was digested for 4 hrs with endoproteinase Lys-C (Sigma) using the protocol set-forth by Perides *et al.* (1987). The sample was desalted using a ZipTip (Millipore, Bedford, MS) and the peptides were eluted into 10ul 1:1 0.1%TFA: ACN for MS/MS analysis.

Database analysis:

Homology search was done for the depicted amino acid sequence with search programs provided both by National Institute of Health (NIH) (<http://www.ncbi.nlm.nih.gov/blast>), using the ‘Search for short nearly exact matches’ option, and by ProteinInfo provided by the Rockefeller University (<http://129.85.19.192/prowl/proteininfo.html>). The NCBI database was used in both cases. The ProteinInfo uses information from mass spectra of peptide maps found in the database.

RESULTS

The best male-attraction activity was found to correspond to a fraction containing a peak-doublet that eluted at 34% ACN (Figs. 4.1a and b). In these figures, a comparison of total male attraction after 3 hrs to un-fractionated female incubate, the peak doublet and fractions from other gradients is shown. In Fig. 4.1a, the z values for 5-10%, 10-20%, 20-30%, 30-40%, 40-50% and 50-60% ACN fractions were, 4.67, 2.77, 5.19, 0.58, 4.94 and

6.17 respectively, and the percent of male attraction to them were 23%, 42%, 38%, 48%, 30%, 25%, and 14% respectively. In Fig 4.1b, which shows the focus on the 30-40% ACN gradient, the z value for 29-32%, 32-34%, 34-36% (peak doublet), 36-38% and 38-41% fractions were 2.94, 5.22, 1.14, 13.51, and 4.88 respectively, and the percent of male attraction to them were 33%, 29%, 46%, 11%, and 19% respectively. The peak doublet was purified under the following running conditions: 20% ACN for 10 min, 20%-33% for 10 min, 33%-35% for 55 min, 35%-90% for 15 min. The doublet always eluted as such (Fig. 4.2) and with no other peaks eluting with it within the 34-36% gradient.

The purity of the eluted peak was checked both by MALDI-TOF and SDS-PAGE. On MALDI-TOF it yielded a single peak of about m/z 9543 (Fig. 4.3). The crude MIF was also checked by MALDI-TOF. It yielded several peaks but the m/z 9543 peak was not among them (Fig. 4.4). For SDS-PAGE gels, the peaks from 15 RP-HPLC runs were pooled and the volume was reduced from about 35ml down to approximately 600 μ l, in presence of 200 μ g albumin, by freeze lyophilization. Aliquots of 10, 5 and 1 μ l were loaded on 16% SDS-PAGE then visualized by silver staining. Fig. 4.5 shows multiple bands instead of one as expected from the result seen on MALDI-TOF. Judging by their MW sizes on the gel, they seemed to be multiples of a low MW protein/peptide. To test this hypothesis, the peak collected from RP-HPLC was subjected to carboxyamidomethylation (CAM) and then checked by MALDI-TOF. The m/z 9543 peak disappeared and two major peaks appeared at about m/z 3000 (Fig. 4.6). The two major peaks were m/z 3265.8 and m/z 3322.8 with a difference of 57 Da between them.

To answer the question of whether the eluted peak is collected from the HPLC in the 9kDa form or the 3kDa form, the crude MIF was subjected to CAM and then run over RP-

HPLC. The hypothesis was if it is in the 3kDa form, then on doing CAM on the crude, the peak will elute at the same time and gradient. If it is collected in the 9kDa form, then on doing CAM it will elute at a different time and gradient. Fig. 4.7 shows that the peak doublet eluted at exactly the same time and gradient. A twin peak accompanying each original peak in the doublet was also seen. The collected peak doublet was then again subjected to MALDI-TOF analysis. Again the two major peaks were detected and the mass at 9 kDa was not observed (Fig. 4.8).

The above results confirmed the hypothesis that the collected peak is a single peptide and that it forms multimers when concentrated. To try and get it in the monomer form the collected peak was subjected to 40 min sonication after concentration, in a water bath sonicator, in presence and in absence of 50 μ g of albumin and then checked by SDS-PAGE and MALDI-TOF. On running a 16.5% Tris-Tricine gel for peptide separation, a single sharp band of about 3kDa was revealed both in the presence and in the absence of albumin (Fig. 4.9). MALDI-TOF showed the presence of a sharp intense peak of m/z 3268 plus three other much lesser peaks of m/z 6596, m/z 9880 and m/z 13168 (Fig. 4.10). The peak of m/z 1634 is the doubly charged form of the m/z 3268 peak.

We first attempted to sequence this purified peptide by Edman degradation. We were unsuccessful in obtaining a definitive N-terminal sequence due to difficulties faced in collecting enough material and in keeping the peptide in solution. The sequence depicted was (W/H) (V) F (A/V) P (E/L/N) (R) N (S) (A) XFNXN (T). Residues between brackets are uncertainties or alternative possibilities and the Xs could not be determined.

As an alternative to Edman degradation, MALDI-TOF/TOF was used. Figs. 4.11 and 4.12 show the resulting spectrum and the identified amino acid residues. Due to the high

mass accuracy of the MALDI TOF/TOF the size of the peptide was more accurately determined to be m/z 3260.5. Only the middle segment of the peptide gave spectra good enough for interpretation. The N-terminus-containing sequence ions include a_9 (at m/z 1151.4), b_9 (1178.9), a_{10} (1278.9), b_{10} (1306.9), a_{11} (1378.0), b_{11} (1405.9), a_{12} (1515.1), b_{12} (1543.0), a_{13} (1652.1), b_{13} (1680.1), a_{14} (1780.4), b_{14} (1808.2), and b_{15} (1936.3). Six residues were thus identified in that segment to be QVHHQK. The immonium ions for histidine (110) and proline were also present. The lysine residue was confirmed by doing a Lys-C digest and then MALDI-TOF. Fig. 4.13 shows the spectra of the digest in which the peptide had been cleaved at two distinct positions. The presence of fragments of m/z 1325.7321 and m/z 1954.0214 confirm the position of the lysine residue at b_{15} . The presence of peptides at m/z 1229.8 and m/z 1560.8 indicates that an additional two residues are present toward the N-terminus of the peptide chain

Table 4.1 shows the homology search results from both NIH and ProteinInfo. Only perfect matches with all six residues or with the first five are shown from both. The NIH search showed 100 hits total while ProteinInfo showed just 7 hits. The mitochondrial superoxide dismutase and the *Drosophila melanogaster* CG13040-PA were the top two hits retrieved in both cases matching all six residues. They were the only perfect matches in ProteinInfo. The other matches shown in the table were retrieved by the NIH program and show matching with the first five residues. Interestingly no matches with *Schistosoma* species or any other worm species was retrieved.

BLASTing using the NIH program against the translated db of all taxa or of the available *S. mansoni* genome project using tblastn, did not yield any hits.

Though unreliable, we did a homology search with the amino acid sequence determined by Edman degradation. We entered our search query as follows: <XXFXPXXNXXXFNXNX> where 'X' replaced residues that cannot be confirmed or were totally undetermined, and it indicated in the search program that it could be any amino acid residue. Only ProteinInfo came back with hits, just 4 of them (Table 4.2).

Fig. 4.1. Total male attraction after 3 hrs to un-fractionated female incubate, and RP-HPLC fractions of <10kDa MIF eluting at different ACN gradients is shown in Fig. 4.1a. Fig. 4.1b represents the focus on the 30-40% ACN gradient and showing the male response to the fractions collected within that range of gradient. The peak doublet eluted at 34% ACN.

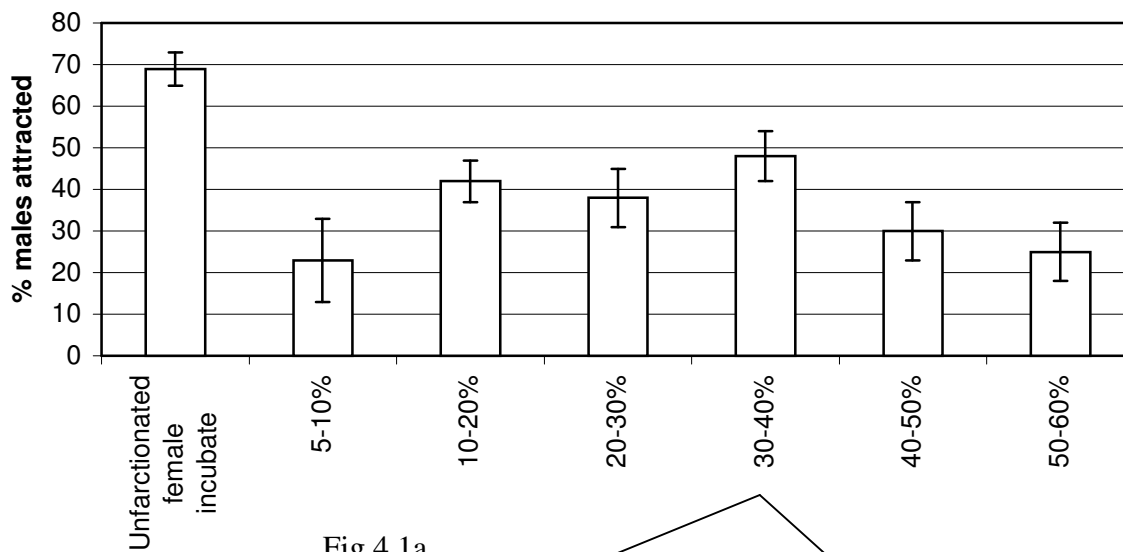


Fig 4.1a

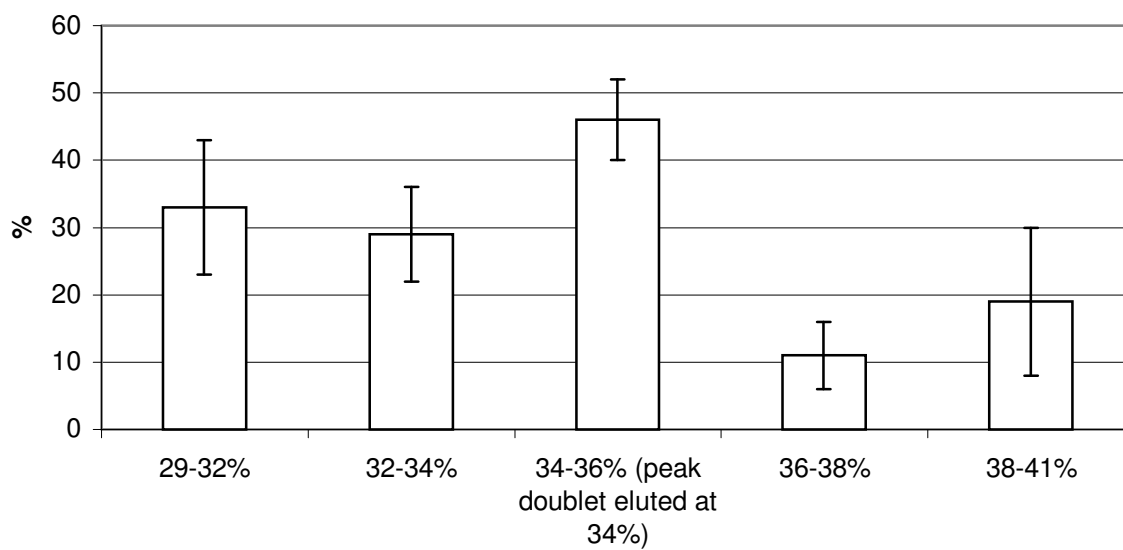


Fig 4.1b

Fig. 4.2: Peak doublet eluted from RP-HPLC at 0.1% TFA:34% ACN.

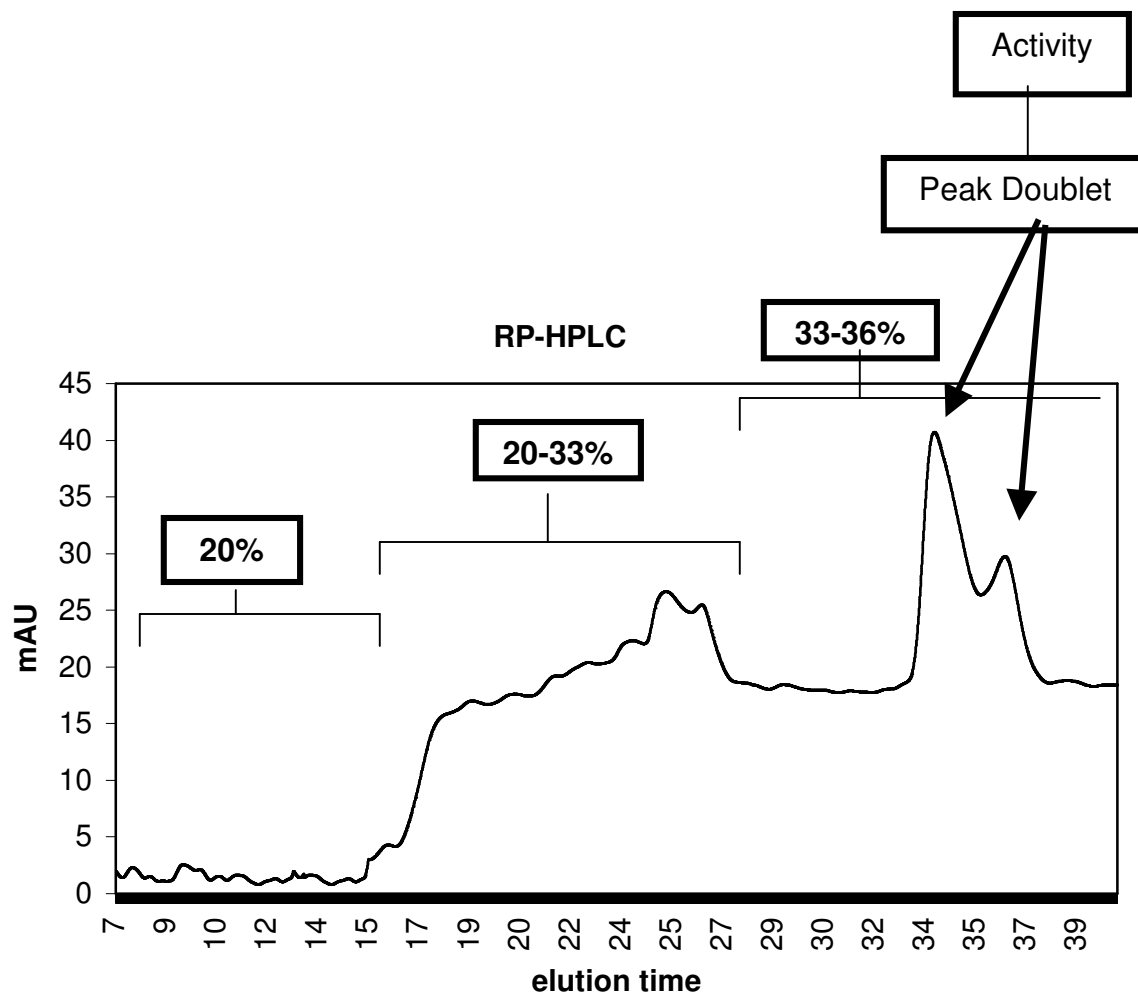


Fig. 4.2

Fig. 4.3: MALDI-TOF MS of the eluted peak doublet after concentration.

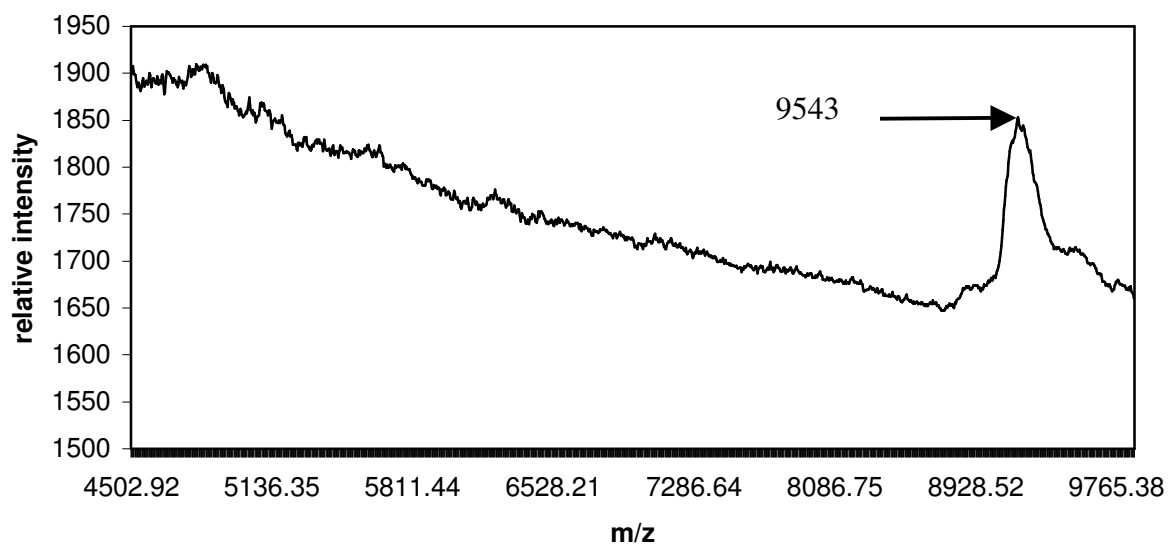


Fig. 4.3

Fig. 4.4: MALDI-TOF MS of MIF showing the absence of the m/z 9543 peak.

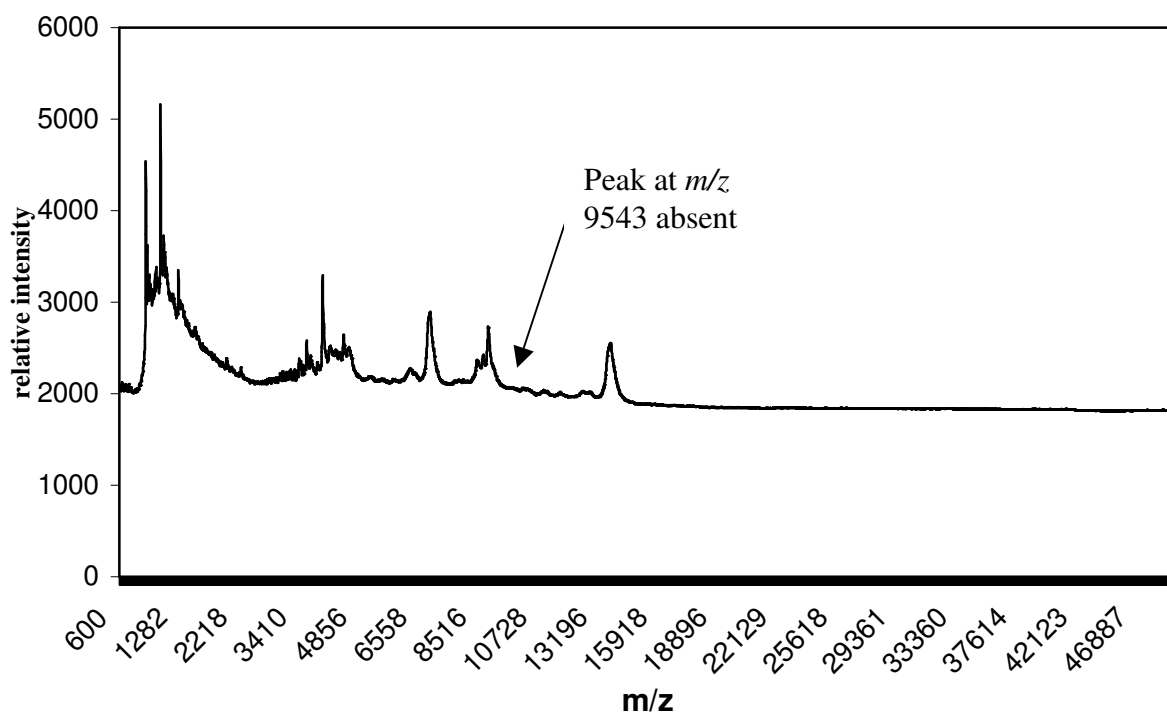
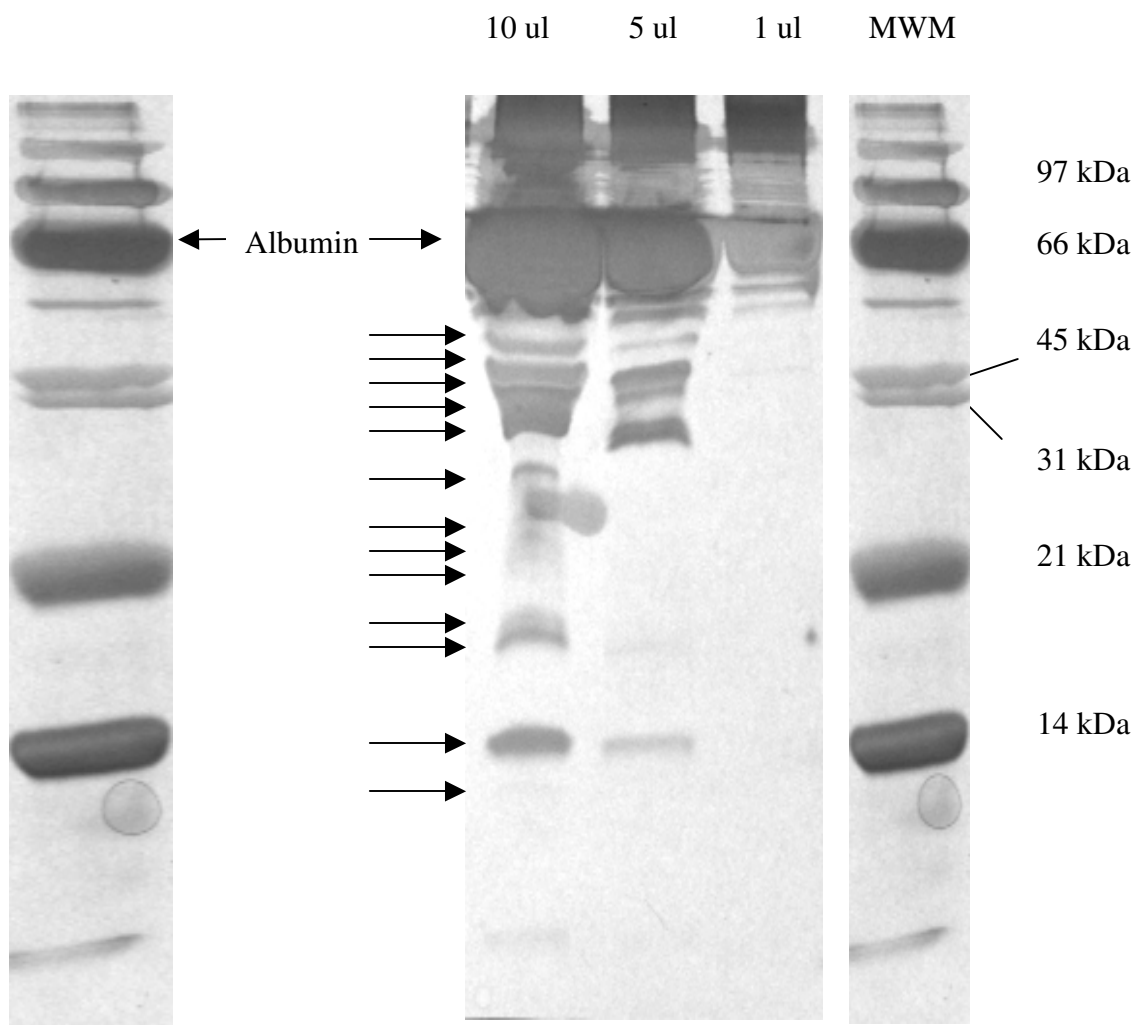


Fig. 4.4

Fig. 4.5: Silver stained 16% SDS-PAGE gel showing multiple bands from highly concentrated peak doublet.



16% SDS-PAGE

Fig. 4.5

Fig. 4.6: MALDI-TOF MS of carboxyamidomethylated peak doublet. It shows two peaks of m/z 3265.8 and m/z 3322.8. The difference between the two peaks was 57 Da, which is the size a carboxyamidomethyl group.

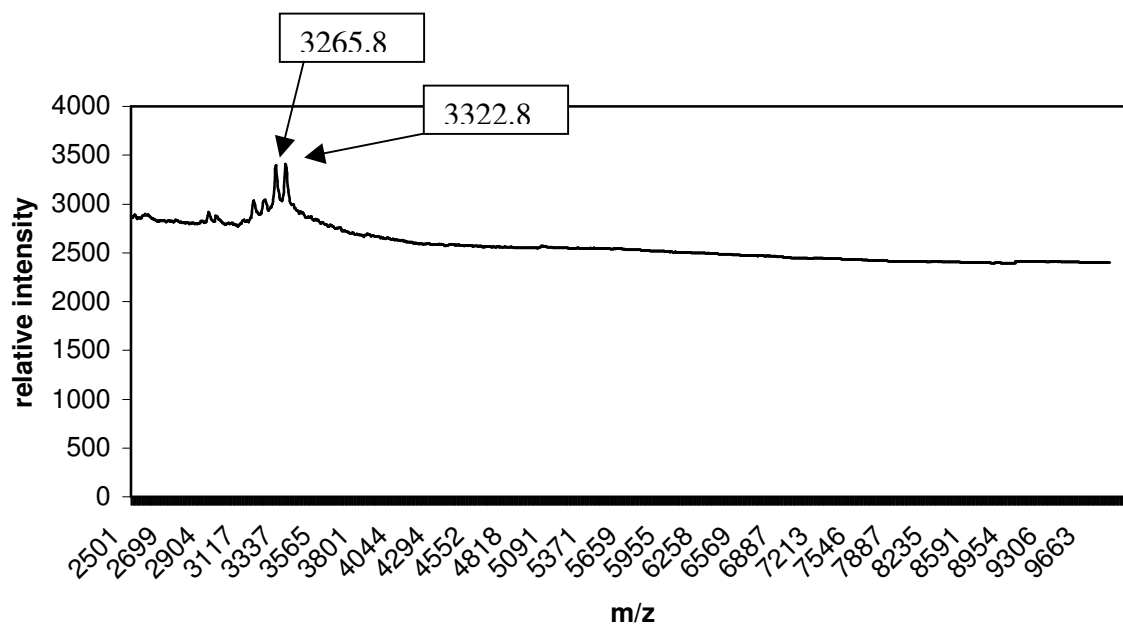


Fig. 4.6

Fig. 4.7: RP-HPLC elution pattern of the peak doublet after carboxyamido-methylation of the MIF. It shows two peaks each eluting adjacent one of the peaks in the peak doublet.

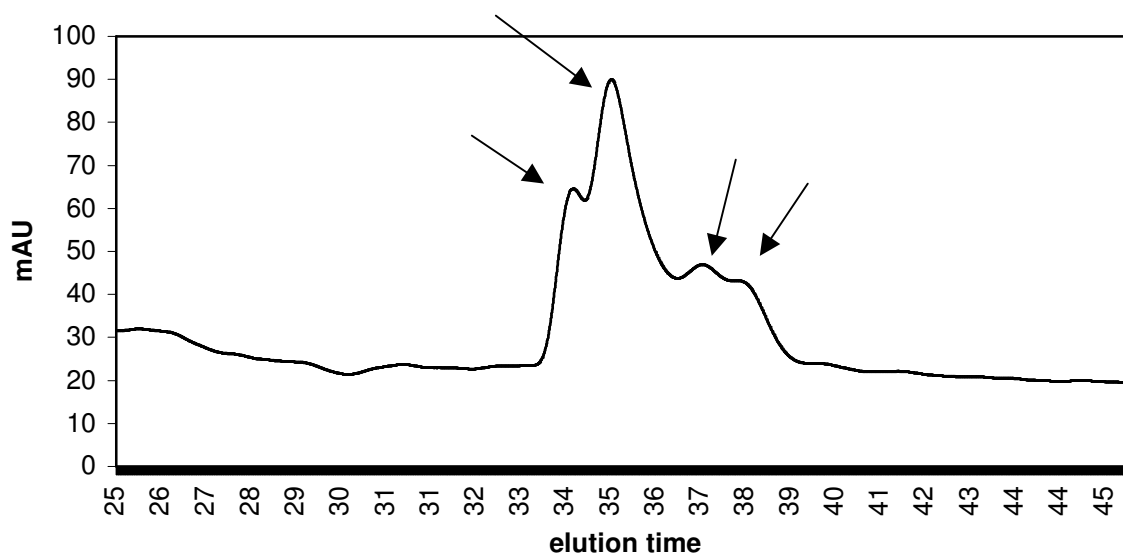


Fig. 4.7

Fig 4.8: MALDI-TOF MS of the peak doublet collected from the RP-HPLC run represented in Fig. 4.7. Again the two peaks at m/z 3265.8 and m/z 3322.8 were observed.

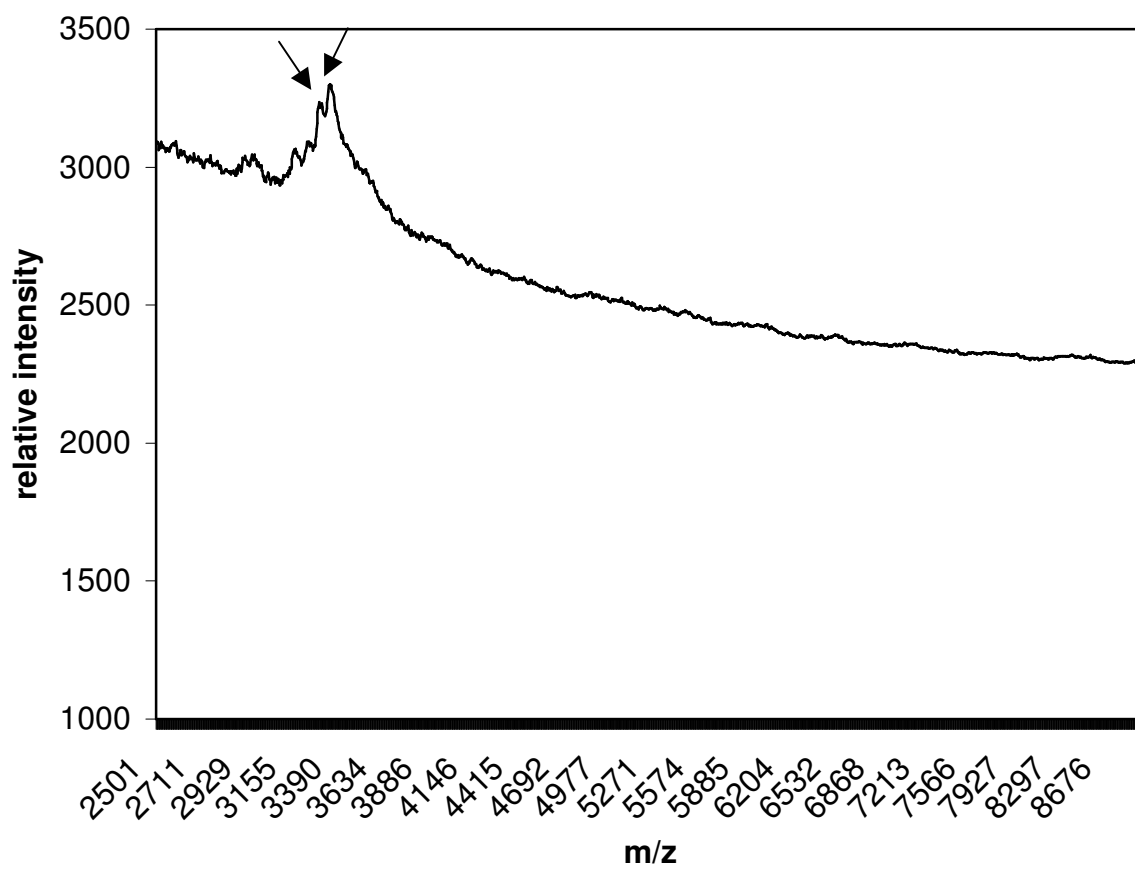


Fig. 4.8

Fig 4.9: Silver stained 16.5% Tris Tricine SDS-PAGE gel showing single sharp band from heavily sonicated RP-HPLC collected peak in presence and absence of albumin.

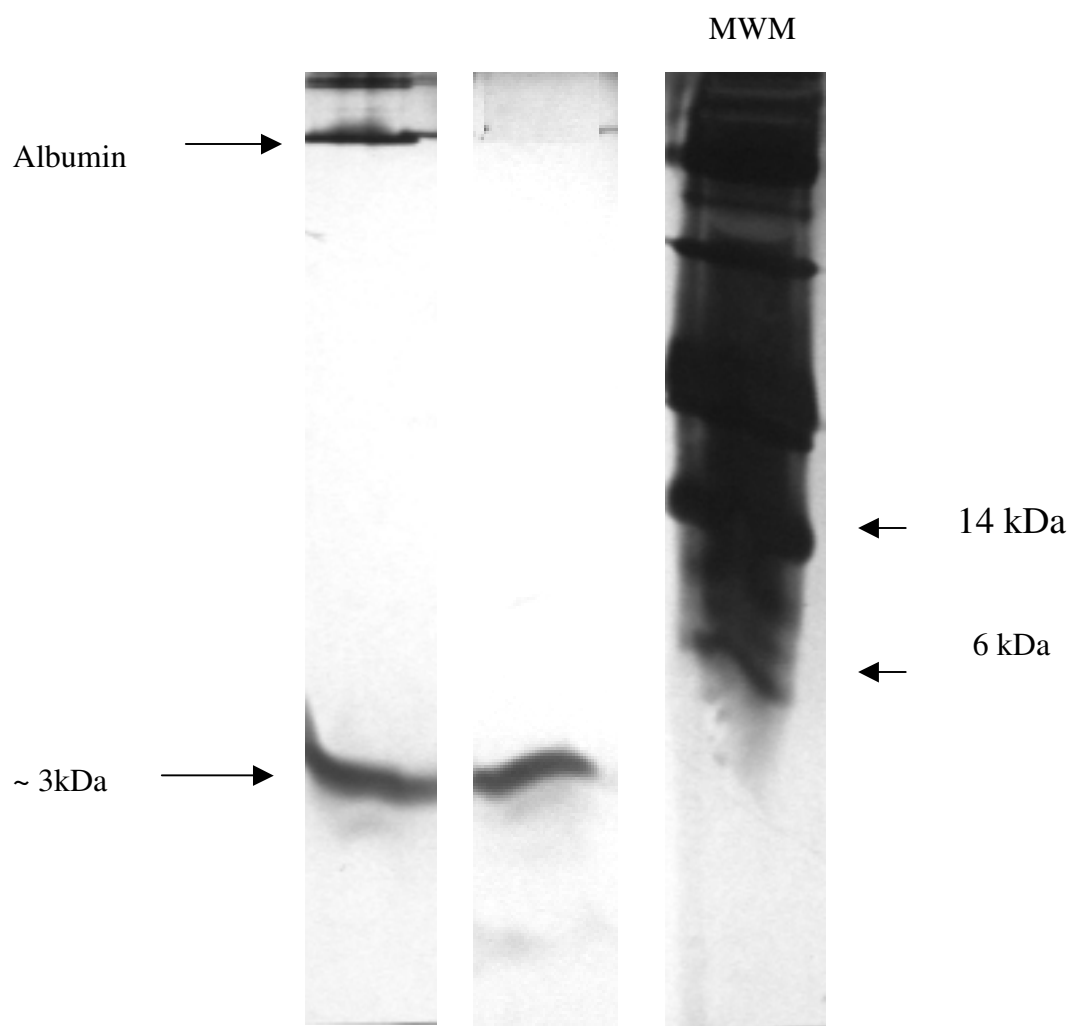


Fig. 4.9

Fig. 4.10: MALDI-TOF MS of heavily sonicated RP-HPLC collected peak

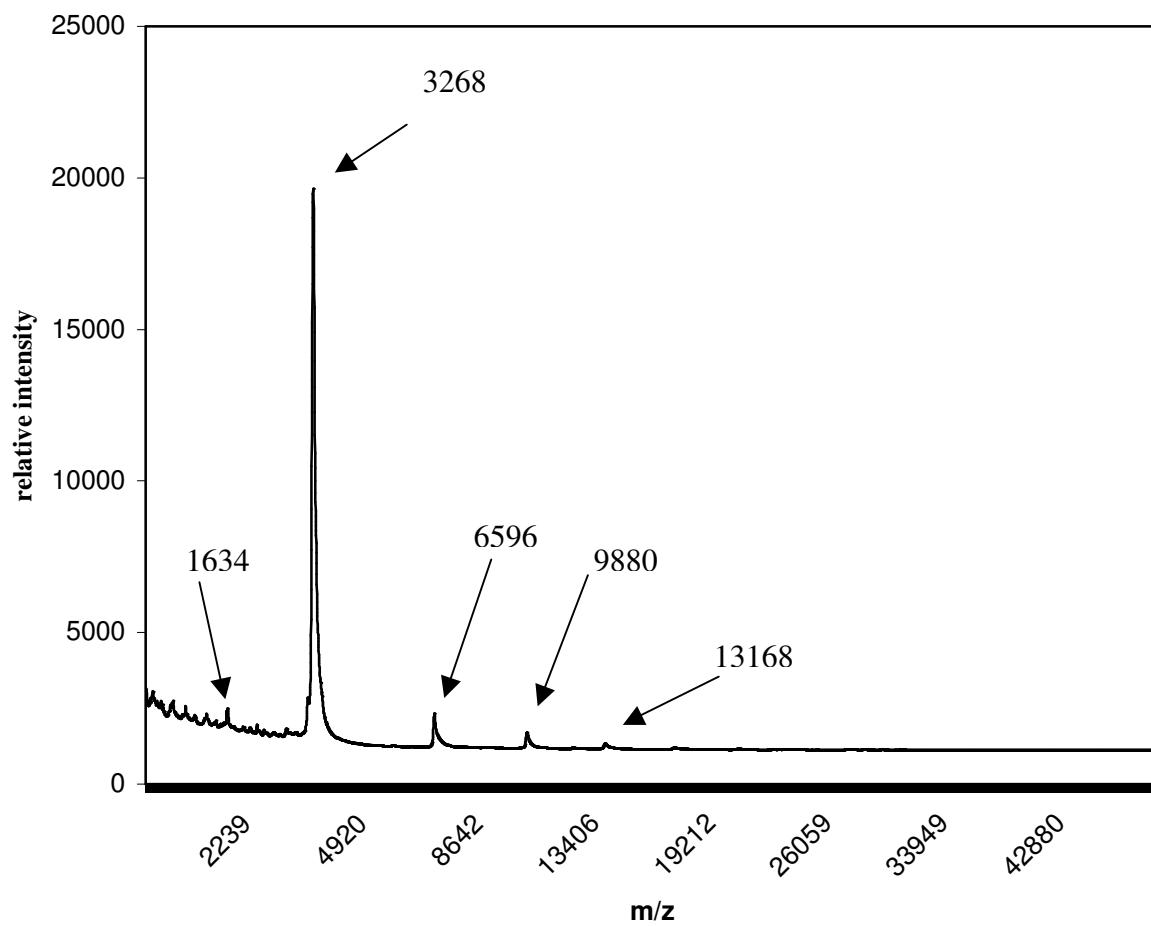


Fig. 4.10

Fig. 4.11: MALDI-TOF/TOF MS of purified peptide.

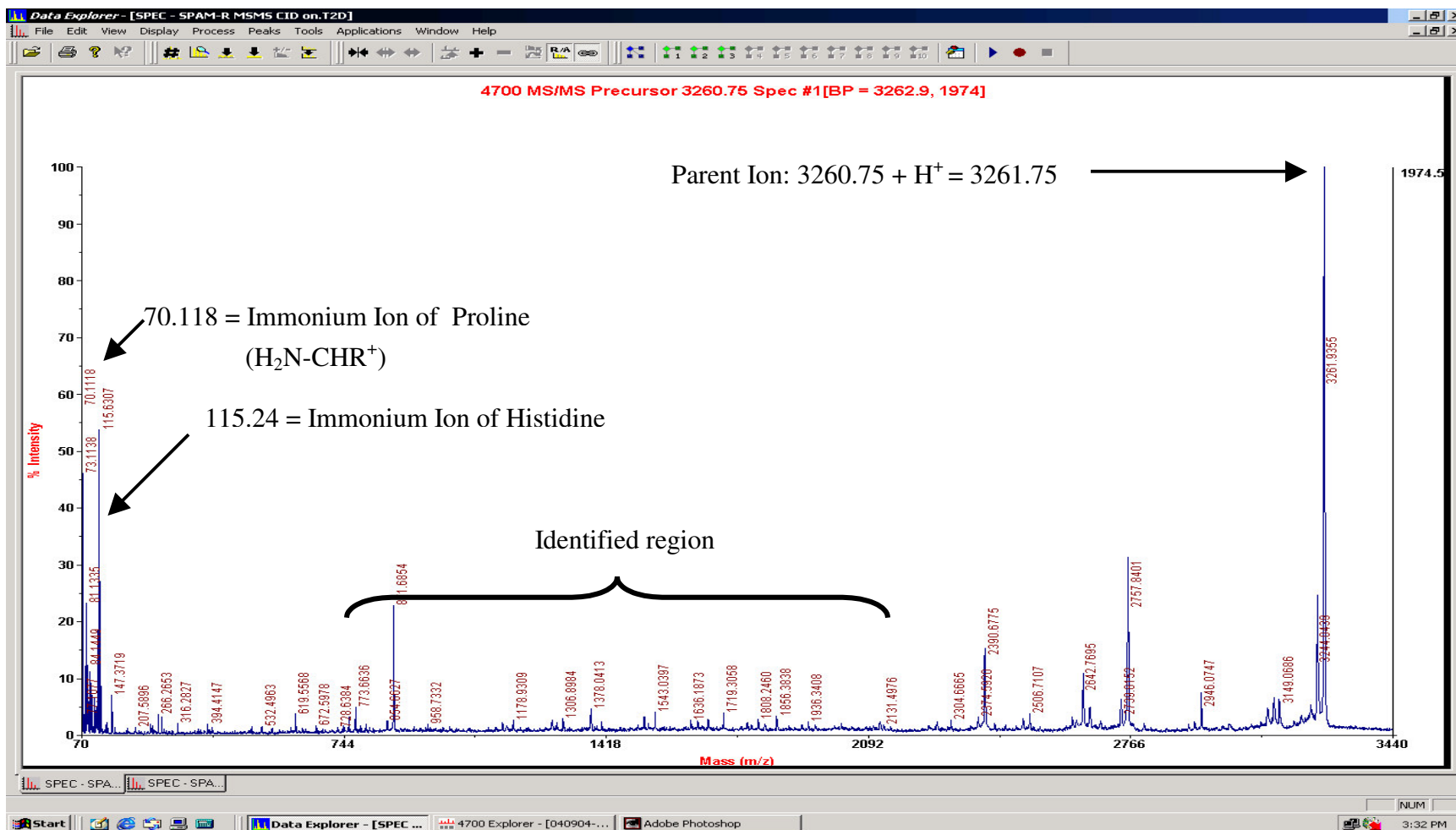


Fig. 4.11

Fig. 4.12: MALDI-TOF/TOF MS of the segment of the peptide of which its amino sequence has been determined.

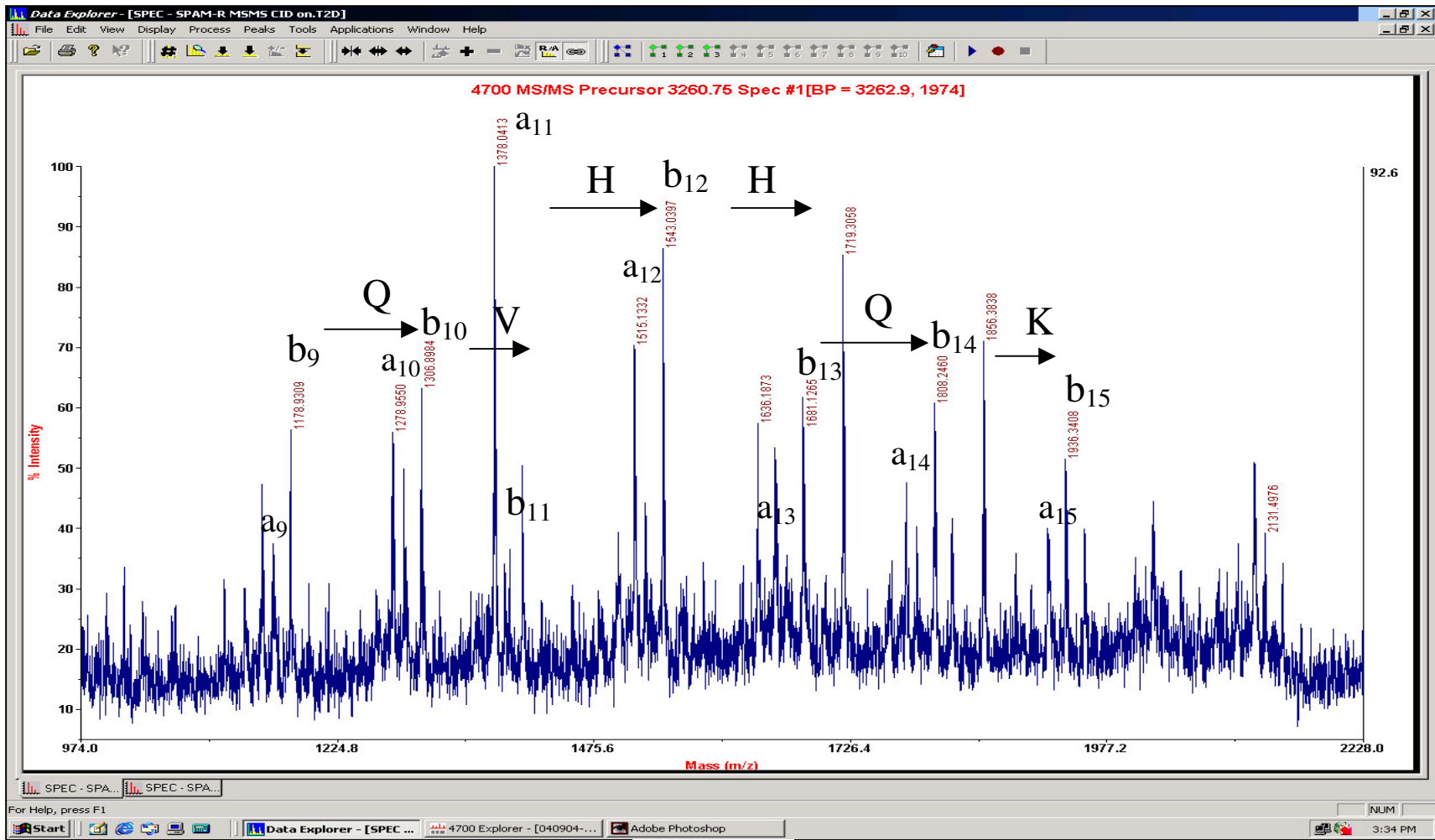


Fig. 4.12

Fig. 4.13: MALDI-TOF MS of the peptide after digestion for 4 hrs with lysine-specific endoproteinase Lys-C showing the MS of the peptide fragments that confirmed the presence of the lysine residue.

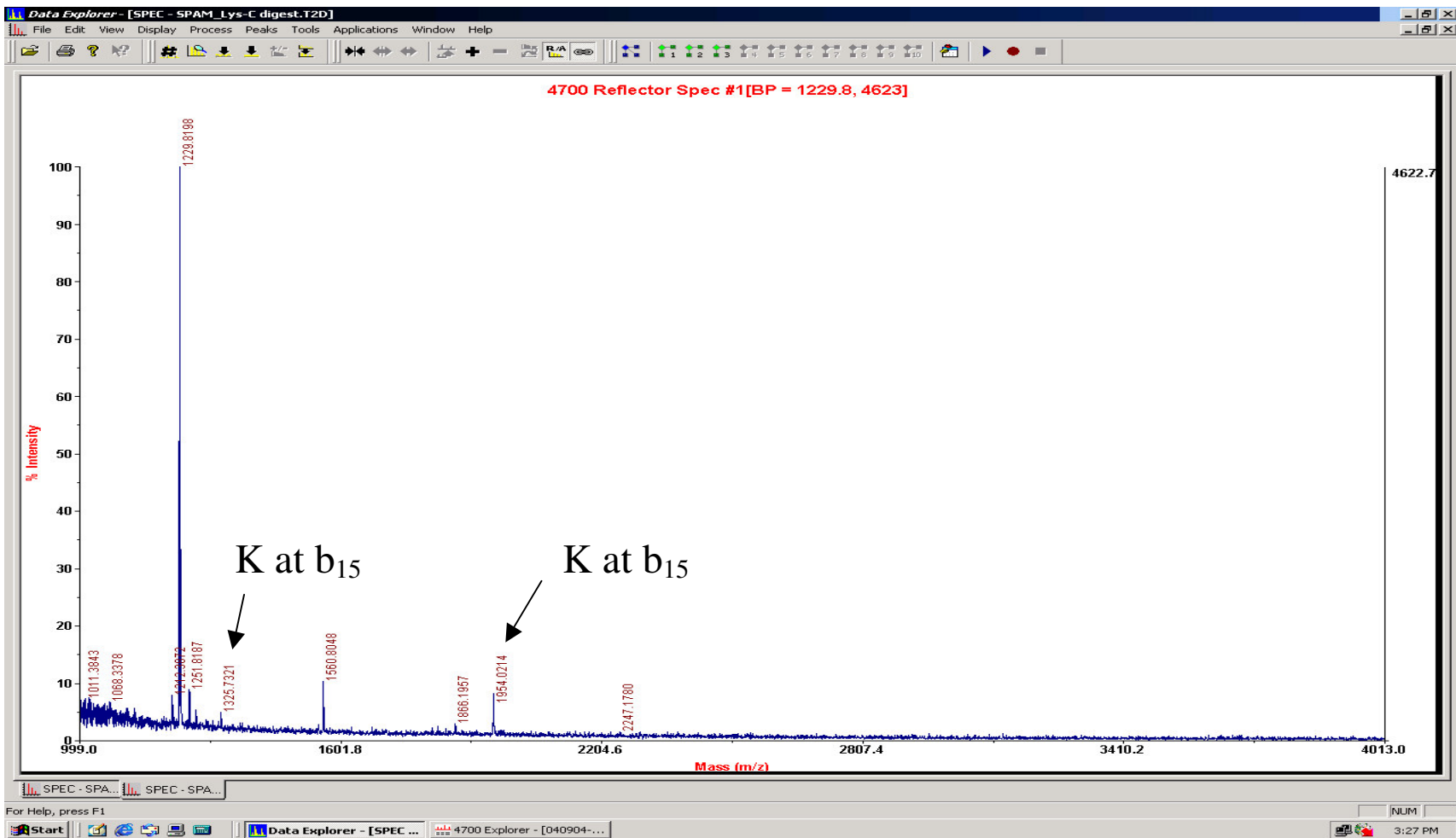


Fig. 4.13

Table 4.1: Results from the homology search done for the peptide sequence QVHHQK depicted by MALDI-TOF/TOF.

Table 4.1

<p>>gil24665205 reflNP_648875.1 CG13040-PA [Drosophila melanogaster] gil7294136 gblAAF49490.1 CG13040-PA [Drosophila melanogaster] Length = 185 Score = 23.5 bits (48), Expect = 336 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 1 QVHHQK 6 Sbjct: 153 QVHHQK 158</p>
<p>gil103687 pirllS23658 superoxide dismutase (EC 1.15.1.1) (Mn) - Florida lancelet (fragment) gil1335607 lembCAA45417.1 superoxide dismutase (Mn-type) [Branchiostoma floridae] Length = 144 Score = 23.5 bits (48), Expect = 336 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 1 QVHHQK 6 Sbjct: 8 QVHHQK 13</p>
<p>>gil5106472 gblAAD39717.1 AF088274_1 Suppressor of Hairless [Drosophila melanogaster] Length = 103 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 41 QVHHQ 45</p>
<p>>gil24641148 reflNP_572669.1 unconventional myosin class XV CG2174-PA gil22832067 gblAAF47983.2 CG2174-PA [Drosophila melanogaster] Length = 2602 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 2033 QVHHQ 2037</p>
<p>>gil15901330 reflNP_345934.1 hypothetical protein [Streptococcus pneumoniae TIGR4] gil15903377 reflNP_358927.1 Hypothetical protein [Streptococcus pneumoniae R6] gil25389153 pirllE95172 hypothetical protein SP1480 [imported] - Streptococcus pneumoniae (strain TIGR4) gil25508988 pirllE98038 hypothetical protein spr1334 [imported] - Streptococcus pneumoniae (strain R6) gil14972971 gblAAK75574.1 hypothetical protein [Streptococcus pneumoniae TIGR4] gil15458980 gblAAL00138.1 Hypothetical protein [Streptococcus pneumoniae R6] Length = 81 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 15 QVHHQ 19</p>
<p>>gil23194451 gblAAN15182.1 MADS box protein GHMADS-1 [Gossypium hirsutum] Length = 236 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 199 QVHHQ 203</p>
<p>>gil15601518 reflNP_233149.1 conserved hypothetical protein [Vibrio cholerae] gil11354813 pirllD82421 conserved hypothetical protein VCA0763 [imported] Vibrio cholerae (strain N16961 serogroup O1) gil9658185 gblAAF96661.1 conserved hypothetical protein [Vibrio cholerae] Length = 552 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 364 QVHHQ 368</p>
<p>>gil23113702 gblZP_00099057.1 hypothetical protein [Desulfitobacterium hafniense] Length = 197 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 193 QVHHQ 197</p>
<p>>gil118684 splP11533 DMD_CHICK Dystrophin gil86249 pirllS02041 dystrophin, muscle - chicken gil63370 lembCAA31746.1 dystrophin (AA 1 - 3660) [Gallus gallus] Length = 3660 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 271 QVHHQ 275</p>

<p>>gil85239 pir A26639 regulatory protein zeste - fruit fly (<i>Drosophila</i> sp.) Length = 555 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 378 QVHHQ 382</p>
<p>>gil3650208 dbj BAA33410.1 LjOtxB [<i>Lethenteron japonicum</i>] Length = 316 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 188 QVHHQ 192</p>
<p>>gil29734752 ref XP_298825.1 hypothetical protein XP_298825 [<i>Homo sapiens</i>] Length = 364 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 109 QVHHQ 113</p>
<p>>gil18057150 gb AAL58173.1 AC093181_19 putative polyprotein [<i>Oryza sativa</i> (<i>japonica</i> cultivar-group)] Length = 1931 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 330 QVHHQ 334</p>
<p>>gil14388446 dbj BAB60763.1 hypothetical protein [<i>Macaca fascicularis</i>] Length = 430 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 59 QVHHQ 63</p>
<p>>gil24586299 ref NP_610297.1 CG2105-PA [<i>Drosophila melanogaster</i>] gil7304194 gb AAF59230.1 CG2105-PA [<i>Drosophila melanogaster</i>] Length = 1379 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 135 QVHHQ 139</p>
<p>>gil18562615 ref XP_094623.1 similar to high affinity nitrate transporter [<i>Oryza sativa</i>] [<i>Homo sapiens</i>] gil13751974 emb CAC37303.1 bA342L8.1 (novel protein similar to C21ORF13) [<i>Homo sapiens</i>] Length = 697 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 59 QVHHQ 63</p>
<p>>gil22094356 gb AAM91883.1 putative mutator protein [<i>Oryza sativa</i> (<i>japonica</i> cultivar-group)] Length = 896 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 216 QVHHQ 220</p>
<p>>gil5106462 gb AAD39712.1 AF088269_1 Suppressor of Hairless [<i>Drosophila melanogaster</i>] gil5106464 gb AAD39713.1 AF088270_1 Suppressor of Hairless [<i>Drosophila melanogaster</i>] gil5106466 gb AAD39714.1 AF088271_1 Suppressor of Hairless [<i>Drosophila melanogaster</i>] Length = 102 Score = 20.6 bits (41), Expect = 2633 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QVHHQ 5 Sbjct: 41 QVHHQ 45</p>

Table 4.1

Table 4.2: Results from the homology search done for the peptide sequence, depicted by Edman Degradation, using search query <XXFXPXXNXXXFNXNX>.

Table 4.2

Query	XXFXPXXNXXXFNXNX
gil15889783 refl NP_355464.1 AGR_C_4574P [AGROBACTERIUM TUMEFACIENS]	
	Matching:SAWGT DYFDPHSNADVFNINK DNGDD (Residues 464 - 479)
gil1086100 pir S55502 MEMBRANE-ASSOCIATED PROTEIN P66 PRECURSOR - LYME DISEASE SPIROCHETE (STRAIN ACAI)	
	Matching:SKKAE DIFDPNGNALNFNKNT ELGIA (Residues 356 - 371)
gil21281463 gbl AAM45350.1 HYPOTHETICAL PROTEIN [DICTYOSTELIUM DISCOIDEUM]	
	Matching:QNQNQ NQFIPHNNNHQFN NNN NNNNN (Residues 107 - 122)
gil23490903 gbl EAA22564.1 HYPOTHETICAL PROTEIN [PLASMODIUM YOELII YOELII]	
	Matching:YHEIN KNFIPSDNILKFNDNH IKNTI (Residues 200 - 215)

DISCUSSION

The fact that this peptide by itself does not attract males as well as the whole female incubate (Fig. 4.1), indicates that there is probably more than one factor involved for optimal attraction to occur, at least when using our *in vitro* system.

The elution of the peak from RP-HPLC as a doublet seems to reflect a property of the peptide rather than being due to two peptides eluting close to each other. This is supported by the results of the MALDI-TOF analysis. It showed a single peak at m/z 9543. The revealing of multiple bands on the gel, that were the multiples of one another, at the MW level, when the collected eluted peak from several RP-HPLC runs was pooled and concentrated, indicate that this peptide tends to form multimers when concentrated. The absence of the m/z 9543 peak from the crude MIF led us to believe that the native peptide is probably collected in a smaller size form than 9543, and that the m/z 9543 form is formed on concentration.

The CAM experiment was done to test the above hypothesis. The results from both SDS-PAGE and MALDI-TOF (Figs. 4.6, 4.7 and 4.8) showed that the peptide's actual size in which it is both found in the MIF and collected from RP-HPLC is actually m/z 3268.6, and that it forms the multimer m/z 9543 on concentration.

The results of CAM also show the presence of cysteine residues in the peptide. The residues probably play a role in the multimerization process of the peptide. The addition of carboxyamidomethyl groups to the sulfide group of cysteine makes the peptide slightly more hydrophilic than its native form, thus causing the appearance of the other peaks adjacent to each of the peaks of the doublet (Fig. 4.7). This could also be due to partial carboxyamidomethylation.

The results from Tris Ticine gel (Fig. 4.10) and MALDI-TOF (Fig. 4.11) confirm both the purity of the peptide preparation and its tendency to form multimers. Its size was determined to be m/z 3268 by MALDI-TOF.

The MALDI-TOF/TOF sequencing was successful both in determining the size of the peptide more accurately to be 3260.75 Da, and in getting the amino acid sequence of a fragment of that peptide. The accuracy in size determination was due to the fact that the MALDI-TOF/TOF machine used is more powerful and advanced than the MALDI-TOF machine and the peak is sharp and intense. The difference in size between the first detected multimer of m/z 9543 and the multimer m/z 9880 is attributed to the fact that the first is of very low intensity and high background noise, and is relatively broad, making it relatively hard to accurately determine its weight. The latter is the more accurate weight of this form of multimer being sharper and with much less background noise. The peptide was determined to be difficult to work with in matter of depicting its amino acid sequence. Several factors have been shown to interfere with the ability to work with and sequence proteins or peptides on MALDI (Amado *et al.*, 1997; Kratzer *et al.*, 1998; Krause *et al.*, 1999; Gobom *et al.*, 2001; Wang and Fitzgerald, 2001; Stevens *et al.*, 2002;). In this work, the important factor seems to be the nature of the peptide as all the other factors (as mentioned in the references above) have been ruled out. This led to elucidating the sequence of just a small part of the peptide. The peptide in its multimer forms tends not to ionize easily on MALDI. We also caution that a reliable and consistent post RP-HPLC concentration method is still needed to successfully keep the peptide in its monomer form and in solution.

Not retrieving any results from BLAST homology search against the *S. mansoni* expressed sequence tags database might not be surprising since, at this stage, there is little or incomplete representation of the *Schistosoma* species in the current database. The schistosomiasis genome project is still an ongoing effort, suggesting that our peptide sequence is likely to be unknown. Other researchers working on other organisms were faced by a similar situation (Yergey *et al.*, 2002). As was described by Franco *et al.*, (2000), the identification of a protein, using MALDI peptide-mass spectrometry and tandem mass spectrometry technologies, is based on comparisons of its partial sequence or the profile of fragments generated by mass spectrometry after digestion of the protein with specific proteases with sequences of known gene products of the organism. For this purpose, a great part or the total genome sequence must have been already determined. This is now a reality for many other organisms. It is yet to be for *Schistosoma*.

In conclusion, we have identified, purified and characterized for the first time a peptide released by *S. mansoni* adult females that plays a role in attracting the males. It is about 3260 Da. We also succeeded, for the first time using MALDI peptide-mass spectrometry and tandem mass spectrometry (MALDI-TOF/TOF) with *S. mansoni*, in determining a partial amino acid sequence <QVHHQK> of that peptide. Although we did not identify a homologous sequence in the available schistosomiasis database, completion of the *S. mansoni* genome will allow eventual identification of the complete peptide.

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CHAPTER 5

CONCLUSION

This research addressed questions about the chemoattraction between adult *S. mansoni* worms and represented one of the sincere attempts to identify and to purify a mate attractant factor from these organisms. The study documented mutual attraction between both sexes, that the attractant factors released by both sexes are probably of different sizes, provided a fresh start in studying pheromonal attraction in *S. mansoni*, and showed that the female worms incubate had a 3260Da peptide that plays a role in male attraction.

The attraction between males and females *in vitro* has been shown using live worms of one sex or worm incubates of one sex as bait to attract the opposite sex. The number of worms attracted to the bait increased by time and peaked at three hours. The worms seemed to be in active search for a mate, and once they detect the right signal they move towards the source, following probably a concentration gradient. The use of multiple worms of one sex as a bait did not enhance the attraction of the worms of the opposite sex, a result that could be due to limited number of receptors present on the worms.

The possibility that male-released factors and female-released factors are of different sizes emerged from what was noticed in the behavior of males and females to defined fractions of female and male worms incubate respectively. The females were attracted to possibly more than one male –released factors; a <30kDa short-range factor

and a >30kDa long-range factor. Males, on the other hand, were attracted to a <10kDa female released factor(s).

No same sex attraction of statistical significance, in particular between males, was detected. It was also shown that boiling the worm incubate of one sex reduced the attraction of the worms of the opposite sex, suggesting that the attracting factors released by both sexes are protein/peptide in nature or have a protein/peptide component.

Mate-attractants leached out of dead worms and the strength of mate attraction was dependent on the method used to kill the worms with males being attracted to ethanol-killed females and not refrigerated ones, and females being attracted to males killed either by ethanol or by refrigeration, though to a greater extent to the latter. These results suggested that the male attractant(s) are probably hydrophilic and that the female attractant(s) are either partially hydrophilic or that some components are hydrophobic and were lost in the ethanol, thus reducing the female attraction to ethanol-killed males.

The use of carbon dioxide inhalation as the method for mice euthanasia could, at least, be partially responsible for the new findings and the differences regarding the dynamics of worm movement in our system as compared to those done before. The use of non-anesthetic methods for mice euthanasia is a factor that needs to be considered when studying the behavior of a living organism.

We documented the existence of a phenomenon in both sexes that we termed the “dip zone”. This zone contained the least percentage of worms at all time points whenever there was attraction, with the exception of the attraction of female worms to defined fractions of the male incubate. This phenomenon could be interpreted as part of the active “search mode” hypothesis mentioned above, in which they are in search for the

factor(s) within a certain range. Once they find it they move straight towards the source, thus creating this “dip”.

The female worms incubate when analyzed by high performance thin layer chromatography showed the absence of lipids and carbohydrates. The treatment of that incubate with different proteases showed that males were attracted to it when it was treated with Proteinase K, while attraction was greatly reduced to that treated with either Pronase E or with trypsin. The female incubate was also treated with methanol and with different concentrations of acetonitril. Males were attracted incubate treated with 5% acetonitril and to the methanol insoluble fraction, not to that treated with 80% acetonitril or to the methanol soluble fraction. The results are consistent with the hypothesis that the female-released male-attracting factor(s) has a protein/peptide component of a hydrophilic nature.

A 3260Da peptide released by *S. mansoni* adult females, that plays a role in attracting the males has been identified, purified and characterized using reversed phase-high performance liquid chromatography and specific chemical treatments. Using MALDI peptide-mass spectrometry and tandem mass spectrometry, partial amino acid sequence <QVHHQK> of that peptide was retrieved. No homologous sequence was identified in the available schistosomiasis database since the schistosomiasis genome project is still an ongoing effort.