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The role of calcium in the host-parasite relationship of *Biomphalaria glabrata/Schistosoma mansoni*.

A thesis completed for the degree of

MASTER OF SCIENCE

by

Joyce A. Thornhill, M. I. Biol.

Division of Biochemistry and Molecular Biology,

University of Glasgow.

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Abbreviations

ARS	Alizarin Red S fluorescent stain
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
C/D/S	Cercariae per day per snail
DCTW	Dechlorinated tap water
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycolbis tetraacetic acid
LSE	Living skin equivalent
PAG	Preacetabular gland
PE	Post parasite exposure
12HL/12HD	12Hour Light/12HourDark maintenance regime

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Summary

This study has indicated that parasitised *Biomphalaria glabrata* snails maintained in a 12 Hour Light (543 Lux) and 12 Hour Dark regime, shed more *Schistosoma mansoni* cercariae than snails maintained in the dark. Of significance is the observation that the pattern of emergence was similar under both maintenance regimes, suggesting that momentary exposure to only 41Lux light intensity was sufficient to stimulate cercarial shedding. Results also suggested that calcium ion (Ca^{2+}) levels of maintenance water may influence cercarial production.

To enable us to compare the calcium content of individual cercariae, a protocol was developed which quantitated the fluorescent emission from the preacetabular glands of cercariae stained with Alizarin Red S, and viewed under the fluorescent microscope. The results suggested that individual cercaria had different amounts of Ca^{2+} in their preacetabular glands. We made the observation that cercariae lost Ca^{2+} from their preacetabular glands after a period of incubation at room temperature (21°C). The arbitrary units of fluorescent emission, although allowing relative comparisons between individual cercariae, do not measure total Ca^{2+} of the cercariae.

Consequently further investigations, employing Atomic absorption and Plasma emission spectrometry, determined the total calcium content of both cercariae and shedding water. These results confirmed that aging cercariae lost Ca^{2+} and that the incubation water gained Ca^{2+} during the period. Shedding snails also lost Ca^{2+} during a 2 hour shedding period.

To investigate whether Ca^{2+} levels affected surface properties of the cercariae, we incubated cercariae with EGTA, and labelled with PKH26. We found that incubation of cercariae in 20mM EGTA prevented tail loss and inhibited membrane damage. Incubating cercariae with the fluorescent PKH26

lipid label clearly highlighted the nervous system of both cercarial body and tail.

The possible effect of calcium loss, by aging cercariae, on successful parasite penetration and maturation is discussed at length in the context of current knowledge.

CHAPTER 1

Introduction.

1.1. Schistosomiasis

It is estimated that 200 million people are infected with schistosomiasis or bilharziasis (after Bilharz, who in 1852 identified the relationship between disease, worms and eggs), causing public health and economic problems. The latter are exacerbated by infection of both domestic and wild animals with *Schistosoma bovis* or *Schistosoma mattheei*. The most commonly found man-adapted schistosome species are, *Schistosoma mansoni* (the most extensively studied species), *Schistosoma japonicum* and *Schistosoma haematobium*. Geographically the disease is found not only in Europe, Asia and Africa but also in the Americas (WHO, 1993).

The clinical and pathological effects of the disease are classified briefly as invasion and maturation, acute schistosomiasis, chronic schistosomiasis and associated complications (von Lichtenberg, 1987). The socioeconomic impact of schistosomiasis is difficult to ascertain. The adverse effect on productivity of infected employed labour is more easily quantified than that of the peasant farmer where the social consequences may be much greater. Conflicting evidence exists concerning the effect of infection on children's scholastic performance (Clarke and Blair, 1966). However it is recognised that schistosomes affect nutrition of children and can result in malabsorption, endogenous nutrient loss and anaemia (Tomkins and Watson, 1989).

Although concerned ultimately with the disease promoting adult stage, the present study, is specifically investigating the larval parasite which develops in the intermediate mollusc host. Of primary interest is the contribution that the invertebrate host , and its laboratory maintenance, may have on both parasite development and resulting parasite properties, such as infectivity. Consequently the introduction describes in detail how *Schistosoma mansoni* locates, matures in and is released from *Biomphalaria glabrata* .

1.2. Life cycle of *Schistosoma mansoni*

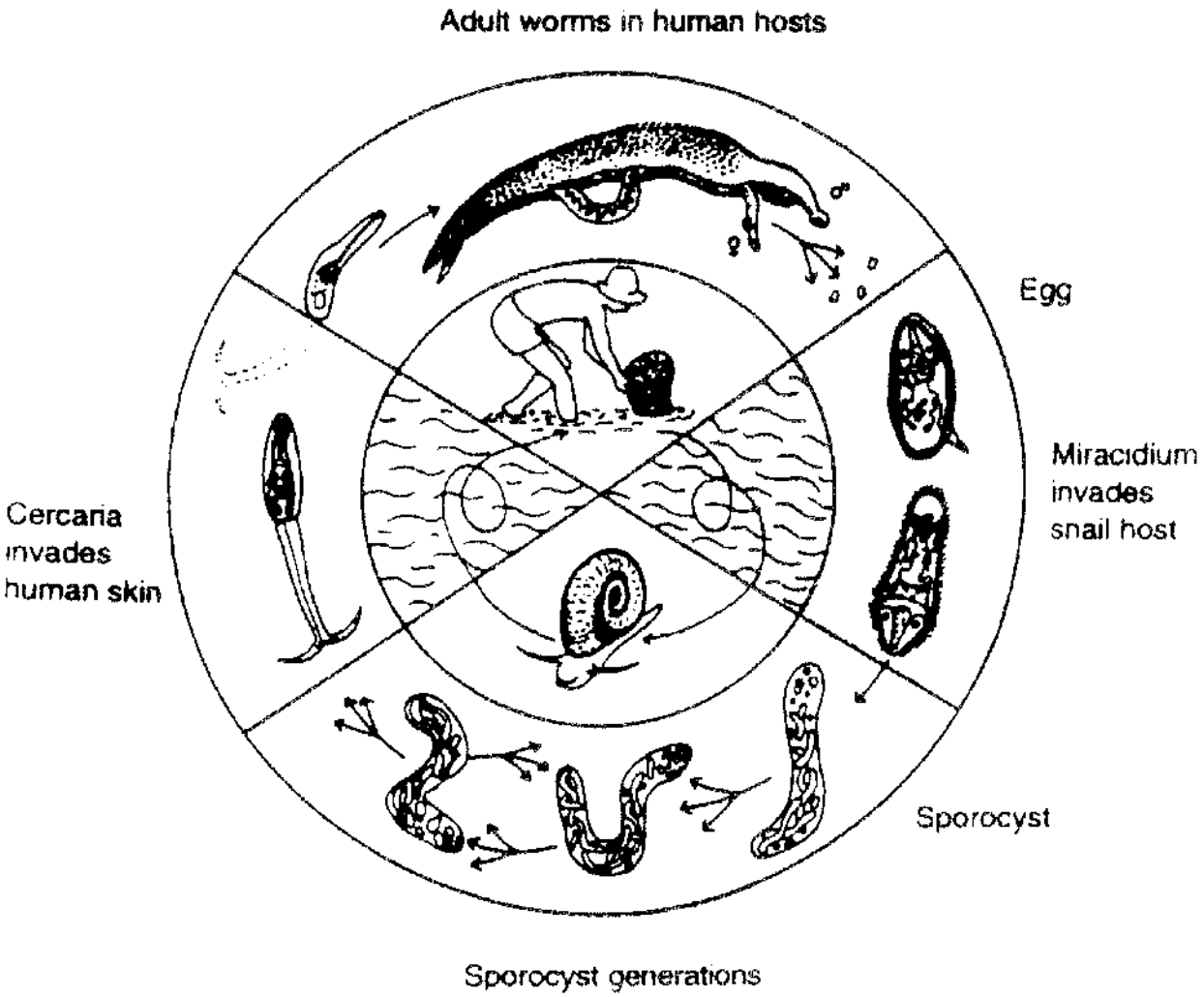
The life cycle of the digenetic trematode *Schistosoma mansoni* consists of an alternation of generations, with sexual reproduction (but no increase in parasite numbers) occurring in the vertebrate host and asexual multiplication in the snail *Biomphalaria glabrata* (Fig 1.1.). There is a degree of synchronisation between different stages in the life cycle to maximise transmission of the parasite, and consequent spread of the disease.
Man, in building dams, bridges and irrigation schemes, contributes to the parasite dispersion by creating suitable habitats for the intermediate mollusc host. In Senegal for instance, an exceptional epidemic of *S. mansoni*, in a non-immune population, followed the introduction of a large scale irrigation programme (Kongs *et al*,1996). Formerly a disease associated with rural areas, it is becoming an increasing urban problem due to migration of infected people into cities. Birrie *et al* (1996), recorded schistosome infection in Ethiopian school children, who had never been out of the city Addis.

1.3. The relationship between *Schistosoma mansoni* and the intermediate host *Biomphalaria glabrata*.

(i) General. There are many schistosome / mollusc combinations, however the compatibility of *B. glabrata / S. mansoni* is regarded the most successful when infectivity, length of prepatent period , cercarial production and longevity of the parasitised snail are considered (Sturrock, 1993). It is a measure of the successful compatibility of this combination that neonatal *B. glabrata* snails , as small as 0.74 mm shell diameter, could survive *S. mansoni* miracidia penetration and could subsequently release normally infective cercariae when only 1.6 mm diameter (Cooper *et al* , 1992).

The susceptible snail is robust and adapts readily to a range of environments. It can tolerate a temperature range of 18-30 ^{o}C, pHs of 5-9 and limited NaCl levels (Chernin and Bower 1971). However it has been shown

Fig.1.1 The life cycle of *Schistosoma mansoni*.



(After Haas *et al*, 1995).

experimentally that these agents are interactive, and quite small variations in any one factor can modify, directly or indirectly, the effect of either of the other two factors (Sturrock and Upatham 1973). The natural habitats exhibit exceptional climatic changes. *B. glabrata* is able to aestivate in dry periods and, if in the prepatent stage of parasitism, can suspend parasite development till more favourable conditions prevail. The opportunistic snail can reproduce when conditions are suitable and it is adapted to recover from catastrophic population crashes such as molluscicide treatment - an obvious advantage for the transmission of the schistosome parasite.

(ii) Calcium. The nutritional requirements of the developing parasites are supplied by the host snail. Of specific relevance to the present study, is the provision of the cercarial calcium reserves. X-ray probe microanalysis of parasitised snails has shown that cercariae, still within sporocysts, have preacetabular glands which are rich in calcium (Davies, 1983). Employing transmission and scanning electron microscopy techniques, together with X-ray analysis, Davies and Erasmus (1984), observed morphological changes in the snail's calcium storage cells, located in the connective tissue of the digestive gland, and also in the inner surface of the shell . By day 40 post infection, these Type A calcium cells were irregular in outline and lacked the calcium carbonate inclusions which were present in non infected control snails. Simultaneously, the nacreous layer of the inner shell surface of the infected snail appeared eroded with pits which extended into the prismatic shell layer. A more recent study indicated that detrimental effects of Type A calcium cells and inner snail shell occurred as early as 48 hrs post infection (Shaw and Erasmus, 1987).

In molluscs the calcium levels of the haemolymph are strictly regulated and, in *Lymnaea stagnalis* the calcium cells and shell are known to be involved in the equilibrium process (de With and Sminia 1980). The calcium requirements of the multiplying schistosome parasites will deplete the calcium

in the haemolymph, which could result in the observed detrimental effects on the calcium reserves of *B. glabrata* (Shaw and Erasmus, 1987).

(iii) Fecundity. Another characteristic of the snail/parasite relationship is the profound effect that the trematode parasite has on the fecundity of the invertebrate host. Snails exposed to *S. mansoni* appear to experience an early reproductive increase whether or not they become parasitised (Minchella and Loverde, 1981., Thornhill *et al*, 1986.).This phenomenon was considered to be a compensatory strategy of the host to offset the cessation in egg laying which may occur later in the patency of the infection. The parasite-induced stimulation of egg laying in host snails increases the potential number of susceptible snails in the indigenous population. However the strategy will benefit the snail too since it is unlikely that all the progeny will become infected. It is noteworthy that not all snails cease egg laying when parasitised, for example an acceptable number of neonatal infected snails simultaneously produced eggs and cercariae (Cooper *et al*, 1992). It is noteworthy that when parasitic castration does occur, these snails can still function as successful males for periods up to 6 weeks after cessation of egg laying (Cooper *et al* 1996).

The characteristics described above indicate not only the complexity of the relationship, but also, how adaptation has resulted in the very successful association of *B. glabrata* as intermediate snail host for the trematode *S. mansoni.*

1.4. Organism associations affecting the *S. mansoni/B. glabrata* relationship.

This section describes organism associations which adversely affect parasite production in the laboratory and the possible effects on parasite transmission in the field are considered.

(i) Rotifers. The source of rotifer contamination in the laboratory is

uncertain but, in our experience, snails in non-aerated water and maintained in the dark are more likely to become colonised. The rotifers establish initially in the centre of the whorl and can develop over the whole shell.

Stirewalt and Lewis (1981), observed a reduced yield of *S. mansoni* cercariae from *B. glabrata* snails colonised with *Rotaria rotatoria* and *Philoderia acuticanis* . The cercariae which did emerge exhibited significantly reduced infectivity compared to that of cercariae from "non rotifer" snails ($p<0.001$ Students *t*-test). It is noteworthy that it is the penetration phase of cercarial infectivity which is adversely affected. The ability of penetrants to mature to adults in mice is not dramatically reduced by rotifer colonisation of the snail host.

Both cercariae from rotifer colonised snails and "non-rotifer" cercariae which had been introduced to rotifer conditioned water, exhibited restricted swimming activity and settled on the bottom of their container. It was suggested that the rotifers emit a factor, which may be a neural or muscular depressant, capable of restricting cercarial motility. However the causal relationship between rotifers and reduced number of cercarial production by infected snails is less clearly understood. Perhaps rotifer colonisation is associated, either directly or indirectly, with an abnormal physiological state in the snail which restricts parasite development.

It is clear from this study that the maintenance of snails in non-aerated water necessitates regular changing of the water and careful observation of the snails. Contaminated snails can be cleaned by gentle brushing with a soft haired brush and copious rinsing with dechlorinated tap water. Laboratory snails , maintained in 2 litres of water, readily browse on each other. This behaviour, even if the snails do not eat the rotifers, may interfere with the ability of the rotifer to successfully colonise the snails.

It is difficult to estimate the significance of rotifer antischistosome effect in natural habitats. If in the field rotifer colonised snails exhibit a similar reduction

in cercarial production, it could be postulated that rotifers are a limiting factor in schistosome transmission. However the factor emitted by rotifers would possibly be diluted to concentrations which would not adversely affect the ability of cercariae to swim and contact their definitive host.

In conclusion rotifers in laboratory maintenance systems can significantly decrease numbers, activity and infectivity of cercariae. To ascertain their importance in natural conditions would be experimentally extremely difficult.

(ii) Annelid worms. Another intimate association exists with snails and the polychaete worm *Chaetogaster limnaei.* Only on one occasion, when a new batch of snails was introduced to our stock, was a contamination of *Chaetogaster* experienced . Fig. 1.2. shows polychaete worms attached to the soft tissue of the head and foot area of a snail.

The worms are rarely found off their hosts and appear to depend on the snail niche providing protection from predators and a food source of algae entering the mantle cavity. Secure attachment to the snail is made possible by fans of chaetae with recurved hook tips for grasping the soft mucus lining of the mantle. The photographs in Fig. 1.3., clearly show the well adapted chaetae both for attachment to the snail and, at the opposite end for directing currents of water containing algae to the worm mouth.

Chaetogaster also feeds on *S. mansoni* miracidia (Khalil, 1961) and cercariae. Clearly these polychaetes could contribute to a) low infectivity rates in snails exposed to miracidia and b) low yields of cercariae from parasitised snails. The *Chaetogaster* were removed by persistent brushing of the colonised area and repeated rinsing of the snail. They were quite difficult to dislodge and care had to be taken to prevent injury to the snail. Attempted culturing of the removed polychaetes proved unsuccessful. Having removed the polychaetes from the snails no further colonisation has occurred in our system.

It is possible that *Chaetogaster limnaei* , by removing emerging cercariae

Fig. 1.2 *Biomphalaria glabrata* snail and *Chaetogaster* worms.

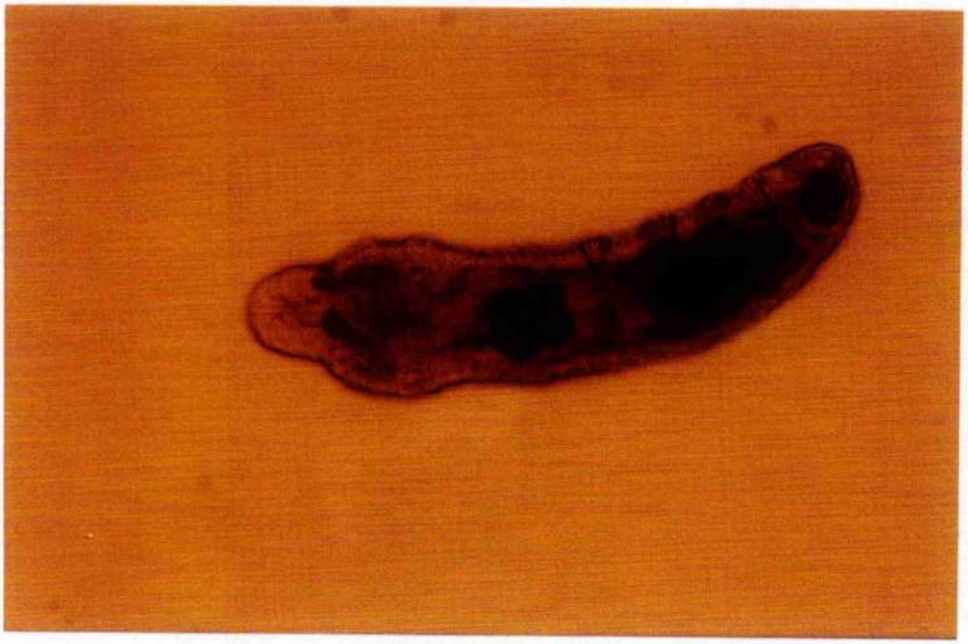
(a) *Chaetogaster* worms attached to *B. glabrata.*

Note: *Chaetogaster* Actual size 7 mm in length.

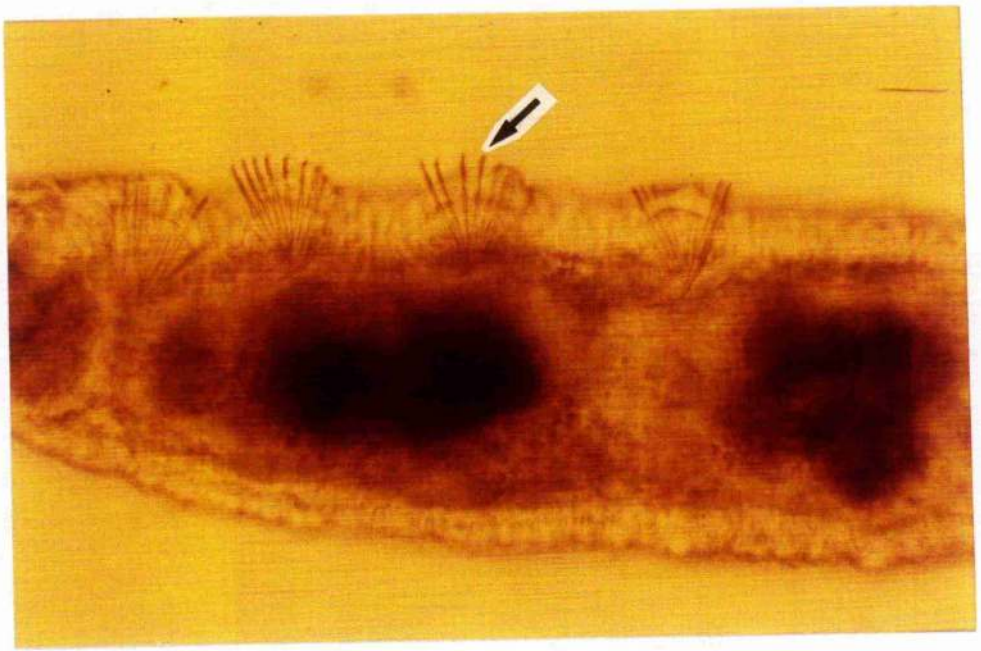
B. glabrata Actual size 12 mm at widest diameter.

Fig. 1.3 *Chaetogaster.*

(a) *Chaetogaster.* (Actual size 7 mm in length).



(b) Attachment hooks.



in the mantle cavity of its host snail, could have deleterious effect on the transmission of *S. mansoni* in the field. It may also, by eating miracidia, contribute to the low level of schistosome infections sometimes found in snails in endemic areas. Again, as in rotifer associations, it is difficult to ascertain accurately what is occurring in the field.

1.5. Intramolluscan development of the parasite

(i) *S. mansoni* eggs. The eggs pass through the wall of the intestine of the vertebrate host, are voided with the faeces, and hatch if deposited in fresh water. The probability of this occurring is enhanced by the social habits of infected people. Children play and adults work in water inhabited by potential mollusc hosts. The eggs, unlike the free living miracidia and cercariae which have a very limited longevity, can remain viable for a week prior to water contact provided they are not subjected to excessive heating or desiccation (Upatham, 1972).

Using a fluorochrome-labelled lectin technique, Linder (1986) demonstrated that the explosive egg hatching event, released the miracidia together with "clouds" of soluble glycoconjugates and secretions which could be visualised at the front end of the miracidium. It was suggested that these secretions aided attachment to and, penetration of the snail host. The fast swimming miracidia (3x faster than *Paramecium*, Haas *et al* 1995), remain infective for a limited period so speed and accuracy in locating the snail host is necessary for successful transmission.

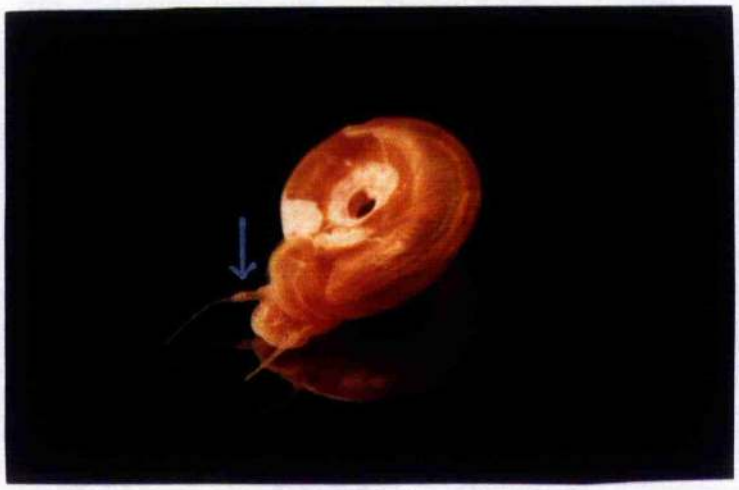
(ii) Location of snail host by miracidia. Detailed studies have indicated that macromolecular cues from the snail host attract the miracidia and stimulate "repeated investigation" behaviour by miracidia (Haberl and Haas 1992; Haberl *et al*, 1995). Fractionation and specific chemical treatment of snail conditioned water, enabled these authors to identify the stimulant as a lysozyme sensitive glycoconjugate with molecular weight >30 kDa.

(iii) Miracidial metabolism. The miracidia after penetration can establish successfully if they survive the snail hosts's defence mechanism, of both cellular and humoral components (van der Knaap and Loker, 1990). An intriguing question is, how does the parasite adjust to the different environments of water and snail haemolymph? In water the miracidia have been shown to possess an aerobic energy metabolism (via Krebs cycle), however they are facultative anaerobes after transformation (Tielens *et al*, 1992). For the first time , these researchers demonstrated in schistosomes the degradation of glucose to lactate and succinate via phosphoenolpyruvate carboxykinase (PEPCK), indicating the ability of the parasite to adjust their metabolism to variable conditions within the snail host.

(iV) The prepatent period is the time from miracidial penetration to release of cercariae. In the field the duration of the prepatent period, which is determined by the ambient temperature, can range from 4 weeks in the summer, to 20 weeks in the winter (Pitchford and Visser, 1965). Cercarial production results from two stages of asexual multiplication, cercariogenesis and sporocystogenesis. Briefly, a miracidium on penetrating a suitable snail host develops into a primary (mother) sporocyst in the subepithelial region of the soft head-foot tissue (Fig. 1.4.). Within 10 to 17 days secondary (daughter) sporocysts have been produced which now migrate to the digestive gland, kidney or rectal ridge. The time range for migration may be due to one primary sporocyst's ability to produce an extensive number , ranging from 34 to 625 , of secondary sporocysts (Jourdane and Théron, 1987). The secondary sporocysts have three developmental choices: 1) directly produce new sporocysts, (sporocystogenesis); 2) initially produce cercariae, (cercariogenesis) and thereafter produce a new generation of sporocysts; or 3) produce at the same time sporocysts and cercariae. In the snail there is integration of sporocystogenesis and cercariogenesis, resulting in a synchronisation of maturing cercariae (Fig.1.5.).

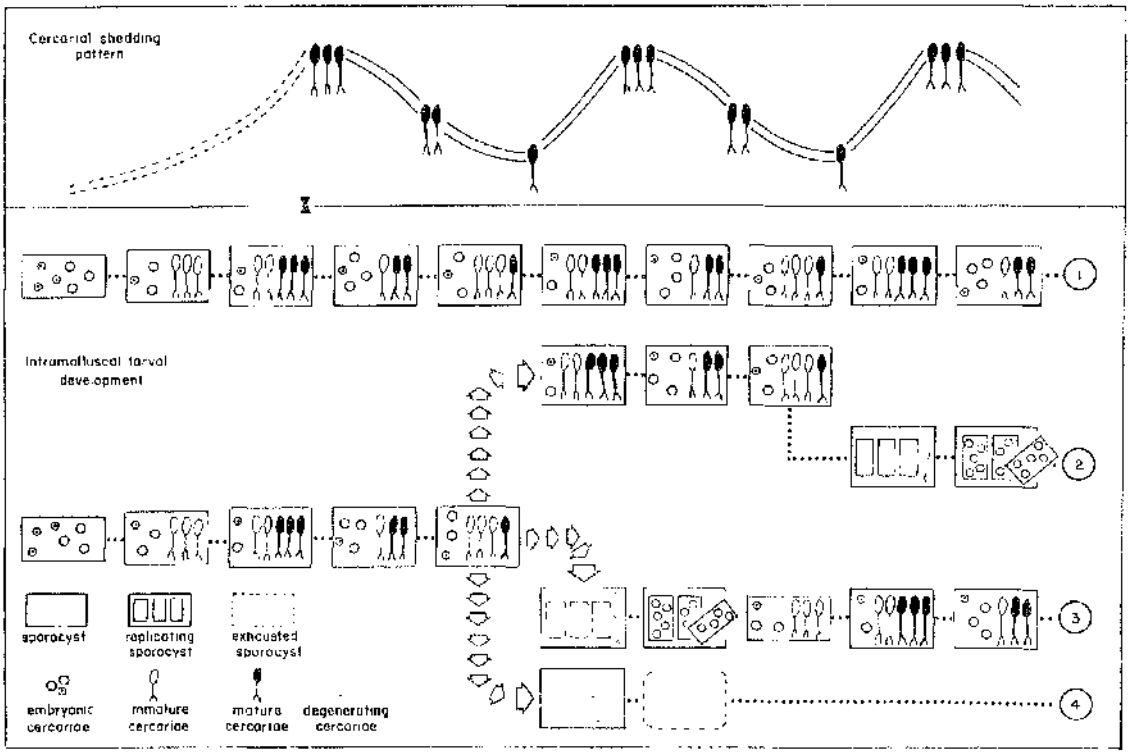
Fig. 1.4 *B.glabrata* infected with *S.mansoni*.

S. mansoni mother sporocyst in snail tentacle.



Note: *B. glabrata* Actual size 12 mm at widest diameter.

Fig 1.5 Dynamics of larval populations of *S.mansoni*.



Model for the larval development of *S. mansoni* in *B. glabrata*.

(1) The daughter sporocyst produces several generations of cercariae.

(2) and (3) The daughter sporocyst ceases production of cercariae after the 2nd or 1st generation and produces sporocysts III.

(4) The sporocyst degenerates. (after Théron 1981 II)

1.6. Cercarial emission.

Cercariae, the infective larvae of *S. mansoni*, are shed from the mollusc host *B. glabrata.* They then have to locate, attach to and finally penetrate the vertebrate host. The free living cercaria has a finite endogenous energy store of glycogen. This glycogen content declines exponentially during aging and is utilised quicker in the tail, where more than half of the total glycogen is contained (Lawson and Wilson, 1980). Given that the cercariae have a limited time to infect their new host, it is imperative that cercarial emergence coincides with the vertebrate host's presence in the water. Cercarial emission has been studied in detail as this transmission stage is considered an appropriate target for parasite control strategies. Two types of emission rhythms have been identified.

(i) The Circadian cycle, exhibits a characteristic maximum emission during the illuminated period of the light cycle (Asch, 1972). Circadian shedding patterns in schistosome species have been well studied and interesting adaptations likely to be related to transmission have been observed. For example two populations of *S. mansoni*, originating from the same endemic Guadeloupean area, have peak emission periods which are 5 hours apart (Théron, 1984). This intraspecific phenomenon may be related to the ecology in both of the sample foci. The early shedding pattern is associated with the urbanised focus where man is the main host. In contrast, the late shedding pattern originated from a sylvatic focus where a crepuscular rat is the main transmitting species. This study of Théron (1984) demonstrated for the first time an intraspecific variation in the chronobiology of *S. mansoni* cercariae and it was interpreted as evidence of host selection pressures influencing expression of the parasite genome.

Pages and Théron (1990), compared two geographical strains of *S. intercalatum* originating from Cameroon and Zaire. The cercariae from the Zaire strain had a mean peak emergence period which was 1hr 45 mins

earlier than the cercariae from the Cameroon strain and this difference was statistically significant ($P<0.05$). Pages and Théron (1990) concluded that the chronobiology of the emergence rhythms of the cercariae appeared to be an additional marker, both to measure genetic variability and to characterise the two strains of *S. intercalatum* .

The adaptive circadian rhythms described above are examples of the complex and specific relationship which exists between the parasite and mollusc host. They provide evidence of the important role that the snail has in the geographical distribution and dispersion of the disease. The results of Pages and Théron (1990), although observed in parasites originating from the field, were nonetheless reproduced in a laboratory situation. Laboratory maintenance conditions were strictly controlled to simulate those in the field. Snails were in constant water temperature of 26°C and in a balanced photoperiod of 12 hours light/12 hours dark, with light intensity gradually increasing at the beginning of the photoperiod while decreasing at the end. The latter condition concerning photoperiod is relevant to Chapter 3 in this thesis and will be discussed later.

(ii) The Circamensual rhythm observed by Théron (1981a and 1981b) has a periodicity of approximately 35 days, and characteristically exhibits alternating periods of high and low cercarial emission. These fluctuations in cercarial production are a consequence of the demography of the intramolluscan parasite stages (Fig. 1.5). Periods of high emission and maximum infectivity correspond with maturation and shedding of cercariae of the same generation, while low emission and minimum infectivity is associated with transition from one generation to another (Théron and Moné, 1984). It is of great interest that two populations of cercariae, collected at these periods of high and low emission, have been identified which possess different success in infectivity. It would be informative to study a range of properties, in cercariae from both high and low emission periods. Concurrent

infectivity studies could perhaps identify a factor which was related to successful parasite infectivity.

The pattern of snail growth also exhibits a circamensual rhythm, negatively correlated to cercarial production. Théron and Moné (1984), demonstrated that high cercarial productivity matched periods of slow snail growth and, conversely, low cercarial productivity corresponded to periods of faster growth in the mollusc host (Fig. 1.6).

Again, as in the studies of the circadian rhythm, the observations concerning circamensual rhythms were obtained under controlled conditions. Of particular interest, is the photoperiod of 12HL/12HD which reflects the natural environment of the field.

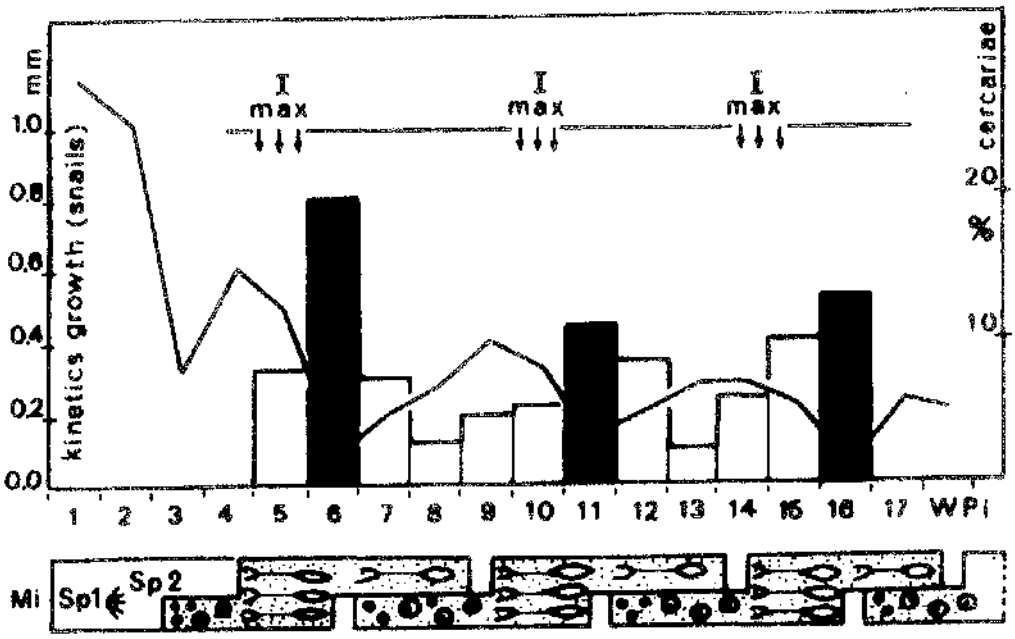
The aquarium housing the snails, used in the study presented in this thesis, is required by many researchers including students. Consequently, as a safety precaution infected snails are maintained in a locked dark cupboard. Thus, the snails used in the present study would normally after parasite exposure be maintained, by necessity in conditions somewhat different to those in the field.

It is routine practice in most laboratory systems, where parasitised snails are maintained in a 12HL/12HD regime, to subject snails to approximately 48 hours of total darkness prior to harvesting a cercarial shed. This is considered an acceptable method of maximising cercarial numbers. However concern that snail maintenance with prolonged unnatural photoperiod may adversely affect cercarial production has prompted the investigation which is described in Chapter three.

1.7. Cercariae

(i) The Morphology. The cercaria which is approximately 0.5mm in length, consists of a body (or head) and a bifurcate tail (Fig. 1.7). The body and tail are covered with an immunologically active glycocalyx, a complex

Fig 1.6 Relations between circamensual rhythm of cercarial production, cercarial infectivity and host growth of *S.mansoni* in *B.glabrata*.



Relations between the growth kinetic rate of infected *B. glabrata* (____ , weekly increase of size), the cercarial production of *S. mansoni* (bars , weekly production percentage of the total production), and the infectivity of cercariae (I_{max}, maximal infectivity) during the course of the experiment (WPI, weeks postinfection).

The scheme below shows the intrasporocystic pattern of *S. mansoni* by successive generations of cercariae. (after Théron and Moné 1984)

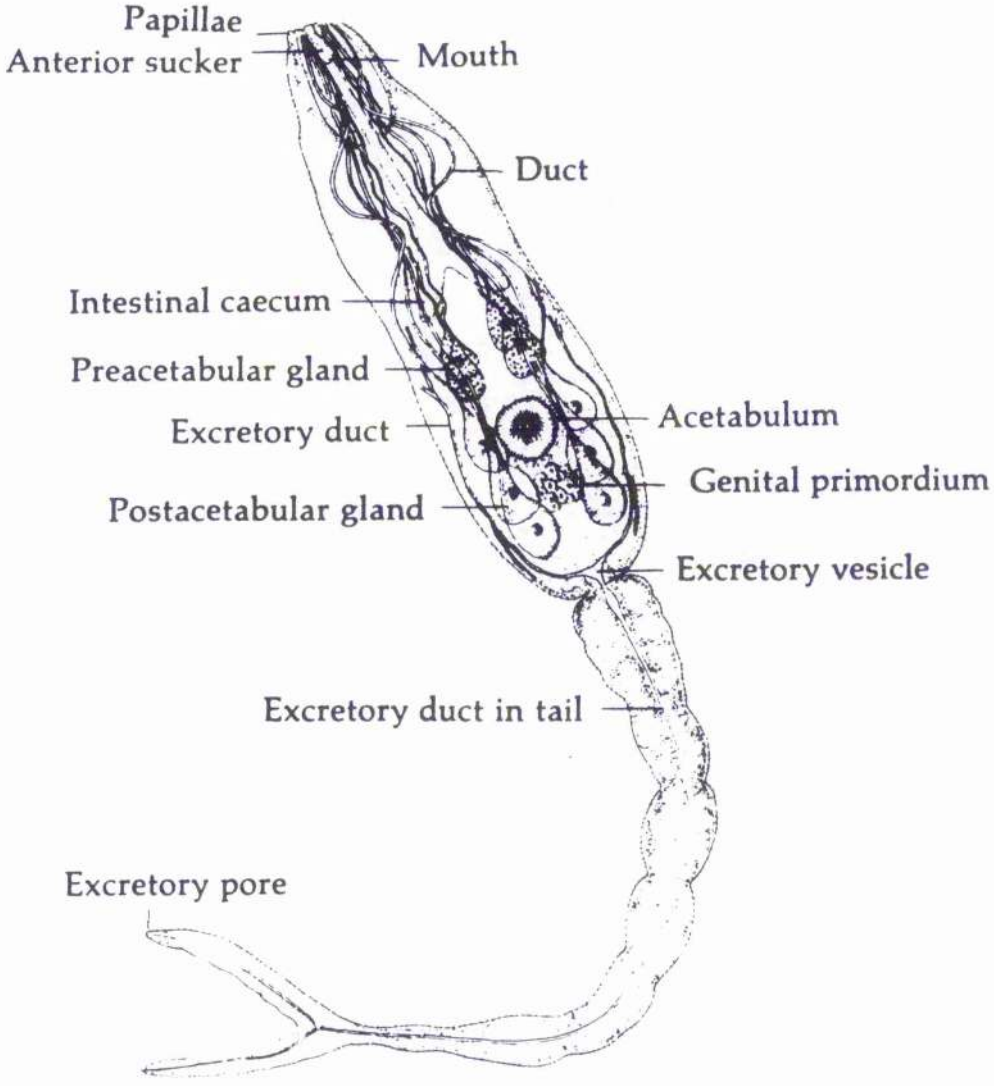
Key

Mi : Miracidium

Sp1 : First generation sporocyst. Mother sporocyst.

Sp2 : Second generation sporocyst. Daughter sporocyst.

Fig 1.7 Morphology of a *S.mansoni* cercaria.



structure composed of oligosaccharides which are probably linked to a peptide (Xu et al , 1994).

The body has one oral sucker, penetrated by a mouth which leads to a primitive Y-shaped gut. The excretory system comprises three pairs of flame cells in the body, connected by tubules to an excretory bladder, and one pair in the tail ducting to the tips of the furca. The ventral sucker (or acetabulum) lies in front of the prominent cephalic glands which occupy most of the body cavity.

The two pairs of preacetabular glands contain proteolytic enzymes, belonging to the serine class, which assist in the penetration of host tissue (Landsperger *et al* , 1982). A protease which is active against elastin has been purified by McKerrow *et al* (1985a). This 30 kDa serine protease was shown to have a calcium dependence of 2 mM. Further studies (McKerrow *et al* 1985b), revealed that the protease degraded fibronectin and laminin and was active against basement-membrane collagens, thus facilitating skin penetration. It is of interest that the proteases were ' ə secreted by cercariae in response to skin lipids.

In vitro studies indicated that calcium levels, similar to those within the preacetabular glands, inhibited the cercarial proteases reversibly (Dresden and Edlin, 1975). The activity of the enzymes within the gland, is considered to be similarly regulated by the calcium, which is present in high levels as calcium carbonate (Dresden and Asch, 1977). Chelation of the calcium, with 0.0125% Alizarin Red S stain, depresses the successful invasion and maturation of the cercariae (Lewert and Hopkins, 1964) indicating the crucial contribution of preacetabular calcium to cercarial infectiveness.

The three pairs of postacetabular glands contain material which swells when released in water, forming a sticky mucus. The mucus assists attachment to surfaces and, by swelling, aids separation of squames during penetration of the horny layer of host skin (Stirewalt and Dorsey, 1974).

(ii) Location and penetration of vertebrate host. The cercariae, which are usually shed within the vertebrate host vicinity (Chapter 1.5.), exhibit a sequence of behavioural phases, each of which is determined by specific environmental and host stimuli (Haas, 1992). Experimentally, cercariae aggregate in response to linoleic acid. In choice chambers they move towards this stimulant and after prolonged stimulation (120 min), they commence irreversible penetration responses (Shiff and Graczyk, 1994). In penetration studies, using a living skin equivalent (LSE), significant increases in penetration rates resulted after LSE had been pretreated topically with linoleic acid (Fusco *et al*, 1993). Interestingly, linoleate also induced cercarial tail loss and a synergistic effect was observed when calcium ionophore was present (Hara *et al*, 1993). Conversely, suppression of tail loss resulted if the calcium ion chelator EGTA, was included in the incubation mixture. Hara *et al* suggested that extracercarial calcium contributed substantially to inducement of tail loss by linoleate.

It is intriguing that linoleic acid attracts cercariae, appears to induce penetration activity and also triggers tail loss.

Penetration of the vertebrate host does not however equate with parasite maturation as only a small percentage of penetrants develop to egg laying adults. Although the glycogen content of cercariae declines exponentially during aging, Lawson and Wilson (1980a and 1983) observed that cercarial ability to penetrate mice remained constant throughout their lives. They suggested that the failure to reach maturity after penetration was age related and may be associated with glycogen depletion during penetraion of the stratum corneum. Could this age related phenomenon be attributed to any other factor?

A possible candidate is calcium. As already referred to [Chapter 1.7. (1)], calcium of the acetabular gland is a prerequisite for successful penetration. It is possible that calcium may decline exponentially as the free-living cercariae

ages and contribute to the death of the parasite during or immediately after penetration. Given the damage exhibited early in patency to calcium storage cells and surface of snail inner shell, it is a further possibility that levels of calcium in newly released cercariae, may decline as patency develops. The studies of Chapter 4 onwards, address the intriguing possibilities concerning calcium levels of the free-living cercariae.

1.8. Aims of this study

This thesis is concerned primarily with the association between the parasite and snail host. Initially the effect of snail maintenance on cercarial production is examined. Thereafter the emphasis is on studying the calcium relationships between cercariae, snail and environment. Consequently the experimentation is in two sections.

Firstly, to investigate the effect of different light regimes on the emission of *S. mansoni* cercariae from *B. glabrata* snails. Secondly, to develop an assay which will permit comparisons of preacetabular calcium in individual cercariae. The application of the assay, in calcium studies of shed cercariae as they age and of cercariae throughout the duration of patency, will be investigated. The total calcium content of both cercariae and the water they were shed in, will also be determined. The results will be discussed in relation to their effect on cercarial production and infectivity.

CHAPTER 2

Materials and Methods

2.1. Life-cycle maintenance

(i) Parasite strain

A Puerto Rican strain of *Schistosoma mansoni* is routinely maintained in an albino strain of the snail host *Biomphalaria glabrata*, both strains were originally obtained from NIMR, Mill Hill, London. Additional stocks have been introduced, and interbred, which were supplied by both York and Bangor Universities.

(ii) Infection of snails

Sorenson's buffer : Solution A : 50mM KH_2PO_4

: Solution B : 60mM Na_2HPO_4

Sorenson's buffer consists of 5% volume solution A, and 95% by volume solution B.

Physiological saline : 0.15M NaCl

Trypsin

Preparation of miracidia. The small intestines of 6-8 week schistosome infected mice were cleaned with physiological saline (0.15M NaCl) and homogenised in 50ml Sorenson's Buffer. The homogenate plus trypsin (1mg trypsin per intestine) and a further 50ml buffer, were incubated at 37°C for 2 hours. After digestion the homogenate was strained through two layers of muslin and centrifuged at 2,000 r.p.m. for 10 minutes. The supernatant was decanted and the sediment was washed with physiological saline and again centrifuged for 10 minutes. This process was repeated and the final sediment of packed eggs was suspended in dechlorinated tap water (DCTW) and hatched in bright light (2,000 lux).

Routine infection and subsequent maintenance of snails. Snails measuring 5-8mm in diameter were placed individually in small glass vials (4x2cm) containing 3ml of DCTW. Using a Pasteur pipette, a small aliquot of

water containing approximately 7 newly hatched miracidia was added into each vial. The snails were left for 24 hours at 26°C before being transferred to plastic canisters in groups of 10 snails per 2 litres of DCTW. Exposed snails, maintained in the dark, were fed three times per week with washed fresh lettuce and had their water changed once a week. After 6-8 weeks, washed snails were exposed to light, 2,000 lux , in a small volume of water to collect cercariae for infection of mice.

(iii) Infection of mice.

Sagatal **:** Anaesthetic 10% (v/v) Sagatal in distilled water : ethanol, 9:1 by volume.

Mice : TO strain of mice was used.

The percutaneous method of exposure to cercariae described by Smithers and Terry (1965a) was used. Aliquots, 50µl, of the cercarial suspension were fixed and stained with small volumes of diluted iodine solution and counted (Olympus Tokyo microscope). Mice were anaesthetised, using Sagatal, at the dosage of 0.9ml anaesthetic per 100g body weight. Anaesthetised mice, with shaved abdomens, were laid on their backs in polystyrene trays. Metal rings of 300µl capacity were laid on the abdomen. Aliquots of cercarial suspension, containing 150-200 cercariae, were introduced into each metal ring. The mice were left undisturbed for approximately 20 minutes to give the cercariae time to penetrate the mouse skin. After post operative recovery, the mice were caged, labelled and given water and food supply. Mice were maintained by staff of the Joint Animal Facility.

2.2. *S.mansoni* cercarial production in *B.glabrata* snails maintained individually in 50ml water at 26±1°C in different light regimes.

(i) Parasite Exposure. *B. glabrata* snails of 5-7 mm diameter were

exposed individually, in glass vials containing 2 ml of water, to 3 freshly hatched *S. mansoni* miracidia. Snails were left overnight in the aquarium which has a controlled temperature of 26±1°C. Exposed snails were thereafter maintained in groups of 8, in appropriate canisters containing 2 litres of water in the following regimes.

(i) Four Clear canisters on an open shelf in the 12HL/12HD aquarium. Light was recorded using a Skye Light Meter (Skye SKP 200), measuring in units of μmol/sec/m^2 (which is equivalent to one μEinstein) .

For conversion. 19.5 μEinstein = 1Klux

(ii) Three Dark canisters (canisters covered in aluminium foil) on the same shelf in the aquarium.

Snails were fed fresh, washed Roman lettuce on alternate days and the water in the canisters was changed weekly for a period of 27 days.

Microscopic examination. On day 27 post exposure (PE), all 12HL/12HD snails and 3 Dark Maintained (DM) snails were examined microscopically to select parasitised snails. Initially, 7 12HL/12HD and 3 DM snails were chosen for study. The 7, 12HL/12HD snails were maintained individually in clear beakers containing 50 ml of water on the aquarium shelf, while the 3 DM snails were maintained in dark beakers (Halfords Satin Black Spraypaint) in a cupboard in the same aquarium. Later, on day 32 PE, four small groups of snails were established. Two clear beakers, each containing a group of 3 parasitised 12HL/12HD snails in 50 ml of water were maintained on the aquarium shelf. At the same time two dark beakers, each with a group of 3 snails from a dark canister were maintained in the aquarium cupboard. Note that in this case the dark snails were not examined microscopically to determine if they were parasitised.

(ii) Cercarial counting. Commencing on day 28 PE, and at the same time each morning, cercariae were collected and counted for a period of 43 days or

until the death of the snail if earlier. On four Sundays the counting procedure was not undertaken.

Daily counting procedure.

1. Water was decanted from each beaker into a 50 ml centrifuge tube (Costar) and the tubes were stored at 0°C.

2. Water was replaced immediately in the beakers, the snails were cleaned if necessary and fed fresh washed lettuce.

3. After 30 minutes at 0°C, the tubes were spun at 1,500 r.p.m. for 10 minutes.

4. The supernatant was carefully removed, initially with an automatic and finally with a Pasteur pipette, leaving approximately 600 µl supernatant of water. The cercariae were then resuspended by flicking the tube.

5. The cercarial suspension was pipetted in droplets onto gridded petri dishes. Each cercarial droplet was carefully stained with iodine and the cercariae were counted microscopically (Olympus Tokyo microscope).

A flow diagram of this protocol is presented in Fig. 3.1.

2.3. *S.mansoni* cercarial production in *B.glabrata* snails maintained individually in 2 litres water at 26±1°C in 12HL/12HD light.

(i) Parasite Exposure. The same procedure as in 2.2. (i), but snail was exposed to only one miracidium. Cercariae emitted from snails exposed to only one miracidium are considered clones since they are the product of one asexually dividing individual. Snails were maintained in groups of 8 snails in 2.5 litres of water at 26±1°C in 12HL/12HD until they were identified as being parasitised. Thereafter 5 snails were maintained individually in 2 litres of water at 26±1°C in 12HL/12HD.

(ii) Alternative cercarial counting procedure.

1. Snails were removed from canisters, cleaned and placed individually in

small glass vials with 2ml of DCTW.

2. After a period of one hour the water was decanted into small 3ml volume , round bottomed glass tubes and stored at 0°C. This procedure was repeated for two more, one hour periods.

3. Snails were returned to 2 litre canister and fed fresh washed lettuce.

4. After 30 mins on ice, supernatant was pipetted from glass tubes leaving a small volume containing sedimented cercariae.

5. Cercariae were resuspended by flicking the tube.

6. Cercarial suspension was pipetted onto gridded Petri dish, stained with iodine (using a Gilson pipette a small volume of iodine was introduced to the edge of cercarial suspension drop to prevent cercariae being scattered) and counted.

7. The recorded emission was the sum of three one hour shedding periods.

The altered method of counting had several advantages. Faecal debris was minimal and no centrifugation was required to sediment cercariae.

2.4. Assessment of calcium, in preacetabular glands of *S.mansoni* cercariae, by Fluorescent Emission Quantitation of Alizarin Red S.

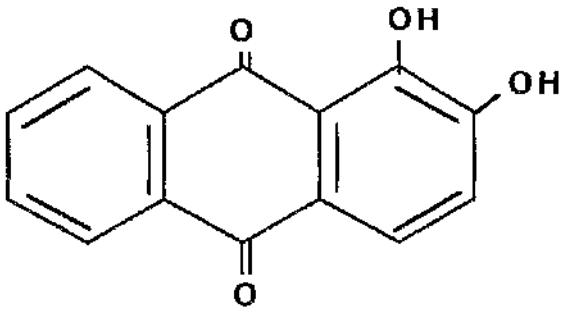
Alizarin Red S. (ARS)

Stock solution 2%ARS.

Dilution used, 0.5µl of 2% stock solution to 1ml Distilled H_2O (0.001% ARS).

(i) Properties of ARS. The emission spectrum for ARS was determined using a SPEX FluorMax (Spex Industries Edison, N. J., U.S.A.) spectrometer. Thereafter the ARS/ calcium standard curve was obtained using a Perkin Elmer LS.5., Fluorescence Spectrometer.

Structural formula of ARS



(ii) ARS staining. Parasitised snails, after washing in DCTW, were exposed individually in glass vials containing 2ml DCTW. After a 2 hour illumination (2,000 lux) period, the cercarial suspensions were decanted into round bottomed glass tubes of 5ml capacity. An equal volume of 0.001% ARS was added to each 2ml cercarial sample. Samples, protected from light in aluminium foil, were incubated at room temperature for 10 minutes. Tubes were then placed in ice for 20 mins to sediment cercariae. Supernatant was removed, cercariae were washed with cooled DCTW and tubes were replaced in ice. Cercariae received 4 washes in total. The final supernatant was removed leaving a cercarial pellet in 200µl DCTW. After resuspension, an equal volume of carbachol was added. Tubes were returned to ice after an aliquot of cercariae had been removed, from one tube, and mounted on a slide. This slide was used to standardise the microscope for quantitation analysis.

This protocol is summarised in Fig.4.5.

(iii) Fluorescence Emission Quantitation. A rectangular area, viewed down the Leitz Laborlux S microscope eyepiece, was adjusted to cover the preacetabular gland region of the cercaria. This area, over which fluorescence values were read, remained fixed throughout the course of the experiment. The cercaria which appeared to exhibit the brightest fluorescence was selected for standardisation of the microscope.

Thereafter, each tube was processed individually. A minimum of sixty cercariae per sample were quantitated. To eliminate prejudice in selection, the cercariae to be quantified were chosen at random as the slide was scanned under bright field. Results were tested for significance using the Student's t-test, where $P < 0.05$ was accepted as significant.

A diagram of the microscope set-up for fluorescence quantitation, is shown in Fig. 2.1.

2.5. Calcium content of cercariae and shedding water.

(i). Total Calcium determination. To determine the calcium content of cercariae and shedding water, a population of snails was shed for a period of 2 hours in DCTW. A sample of stock DCTW was reserved as a control. The faecal free, cercarial suspension was decanted into a 50 ml plastic tube (NUNC) for transportation to the laboratory. Thereafter 10 ml volumes of cercarial suspensions were transferred into glass conical tubes (Volac). This was necessary because the cercarial pellet is dissolved in nitric acid prior to calcium analysis. Aliquots (3x100µl) were removed, cercariae were counted and the number of cercariae per 10 ml sample was calculated.

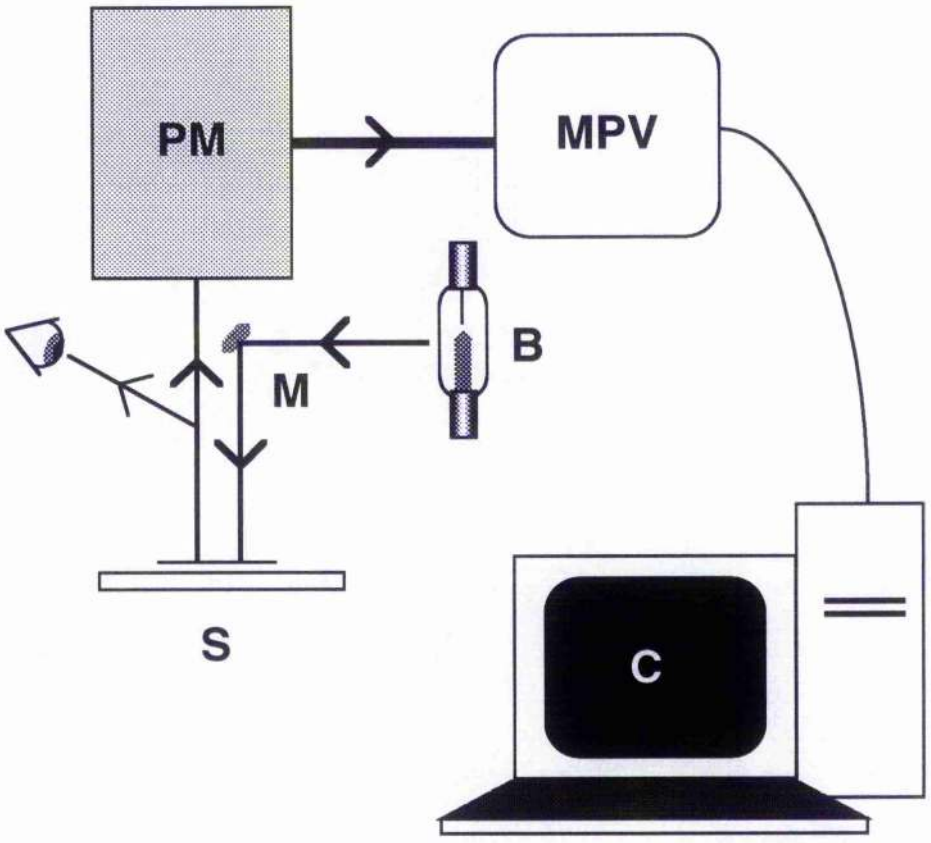
The samples were either directly incubated on ice to sediment or, left at room temperature in the light prior to sedimentation, if the effect of aging on calcium reserves was to be investigated. After sedimentation on ice the samples were spun at 2,000rpm. The supernatant was transferred to plastic universals, frozen and reserved for calcium determination. The cercarial pellets were freeze dried prior to storing.

A summary of this protocol is presented in Fig.5.1.

The calcium of the DCTW and supernatant shedding water was determined by either Plasma Emission Spectrometry (Thermo Jarrell Ash Spectra 16) or Atomic Absorption Spectrometry (Perkin Elmer 1100B. Hall cathode lamp specific for Calcium). The cercarial pellet was only analysed by Plasma Emission Spectrometry.

Fig. 2.1.

Microscope set-up for Fluorescence Quantification



KEY

B	3-1LPLEOMOPAK fluorescence illuminator
M	Leitz Laborlux S microscope (Leica, UK)
S	Stage of microscope
PM	Leitz photomultiplyer
MPV	PMPV-STAT software
C	Computer PC (Silicone Valley)

2.6. Cercarial incubation in EGTA. (ethylene glycolbis tetraacetic acid) **EGTA.** (A calcium chelating agent). A 1ml cercarial suspension was incubated at 22°C, for a prescribed time, with 110µl of 100mM EGTA, final dilution 10mM EGTA.

PKH26. (A fluorescent lipid probe). A 50µl cercarial pellet/suspension was made up to 300µl with the manufacturers diluent C. The cercarial suspension was gently agitated while 3µl of the PKH26 probe was added. Incubate at 22°C for 10mins. Spin at 3,000 rpm for 2 mins. remove supernatant and wash cercariae 3 times with distilled water.

Calcium chloride. A 1ml cercarial suspension was incubated at 22°C, for a prescribed time, with 20µl of 500mM $CaCl_2$, final dilution 10mM $CaCl_2$.

2.7. Microscopic Photography

(i) Leica Camera WILD MPS52

(ii) Kodak 5040 EPH film was used for coloured photography. When photographing ARS stained or PKH26 labelled parasites a Rhodamine filter was applied.

(iii) Ilford XP2 film was used for black and white photography.

CHAPTER 3

Cercarial production from snails in a) 12HL/12HD and b) Dark maintenance regimes.

3.1. Introduction

Shedding of *Schistosoma mansoni* cercariae by *Biomphalaria glabrata* snails infected with a single miracidia, has been shown to be irregular when measurements were made on a daily basis. Alternate periods of high and low emission were observed when cercarial production was monitored over an extended period of time (Théron, 1981a). The observations of Théron were obtained from a group of ten *B. glabrata* snails maintained in 2.5 litres of water at 26±1°C, in a regime of 12Hour Light/12 Hour Dark (12HL/12HD).

In the present study cercarial production, from individual snails in 50ml of dechlorinated tap water (DCTW), and maintained under different light regimes, is presented. It should be noted that the total daily emission of cercariae was recorded, the daily circadian rhythm was not determined. Complementary observations are included of cercarial emergence from snails maintained in a larger volume of water.

A 71 day study was undertaken to compare the cercarial production of individual snails maintained in a 12HL/12HD regime with snails maintained in the dark . It should be noted that snails maintained in 12HL/12HD were exposed to 12 hours light intensity of 543 lux, and dark maintained snails may have been exposed momentarily to light intensity of 41lux when water was removed for cercarial estimation.

The effects of maintenance on a) the length of the prepatent period, b) the number of cercariae produced and c) the pattern of cercarial emission are presented and discussed in this chapter.

3.2. Cercarial production from snails in a) 12HL/12HD and b) dark maintenance regime. Maintenance water per snail was a volume of 50ml at a temperature of 26±1°C.

The experimental procedure to study cercarial production in snails maintained in two different light regimes is described in Materials and

Methods (2.2.), and is summarised in a flow diagram (Fig.3.1.).

It should be noted that the six snails selected for groups 12 and 13, were all known to be parasitised. However, the six snails comprising groups 14 and 15 were selected from dark canisters and were not identified as being parasitised. Over the experimental period snails from the latter two groups died but, as no corresponding drop in cercarial production was detected, it was assumed that only one snail in each of groups 14 and 15 was parasitised. Consequently in the results they are referred to as snail 14 and snail 15, although they were initially in a group of three. It should also be noted that the time clock in the aquarium was over ridden by another researcher and for a short period immediately after exposure, the snails in the 12HL/12HD regime were exposed to continual light for approximately five days .

Cercarial Emission Results

(i) Prepatent period. All of the 12HL/12HD snails and three of the dark maintained snails were examined microscopically on day 27 post exposure (PE), and it was noteworthy that there was no observable difference in the appearance of the snails. All parasitised snails, including those maintained in the darkness, had very active cercariae in their tissues and, after examination, free swimming cercariae were present in the water. On day 28 PE, the first day of counting, the average number of cercariae per 12HL/12HD snail was 62±27; while that per dark maintained snail was 110±33.

(ii) Number of cercariae. Tables 3.1.a) and b), show the total number of cercariae shed from each snail, together with the average number of cercariae per shedding day. The variation in total output per snail is clearly seen , ranging from as little as 732 to 4267 cercariae.

(iii) Snails maintained individually. In more detail, the daily number of cercariae emerging from four of the individual 12HL/12HD snails is plotted as column graphs (Fig. 3.2.). The snails all shed on day 28 PE and

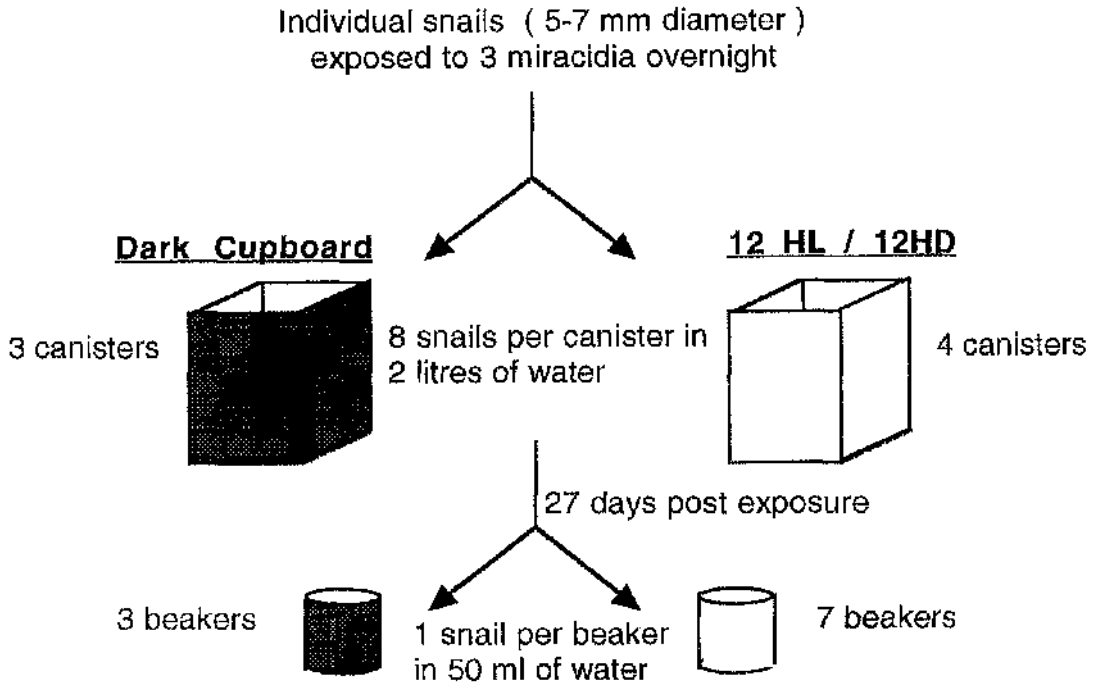
Note Fig. 3.1

At day 33 post exposure four groups of snails were set up.

a) 2 groups of 3 parasitised snails / 50 ml beaker in 12HL/12HD.

b) 2 groups of 3 snails from dark canister / 50 ml dark beaker in dark cupboard.

Fig 3.1 Protocol for monitoring cercarial production



Water decanted from each beaker into 50 ml centrifuge tube* and stored at 0°C for 30 min.

↓

Water replenished in beaker. Snail cleaned and fed lettuce.

↓

*Centrifuge tubes spun at 1500 - 2000 rpm for 10 min.

↓

Supernatant removed leaving approximately 600 µl of water and cercarial pellet.

↓

Resuspend cercariae by flicking tube.

↓

Pipette cercarial suspension onto gridded petri dish. Carefully stain and immobilise cercariae with iodine.

↓

Count cercariae using microscope

} Daily Procedure

Table 3.1. a) ***S.mansoni*** **cercariae shed from** ***B.glabrata*** **snails maintained individually in 50ml water.**

Snail	Number of Days Shed	Total Number of Cercariae	Mean Number of Cercariae per Shedding Day ± St.Dev.
1	**28**	**2101**	**75 ± 65**
2	**44**	**2355**	**53 ± 45**
3	**8**	**119**	Died on Day 35 PE
4	**44**	**4796**	**109 ± 75**
5	**39**	**2005**	**51 ± 35**
6	**44**	**2933**	**66 ± 40**
7	**14**	**471**	Died on Day 41 PE
9	**35**	**1810**	**51 ± 43**
10	**30**	**1043**	**34 ± 41**
11	**44**	**1709**	**38 ± 36**

Key Snails 1 - 7 maintained in 12HL/12HD.
Snails 9 - 11 maintained in continual darkness.

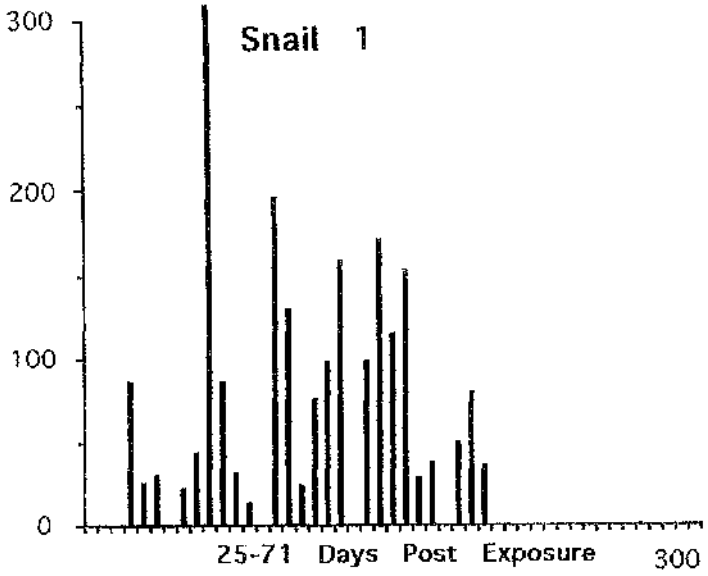
Table 3.1.b) ***S.mansoni*** **cercariae shed from** ***B.glabrata*** **snails maintained in groups of 3 in 50ml water.**

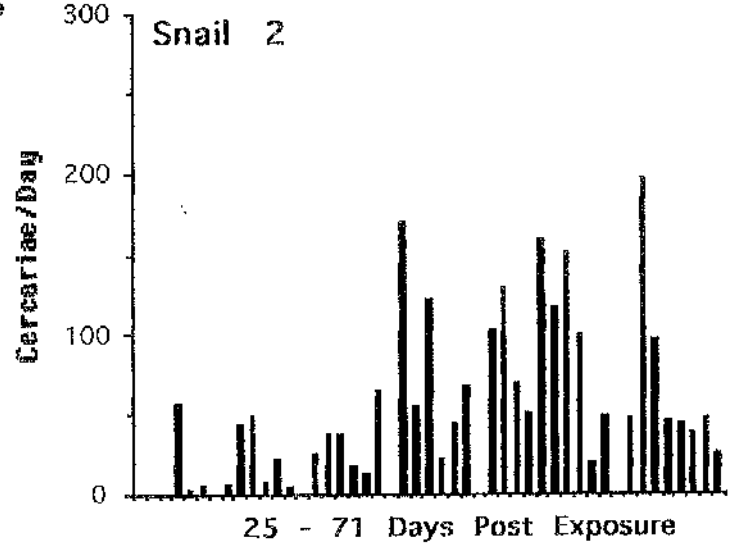
Snail	Number of Days Shed	Total Number of Cercariae	Mean Number of Cercariae per Shedding Day ± St.Dev.
12*	**39**	**4203**	**107 ± 65**
13*	**39**	**4267**	**109 ± 56**
14**	**39**	**732**	**18 ± 17**
15**	**39**	**1989**	**51 ± 47**

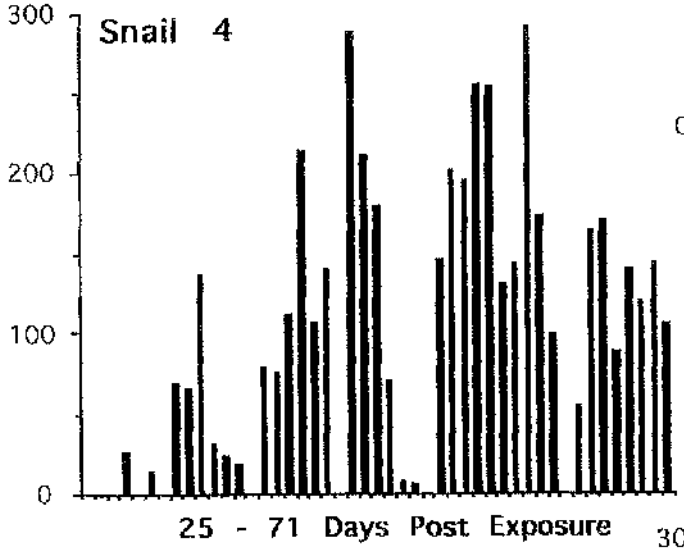
Key * Average of three parasitised snails maintained as a group in 12HL/12HD.

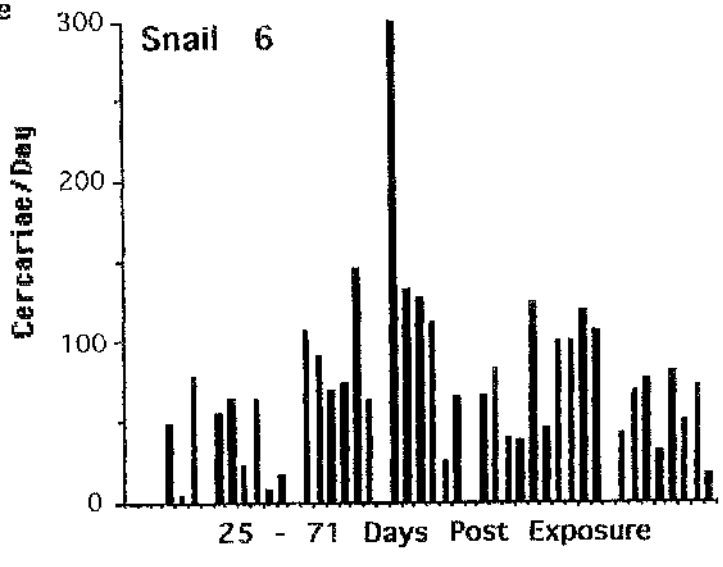
** One parasitised snail maintained with two non-parasitised snails in a group in continual darkness.

Fig. 3.2 **Cercariae emitted per day from 4 parasitised snails maintained individually in 50 ml of water in 12HourLight/12HourDark regime.**


Snail 1
300
200
100
0
25-71 Days Post Exposure


Snail 2
300
200
100
0
Cercariae/Day
25 - 71 Days Post Exposure


Snail 4
300
200
100
0
25 - 71 Days Post Exposure


Snail 6
300
200
100
0
Cercariae/Day
25 - 71 Days Post Exposure

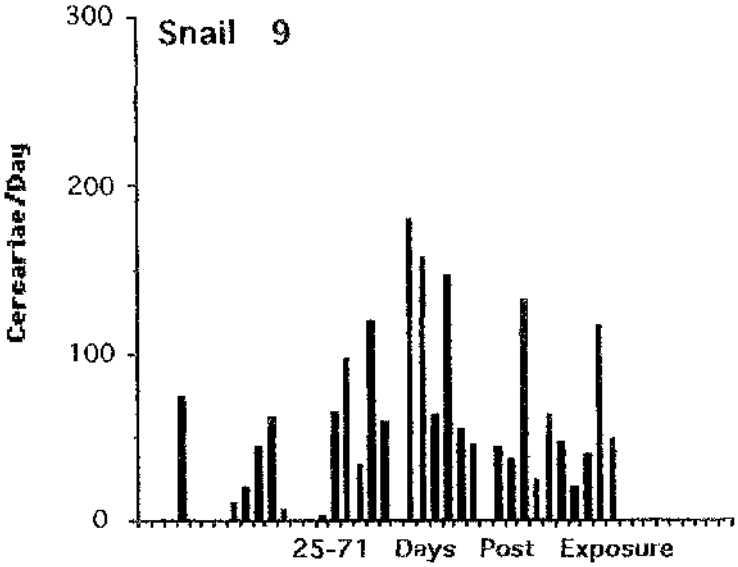
thereafter exhibited irregular daily emissions of cercariae with the average daily output per shedding day ranging from, 51±35 cercariae Snail 5, to 109±75 cercariae Snail 4.

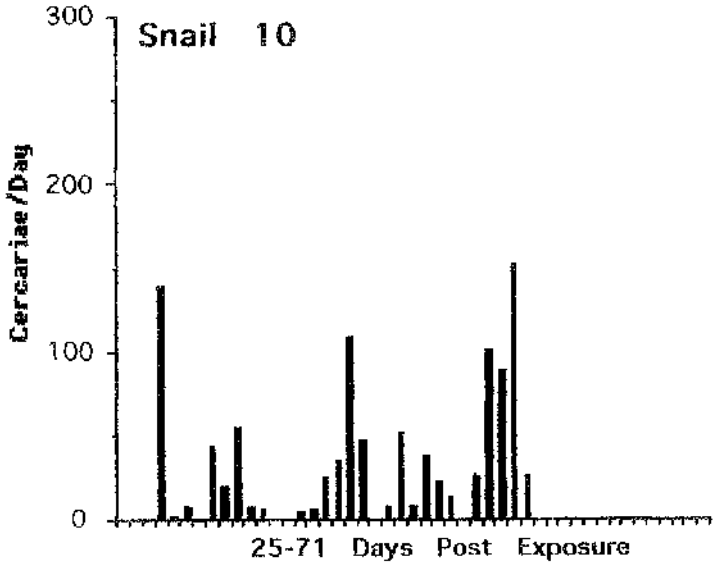
In comparison cercarial emission was much reduced in snails maintained in the dark (Fig. 3.3.). Here the average cercarial output per shedding day ranged from, 34±41 cercariae Snail 10, to a maximum of 51±43 cercariae Snail 9.

(iv) Snails maintained in small groups. A difference in cercarial production was also recorded under different light regimes, when snails were maintained in small groups (Fig. 3.4.a. and b.). The snails in groups 12 and 13 maintained in 12HL/12HD shed on average 107±65 and 109±56 cercariae per shedding day, compared to a lower average of 18±17 and 51±47 cercariae per shedding day, when groups were maintained in the dark (Table 3.1. b).

(v) Pattern of cercarial emission.When the data was pooled (ie. 11, 12HL/12HD snails and 5, dark maintained snails), and the average number of cercariae per day per snail was plotted, the difference in cercarial output from snails in the two maintenance regimes was clearly demonstrated (Fig. 3.5.). With the exception of two days, day 28 and 50PE, the 12HL/12HD maintained snails produced more cercariae than the snails in continual darkness. However, it is very apparent that the pattern of emission is very similar in both maintenance regimes. For example, at days 34, 47, 56 and 65 PE, both maintenance groups of snails showed increases in cercarial output whereas at days 37, 53 and 64 PE there was a noticeable decrease in emission of cercariae by snails from both maintenance regimes.

Fig. 3.3 Cercariae emitted per day from 3 parasitised snails maintained individually in 50 ml of water in dark conditions.


Snail 9
300
200
100
0
Cercariae/Day
25-71 Days Post Exposure


Snail 10
300
200
100
0
Cercariae/Day
25-71 Days Post Exposure

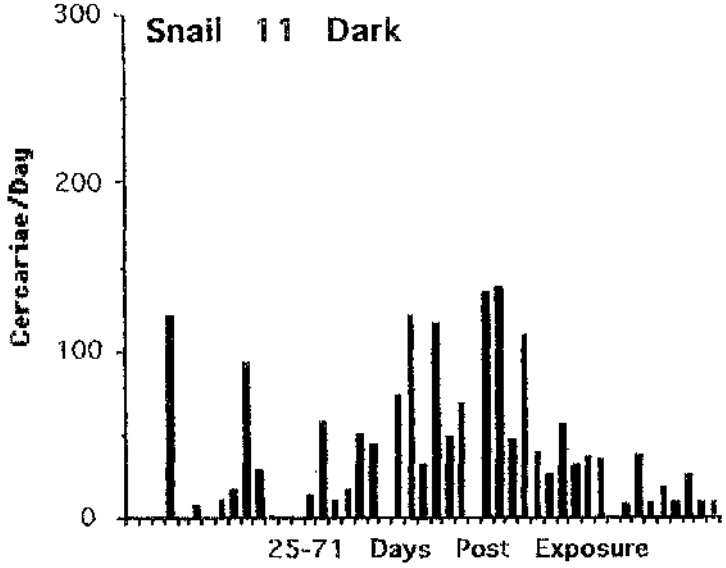

Snail 11 Dark
300
200
100
0
Cercariae/Day
25-71 Days Post Exposure

Fig. 3.4.(a) Group maintained snails

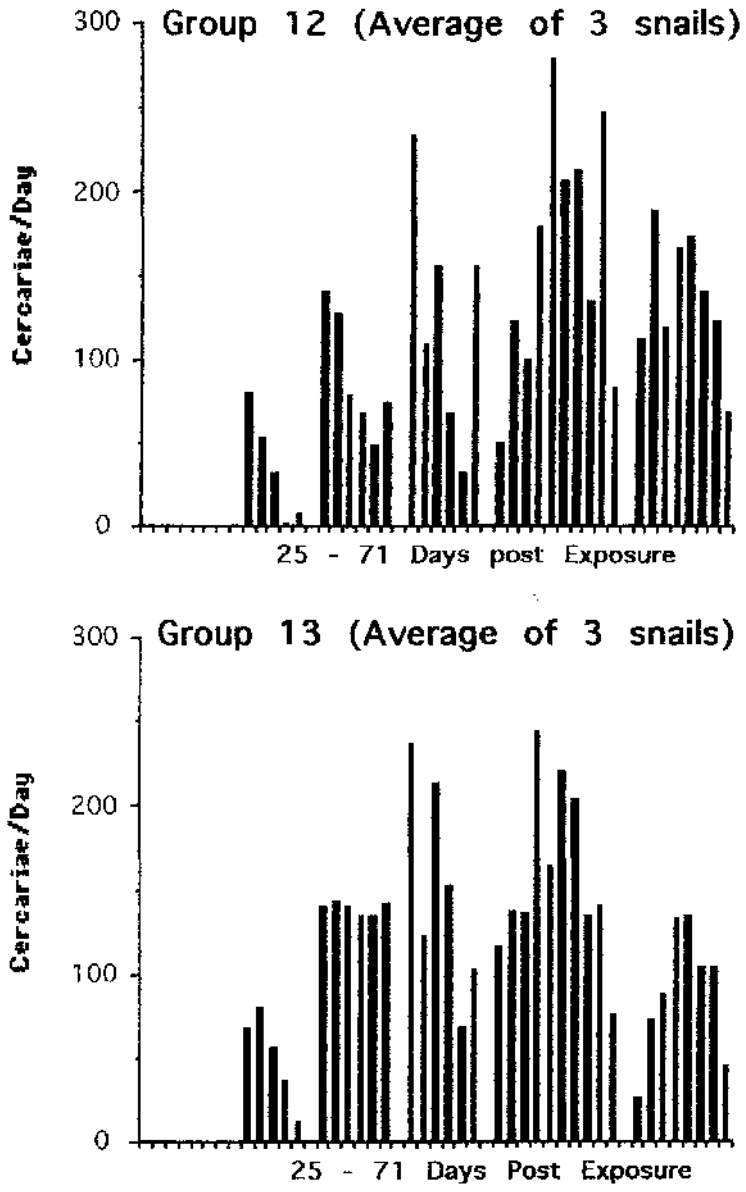


Fig. 3.4.(a) Average number of cercariae emitted per snail per day from parasitised snails maintained in 2 groups of 3 snails per 50 ml of water in 12HL/12HD regime.

Fig. 3.4.(b) Group maintained snails.

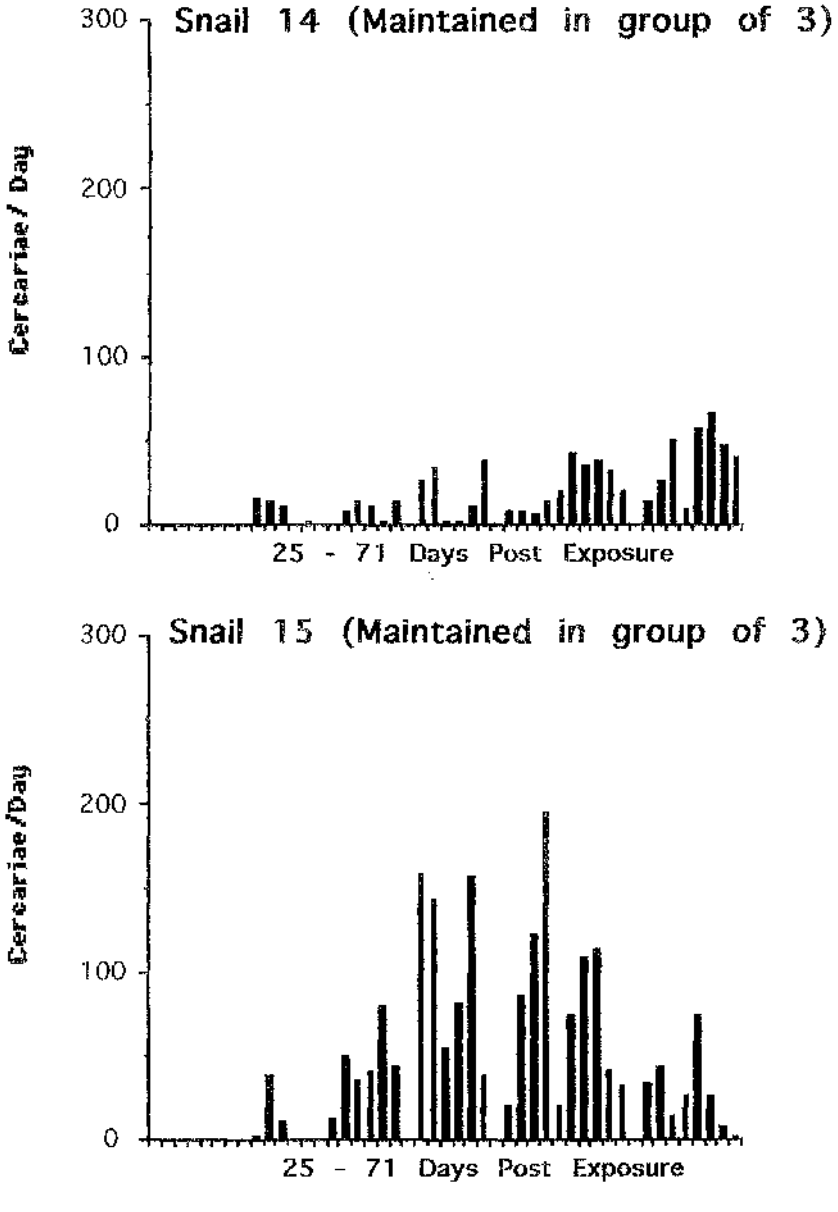
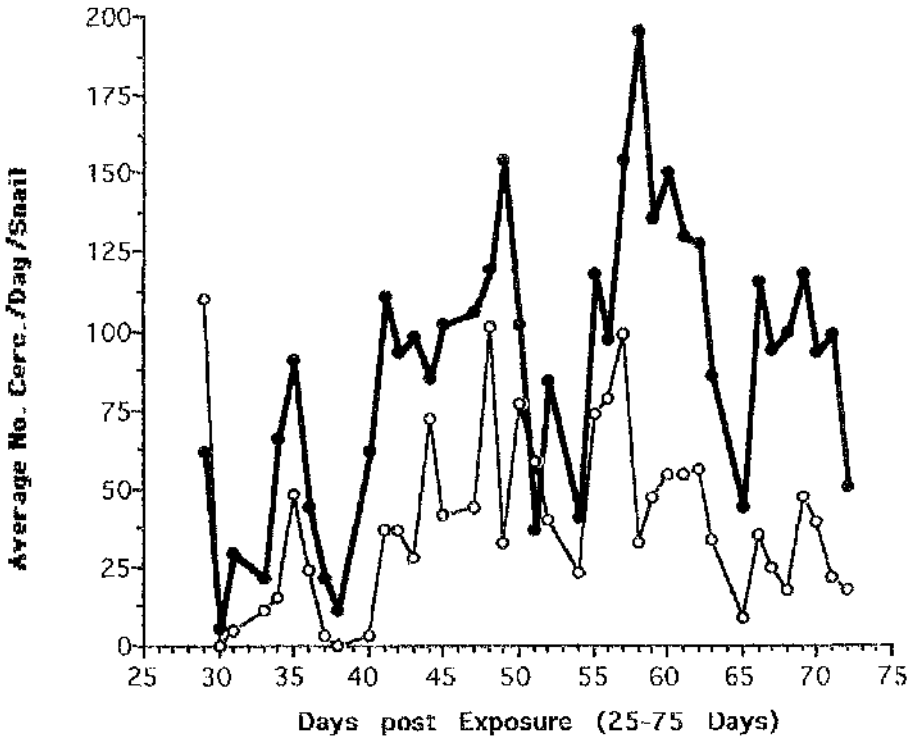


Fig. 3.4.(b) Cercariae emitted per day from 2 groups of 3 snails, each maintained in 50 ml of water in dark conditions. Each group consisted of 1 parasitised and 2 non-parasitised snails.

Fig. 3.5. Average number of cercariae emitted per day from snails maintained in a 12HL/12HD or a dark regime.



Key

——●—— Average of 11 snails maintained in a 12HD/12HL regime.

——o—— Average of 5 snails maintained in dark beakers in a dark cupboard..

3.3. Cercarial production from snails maintained in 12HL/12HD regime. Maintenance water per snail was a volume of 2 litres at a temperature of 26±1°C.

The experimental procedure to study cercarial production in snails maintained in a larger volume of water is described in Materials and Methods (2.3.). The values recorded in this study, were the number of cercariae emitted over a limited period of three hours and only on three separate occasions.

(i) Number of cercariae. Table 3.2. indicates that, although the values do not represent the total daily emission, on the occasions that cercariae were counted, emission was much higher than that recorded in the previous study (Chapter 3.2). The snails with lower emissions died after the second count. Although these counts were sporadic rather than sequential, as in study 3.2., the values of, for example 575, 778, 572 and 863 far exceeded the highest daily value of 309 cercariae recorded for Snail 1 on day 34 PE in the previous study (Fig. 3.2.)

3.4. Discussion

It should be emphasised that the daily production was monitored by collecting the water from the snails at the same time each morning and that the water contained cercariae shed in the previous 24 hour period. The circadian cycle was not being studied.

(i) Prepatent period. Under our experimental conditions, the length of the prepatent period of the parasite *S. mansoni*, developing in *B. glabrata* snails, appeared to be unaffected by maintenance in the dark. Both groups of snails, 12HL/12HD and dark maintained, had parasites which appeared at the same stage of development when snails were examined microscopically on day 27PE. The emergence of cercariae on day 28PE in both groups, may be evidence that prepatent development of the parasite is not exclusively light dependent.

Table 3.2. *S.mansoni* cercariae shed from *B.glabrata* snails maintained individually in 2 litres water.

Snail	10th. Jan.	26th. Jan.	29th. Jan.
1	44	7	Died
2	345	87	Died
3	57	575	778
4	267	572	156 *
5	298	369 **	863

* Snail bled between readings.
** Incomplete count, only two one-hour periods instead of three as in others.

Exposure to light during microscopic examination, may have stimulated, in dark maintained snails, emission of cercariae which had matured but remained within the snail. Whereas, snails exposed to 12HL/12HD regime may have been emitting cercariae prior to day 28PE when counting commenced. This would explain why at first dark individually maintained snails shed an average of 110 cercariae compared to the lower average of 62 cercariae from a 12HL/12HD snail. It is of interest that the groups of dark maintained snails did not exhibit this comparatively higher emission of cercariae on the first day of group counting (day 34 PE, Fig 3.4.(b)). On this occasion the dark maintained snails had not been previously examined for patency and consequently were not exposed to the microscope light. This may be supportive evidence that it was the microscope light which stimulated the apparent initial increase in cercarial emission in the individual dark maintained snails (Fig. 3.3.).

The effect of maintaining *B. glabrata* snails, which had been exposed to one miracidium of *S. mansoni* , at two temperatures of 26 and 24°C during the prepatent period has been studied (Théron , 1981). Both groups, in a 12HL/12HD regime, commenced emitting cercariae on day 33 and 32 PE respectively. It therefore appears that the drop in temperature during the prepatent period only, did not affect significantly the prepatent maturation of the parasite. However, Théron observed that in the first two weeks of shedding, the number of cercariae emitted from snails maintained prepatently in the lower temperature of 24°C was reduced by 33%, compared to production of snails in 26°C. It should be noted that the temperature was raised to 26°C once cercarial emission had started, and that the drop in production was temporary.

(ii) Number of cercariae. Individual snails which had received similar exposure and maintenance conditions produced in total, variable

numbers of cercariae. When results are pooled (Fig.3.5) the observed daily variation in cercarial emission is in accordance with the irregular cercarial output reported by Théron (1981), from a group of 10 snails maintained in 2.5 litres of water.

It is of interest that within the same light regime, maintaining snails in groups of three did not appear to have any significant effect on cercarial production (compare Table 3.1.a. and b.). Yield of cercariae was similar whether snails were maintained individually or in groups. Perhaps limited information can be extracted from the dark maintained group of snails since it is deduced that only one snail per group was parasitised. However the apparent reduction in cercarial production between the two light regimes is repeated in the group maintained snails, with the dark snails seemingly emitting fewer cercariae.

The low production, even by our 12HL/12HD snails, of 90±44 cercariae per day per snail (C/D/S) compares unfavourably with the higher 160 C/D/S observed by Théron (1981). Two factors, light intensity and volume of snail maintenance water, are considered which may contribute to the lower yield of cercariae observed in this study.

The light intensity in the present study, measured at the shelf level in the aquarium using a Skye Light meter, is 543 lux. In other studies (Nojima and Sato, 1982 and Théron, 1984) the light intensities measured were 1,500 and 2,000 lux respectively. Clearly the light regime in our system is much less intense than that referred to in these studies and could contribute to the lower yield of cercariae.The light intensity is most probably a critical factor in the snail maintenance and may affect biophysical properties of the snail and / or development of the parasite.

It should be noted however that out with this study, infected snails which are maintained in darkness are exposed to light intensity of 2,066 lux when shedding cercariae.

The volume of snail maintenance water may influence cercarial production. In the present study snails were maintained either individually or in groups of three, in only 50 ml of water whereas in the study of Théron, a group of 10 snails was maintained in 2.5 litres (an equivalent of 250 ml/snail). Supportive evidence for this hypothesis was gained when an alternative counting procedure (Chapter 2.3.ii.) was being developed. Although the light intensity was still 543 lux , much higher emission values of 575 and 778 cercariae were recorded in monomiracidial exposed snails, maintained individually in 2 litres of water (it should be noted that these values represent cercariae emitted over a three hour period only and are not the total daily emission). These snails were used to test the counting method because they were patent at the appropriate time. It is emphasised that this was not a controlled experiment but, the observations were considered relevant to the present study and consequently included. The snails with lower emissions (Table 3.2.) died after the second count and were obviously not so healthy. The cause of death is unknown and may or may not be parasite associated.

The modified method of counting produced a much cleaner preparation and may have resulted in a more accurate numerical assessment. However, the much increased emission of cercariae recorded using this counting method, could be considered a consequence of the snail maintenance. The larger volume of water may be more environmentally conducive to producing healthier snails which can in turn then host more numerous parasites.

Although it is suggested that the maintenance of individual snails in 2 litres of water, as opposed to the smaller volume of 50 ml, caused the increased cercarial production, other factors may have influenced the observed results and are discussed below.

(a) **Monomiracidial exposure.** It has been established (Touassem and Theron, 1989) that no significant difference resulted in cercarial production when B. glabrata was exposed to either one or several miracidia

of *S. rodhaina* . However this result differed from Theron's observations in the *S. mansoni / B. glabrata* combination in which cercarial production was higher for plurimiracidial infections (cited Touassem and Théron, 1989). Consequently we consider we can discount our monomiracidial exposure method as the causative factor in increased cercarial emission.

(b) The **number of snails** observed was limited and no control snails, maintained for example in a smaller volume of 50 ml of water, were included in the study. Nevertheless much higher daily yields of over 500 cercariae were recorded on several occasions (Table 3.2.). An experimental design for future investigations, to test the hypothesis that maintenance of snails in a larger volume of water increases cercarial yield, is described later in the discussion.

(c) The **altered counting** method, involved a different method of harvesting cercariae. Individual snails were exposed in 2ml of water per period of one hour, over a period of three consecutive hours. Could for example, this exposure procedure cause stress related changes within the snail which would result in higher cercarial emergence?

It is perhaps appropriate at this point to consider briefly some of the physiological consequences of parasitism. For example parasitised snails have a significant reduction in blood glucose levels (Michelson and Dubois, 1975) ; depletion of carbohydrate reserves (Mohamed and El Fiki, 1980; Christie *et al* , 1974) and obvious changes in the protein metabolism (Gress and Cheng, 1973; Lee and Cheng, 1972). From various studies on the aerobic and anaerobic metabolism in infected snail hosts, it was concluded in a review by Nabih and Ansary (1992), that both host and parasite have a similar pathway for anaerobic degradation of glucose. Specifically with respect to the present study, Wolmarans (1987), observed no differences in the concentrations of organic acids and calcium in snail haemolymph under different aerating conditions. He also reported that artificial crowding did not initiate anaerobic respiration. He concluded that only a low temperature of

4°C and total absence of oxygen in the water resulted in anaerobic respiration in the snail. We therefore suggest that shedding an individual snail in 2 ml of water for periods of only one hour would not, according to Wolmarans (1987), initiate anaerobic respiration. On this basis, it is concluded that respiratory metabolism was unaffected while shedding individual snails in 2ml of water for periods of one hour.

It is questionable whether any temporary environmental change, caused perhaps by concentration of snail excretory products, even if it resulted in stress to the snail, could stimulate increased cercarial production over a longer period of time in snails which, between counting were maintained individually in the larger 2 litres of water. We consider it unlikely therefore that the altered counting method was responsible for the increased production.

Having discussed possible contributing factors, monomiracidial exposure, limited number of snails and altered method of counting, the increased cercarial production is considered to be due to maintenance of individual snails in 2 litres of water, as opposed to the 50ml in the 71 day study. It should be noted that light intensity was a constant 543 lux, in the aquarium and would not therefore contribute to the observed differences in cercarial production when snails were maintained in a larger volume of water.

It is recognised that other factors influence cercarial production by the snail. A decrease in temperature from 26°C to 24°C during the prepatent period reduces cercarial production by 33% (Théron, 1981). However, temperature also remained constant throughout the present study and can be discounted as a contributing factor.

To resolve the problem, an improved experimental design, where volume of maintenance water is the only variable, is briefly outlined in Fig.3.6. This would indicate the most suitable volume of maintenance water for maximum cercarial production from host snails. Thereafter, the effect of

Fig. 3.6. Experiment to establish if maintaining snails in 2 litre of water increases cercarial production.

1. Expose size and age matched snails to one miracidium in 2ml of water.
2. Maintain snails in batches of 8 per 2.5 litres of water at 26°C in 12HL/12HD until parasitism can be identified.
3. Thereafter divide parasitised snails into two groups.

   Group a) maintain snails individually in 2 litres of water.

   Group b) maintain snails individually in 50 ml of water.
4. Maintain both groups at 26°C in 12HL/12HD.
6. Monitor cercarial production by placing snail in 2 ml of water as described in Chapter 2.3.(ii).

Any differences observed in cercarial production under these conditions could then be confidently attributed to the volume of snail maintenance water.

maintaining snails in a light intensity of 2,000lux, compared to the present 543 lux could be investigated.

(iii) Pattern of emission. It was noteworthy that the pattern (or perhaps more accurately, the irregularity) of emission observed in this 71 day study appeared similar in snails of both 12HL/12HD and dark maintenance regimes. It could be concluded therefore that cercarial emission in the snail was not regulated by prolonged light exposure. The exposure of dark maintained snails to the very low light intensity of 41lux, even for a minimal period when removing cercarial suspensions, appears to have been sufficient to stimulate daily cercarial emission.

The causative agent of the high and low numbers in daily cercarial emissions may be unknown, but it is extremely interesting that both the 12HL/12HD and dark maintained snails exhibited corresponding peaks and troughs . It is probable, as previously suggested by Théron (1981), that the daily irregularities are a consequence of some developmental stage of the parasite and reflect the maturation process of the cercariae. If this be so, our results may be further evidence that the rhythm of cercarial production (not to confused with circadian rhythm) is not solely light dependent, but a process that once triggered, perhaps by some snail component, is a continuous cycle. In this 71 day study we cannot confirm the interesting circamensual rhythm, of 35 days periodicity, reported by Théron (1981).

An intriguing question is, given it is postulated that the rhythm of cercarial production is not light regulated, but a consequence of "the dynamics of the intramolluscan larval stages of the parasite during the course of infection" Théron and Moné (1984), why then do snails maintained in the dark produce fewer cercariae? Perhaps the minimal exposure to 41 lux light is sufficient to stimulate emission, but inadequate for some other requirement of cercarial production. In the introduction the importance of the snail in the parasite / host relationship was emphasised. The reduced yield of cercariae

from dark maintained snails may indicate deficiencies in the snail. Snails maintained in such a low light regime, and so unrelated to conditions of the field, could be lacking some factor vital to the schistosome.

Another scenario is that lack of a prolonged photoperiod may have some adverse effect on a biochemical or physiological process affecting cercariogenesis. Prolonged light of intensity 543 lux, may initially stimulate development and/or emission of cercariae to the extent that sporogenesis occurs in sporocysts previously producing cercariae. If so, a cascade of sporogenesis would result in higher yields of cercariae. This speculation could be tested by microscopic examination of parasitised snails from both light regimes to count the stages of sporocysts present in the tissues.

Although the main factor being investigated was light, other agents which may affect cercarial production were alluded to. The influence of temperature and volume of maintenance water on cercarial yield were considered. In the introduction the importance of calcium in the host - parasite relationship was discussed [Ch. 1.3.(ii)]. It has been observed that calcium levels of snail maintenance water can affect cercarial emergence (Mishkin and Jokinen 1986). The calcium concentrations in the DCTW in the present study may consequently be of relevance and are investigated later in this study. The possible contribution, of the maintenance water, to cercarial production will be considered in Chapter 6.

The findings of this study have indicated that the routine method of snail maintenance may not yield the full potential of cercarial numbers from the parasitised snails.That snails in both light regimes exhibited a similar pattern of emission was a most interesting observation, lending support to the theory that cercarial production rhythms are not dependent solely on a 12 hour photoperiod. The results suggest that cercarial production is dependent on both exogenous and endogenous factors. The results are summarised on the following page.

3.5. Summary

The following conclusions are drawn from the results of this study.

(i) The total production of *Schistosoma mansoni* cercariae varied in individual *Biomphalaria glabrata* snails.

(ii) The daily emission of cercariae from individual snails was irregular.

(iii) The "pattern" of emission, over the 71 day period, was similar in snails maintained in both a) 12HL/12HD and b) Dark maintenance regimes.

(iv) However 12HL/12HD snails consistently produced more cercariae than dark maintained snails.

(v) 12HL/12HD snails maintained individually in a larger volume of water exhibited a much higher yield of cercariae.

(vi) The light intensity of 543 lux is lower than what may be optimum conditions for cercarial production.

(vii) Momentary exposure, once per day, to low light intensity of 41 lux appears sufficient to stimulate cercarial emission.

It is concluded that dark maintained snails were slightly disadvantaged in their ability to produce cercariae. Production would be enhanced in our laboratory if snails were maintained in 12HL/12HD and only placed in darkness for a short period of 48 hours prior to shedding.The possibility that increased light intensity and a larger volume of maintenance water may enhance cercarial production requires further investigation.

CHAPTER 4

Assessment of calcium in preacetabular glands of *S. mansoni* cercariae by Fluorescent Emission Quantitation of Alizarin Red S.

4.1. Introduction

The importance of calcium in the *B. glabrata/S. mansoni* relationship has already been discussed in Chapter 1.3.(ii). It has also been established in the literature that calcium of the preacetabular glands is a prerequisite for successful penetration of the vertebrate host (Lewert and Hopkins, 1964). To our knowledge, the possibility that different amounts of calcium in the preacetabular gland could contribute to cercarial variation in infectivity has not been addressed. A reliable method of assessing calcium in the glands, which would allow comparisons between individual cercariae, would be a useful asset in such an investigation.

The calcium in the preacetabular glands of *S. mansoni* cercariae is in carbonate form. Previously it has been shown that the vital stain Alizarin Red S (sodium alizarin monosulfonate, ARS), has an affinity for calcium, forming insoluble lakes with the calcium within the preacetabular glands which stain red (Lewert, Hopkins and Mandlowitz, 1966). It is emphasised that, both in this and subsequent chapters, the calcium stained with ARS and measured using both Plasma Emission Spectrometry and Atomic Absorption Spectrometry, is free calcium ions. The calcium will then be referred to as calcium ions or Ca^{2+}.

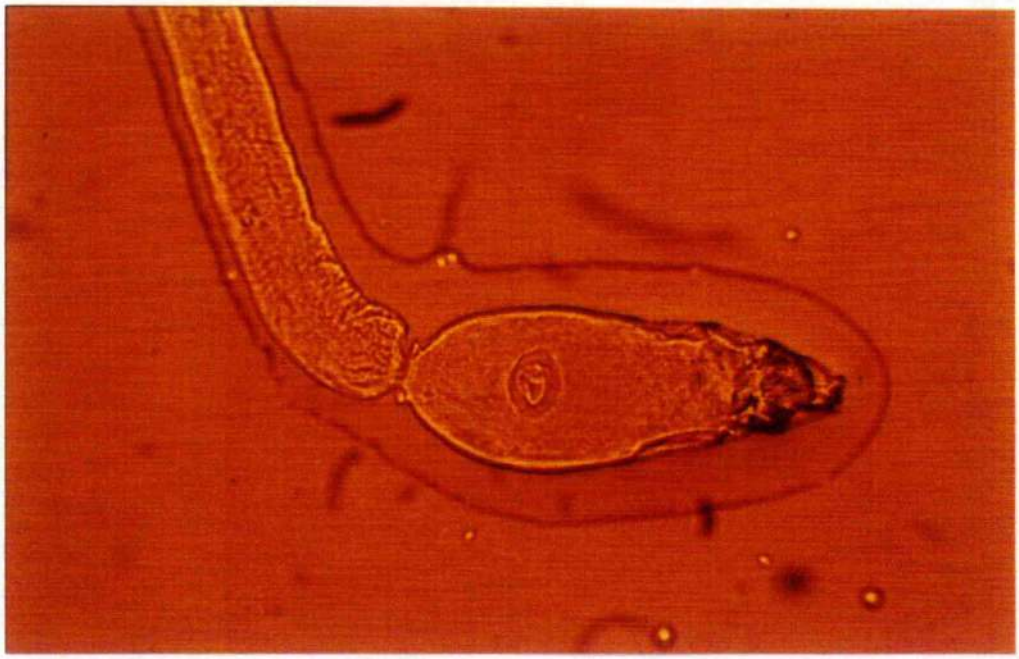
The aim of the present study was initially to determine the potential of Alizarin Red S as an indicating agent in fluorescent quantitation studies, and then to develop a protocol for measuring fluorescent emission as an indicator of calcium levels in preacetabular glands of cercariae.

4.2. Properties of Alizarin Red S.

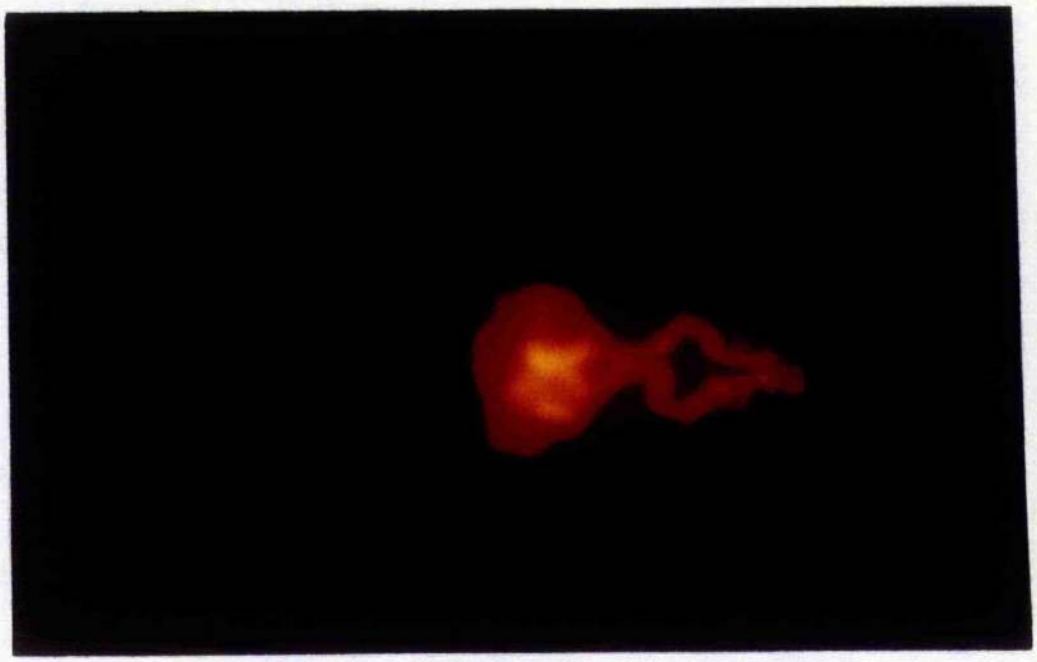
When stained with ARS, the preacetabular glands of *S. mansoni* emit a red fluorescence which can be observed microscopically using a Rhodamine filter (Fig. 4.1.). To confirm that there was a correlation between the fluorescent value and amount of calcium present, it was necessary to test standard solutions of known calcium concentration to prepare a standard curve. To use

Fig. 4.1 Alizarin Red S stained *S.mansoni* cercaria.

(a) Bright field exposure. (——— Represents 35.6 μm).



(b) Dark field exposure.



the fluorescence spectrometer for this purpose, the excitation / emission spectrum for ARS was required.

(i) Emission spectrum for Alizarin Red S. To determine the emission spectrum, 5µl of 2% ARS was added to 10 ml of 0.5% $CaCl_2$ and processed. The equipment used is detailed in Chapter 2. The spectrum (Fig 4.2.), indicated that for the preparation of the standard curve, with Alizarin Red S and Calcium, the appropriate excitation and emission settings were 470 and 588nm respectively.

(ii) Alizarin Red S / Calcium standard curve. For this analysis several dilutions of $CaCl_2$ (0.1, 0.25, 0.5, 1, 2 and 3%), were processed together with a blank distilled water sample which acted as a control. Each cuvette containing the appropriate test solution, had 5µl of 2% ARS added prior to processing. As can be seen in Fig. 4.3. there appears to be a positive linear relationship between the amount of calcium present and fluorescence recorded. A sample of dechlorinated tap water was also processed giving an emission value of 7.7, indicating that, as expected, there is more calcium present in the dechlorinated tap water than in the distilled water (emission value of distilled water was 1.8, Fig. 4.3.).

4.3. Development of Quantitation Protocol

(i) Alizarin Red S stained cercariae of *S. mansoni*. Initial observations of cercariae stained with 2% Alizarin Red S (ARS) revealed, under bright field microscopic examination, heavily stained preacetabular glands together with a dark exudate from two duct openings at the tip of the cercarial head. It was of interest that, when viewed using a Rhodamine filter, although red fluorescence of the glands was clearly observed, under these conditions the exudate could not be seen. The heavy staining and production of exudate suggested that the stain required further dilution. It was also apparent that the staining was time dependent and that over exposure

Fig. 4.2. Emission spectrum for Alizarin Red S.

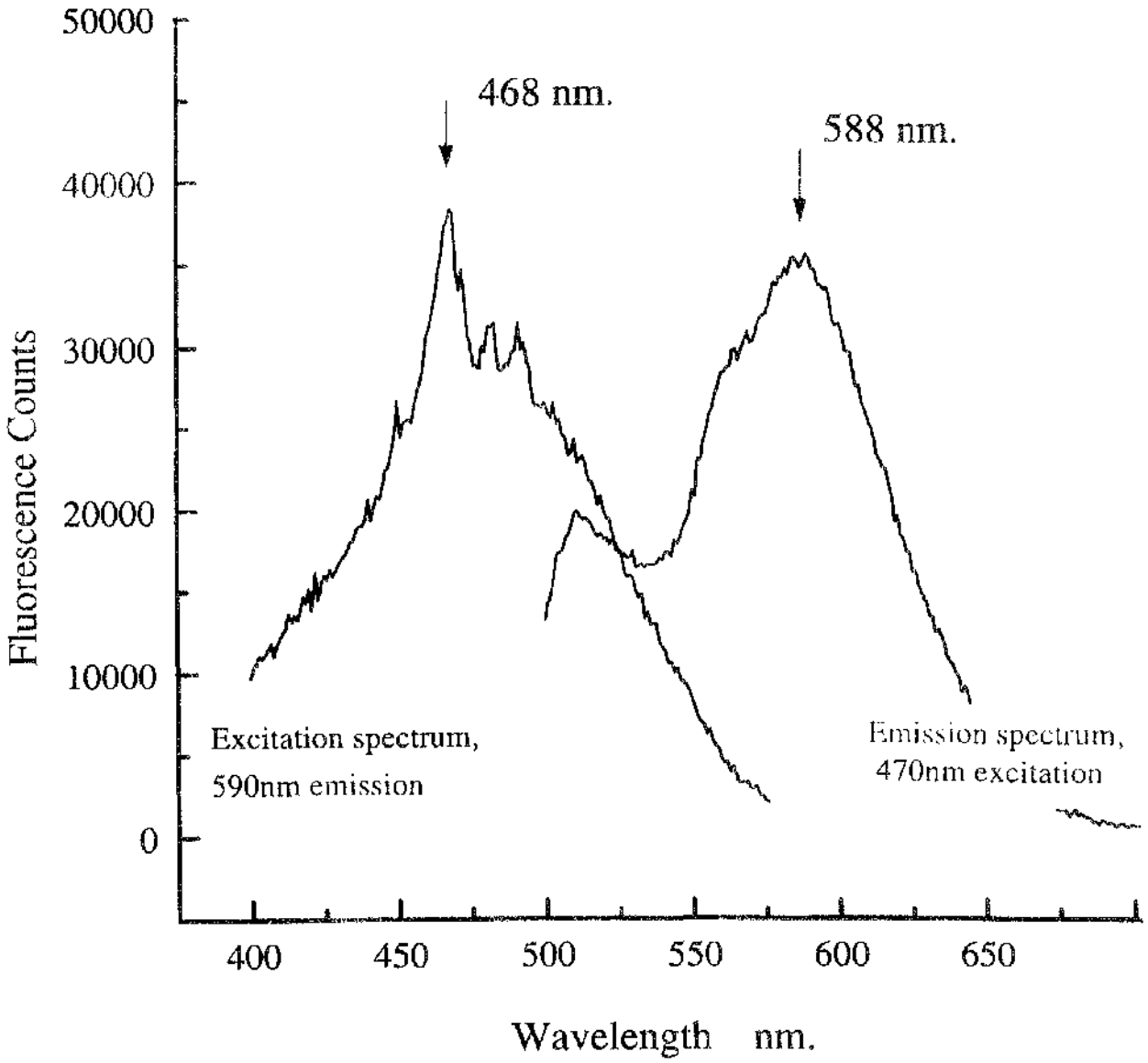
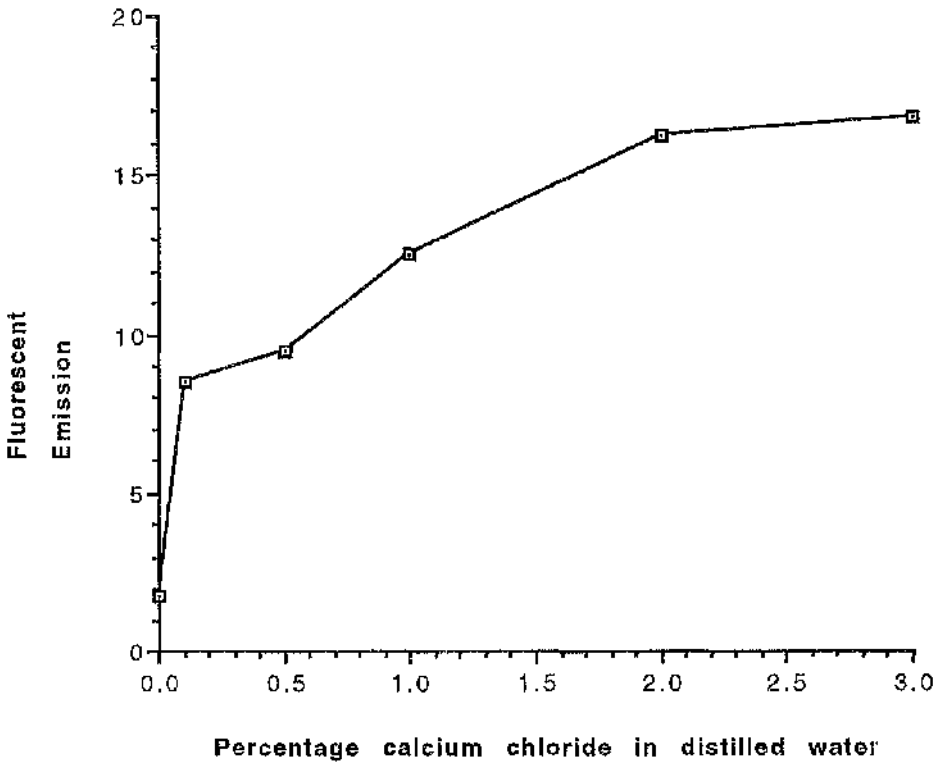


Fig 4.3. Standard Curve for Alizarin Red S/Calcium



Note : 5µl of 2% Alizarin Red S was added to each cuvette containing appropriate test solution, and emission was recorded.

resulted in dark, almost purple staining which made the cercariae unsuitable for fluorescent quantitation.

A range of stain dilutions and exposure times were investigated to determine the most suitable method of preparing stained cercariae for fluorescent quantitation. A concentration of 0.001% ARS, exposure time of 10 mins, followed by 4 washes in cooled declorinated tap water (DCTW is the maintenance medium for our snails) , was selected as an appropriate protocol since it resulted in clearly defined fluorescent glands and ducts, with no granulation in the body or tail of the cercariae. Material prepared by this method was suitable for fluorescent quantitation.

(ii) Carbachol immobilisation of ARS stained cercariae. Two immobilising agents, formaldehyde and carbachol, were tested for suitability. Carbachol (10mg/ml distilled water), was the more effective since it conserved the integrity of the stain and allowed cold storage of stained material.

The stained and immobilised samples were covered in aluminium foil to minimise deterioration of the stain by light. If necessary samples could be quantified on the day after preparation as cercariae were still alive with clearly defined glands and ducts. It should be noted however that in the experiments described in section 4.4., quantitation was carried out on the day of shedding the cercariae.

Longer cold storage of stained cercariae, 36 hours, had detrimental effect on the quality of material. Clear organelles could be recognised under bright field observations with some fluorescence in the gland area when the green filter was used. However the body and tail of the cercariae were now exhibiting a granular fluorescence. The material was now unsuitable for quantitation.

It is noteworthy that variation in cercarial appearance and behaviour was sometimes observed after staining and immobilisation. Cercariae appeared

Cercarial clone. Cercariae harvested from a single snail which has previously been exposed to only one miracidium are termed clones. Since these cercariae are the product of one asexually dividing miracidium they are considered to be genetically identical.

either "smooth" or "wrinkled" . Both types were alive and fluorescence could be quantified. No significant difference was observed in the fluorescent emission from both types. However when tested, the sample size of wrinkled was very small compared to that of smooth cercariae, consequently no reliable conclusions could be made from the data. Further investigations are required to elucidate the significance, if any, of this difference in cercarial appearance.

It was also apparent that individual cercariae responded differently to carbachol immobilisation. Some cercariae resisted carbachol immobilisation and remained active for much longer periods.

(iii) Fluorescent quantitation. During these preliminary studies, before a standard protocol was finally developed, encouraging results were obtained which illustrated that the method could identify varying concentrations of calcium ions in the preacetabular glands of cercariae.

In Fig.4.4., graphs are plotted showing the fluorescence emitted from clones of cercariae, shed from snails on three different occasions. It should be noted that the scale, on the y-axis, of the fluorescent emission is arbitrary and that comparisons cannot be made between these experiments which were performed on different days. These preliminary results simply indicate the sensitivity of the quantitation process. Variation of fluorescent emission from the preacetabular gland is detectable within and between clones of cercariae. This variation in fluorescent emission is interpreted as an indication of different concentrations of calcium ions in the preacetabular glands.

The protocol, which was finally adopted, is summarised in the flow diagram in Fig. 4.5., and it was used in the studies described in the following three sections.

Fig. 4.4. Fluorescent emission from ARS stained *S.mansoni* clones of cercariae.

Key ○ Fluorescent emission value from one cercaria of clone 3 .

● Fluorescent emission value from one cercaria of clone 6.

Note The scatter graphs on the opposite page illustrate differences in fluorescent emission between the two clones. On all three occasions the difference was significant ($P < 0.001$, Student's t-test).

However it should be emphasised that these results were obtained in experiments performed while developing a protocol to quantitate fluorescence. Although the concentration of ARS was the same as that in the final protocol (0.001%), the incubation period was 15 mins in Ex. 3 and 9, but 25 mins in Ex. 4.
The adopted protocol outlined in Fig. 4.5. has a defined incubation time of 10 mins.

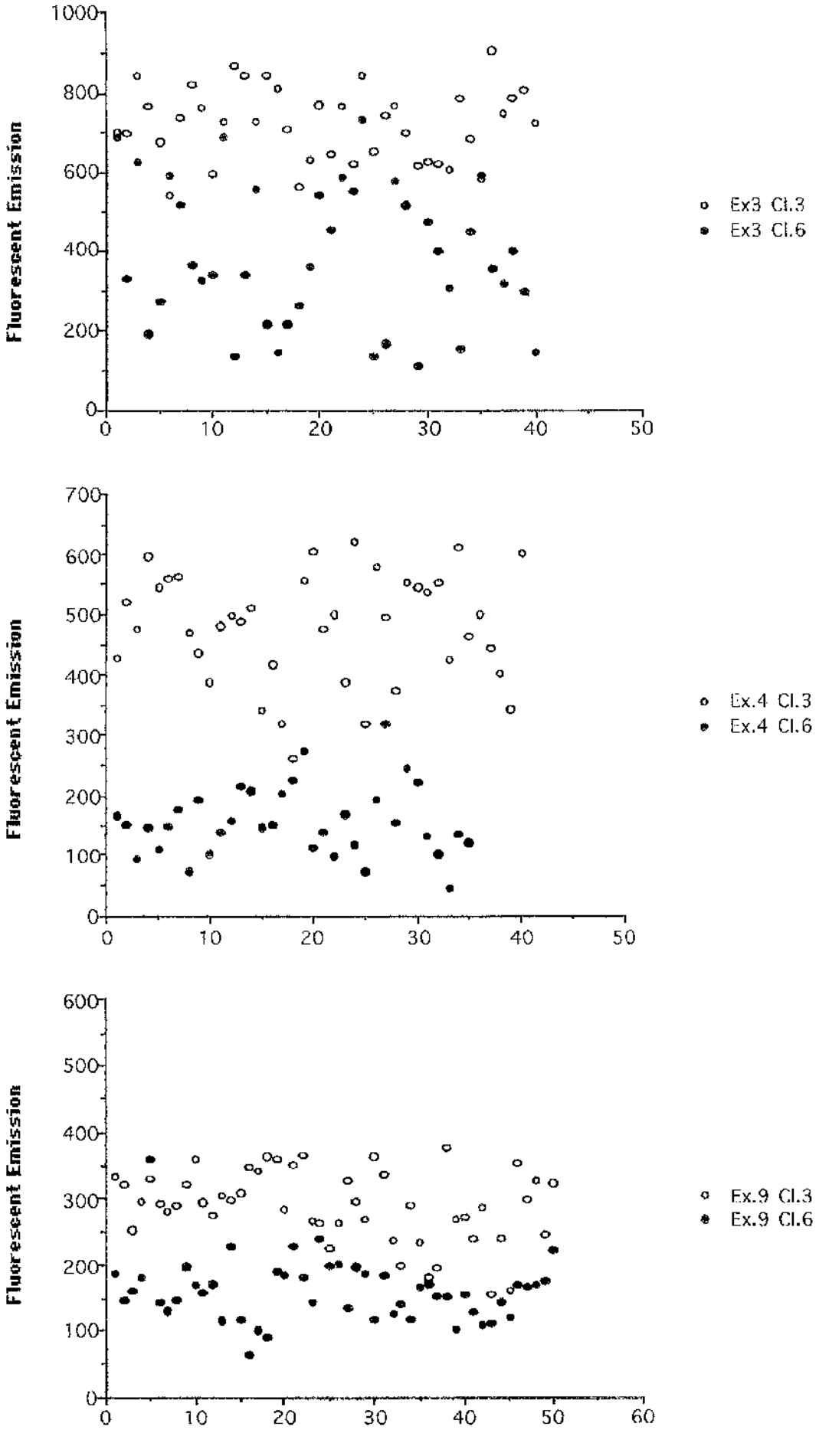
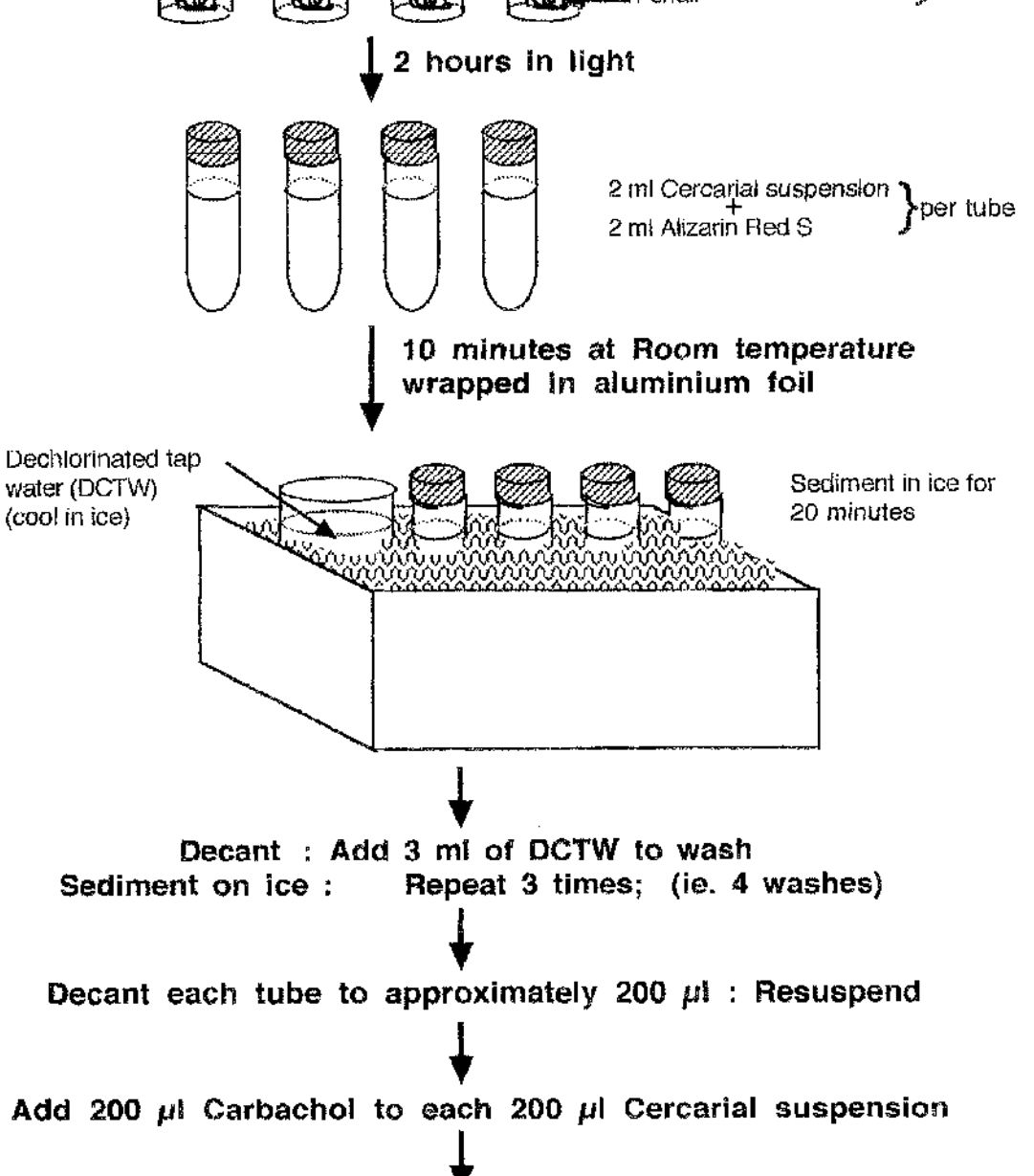

Fluorescent Emission
1000
800
600
400
200
0
0
10
20
30
40
50
Ex3 Cl.3
Ex3 Cl.6
Fluorescent Emission
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Ex.4 Cl.3
Ex.4 Cl.6
Fluorescent Emission
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Ex.9 Cl.3
Ex.9 Cl.6
Number of cercariae measured

Fig 4.5. Protocol to quantify fluorescence.



4.4. Studies using the Quantitation Protocol.

(i) Cercarial sample number. It was necessary to test that a sample size of 60 cercariae was sufficient to represent accurately a population of shed cercariae. A batch of cercariae harvested from a pool of snails was divided into two aliquots and processed simultaneously. The fluorescent emissions, when plotted as a scatter graph together with a simple curve fit, appear very similar (fig.4.6.) Processing of the data showed that there was no significant difference in fluorescence emitted from the two samples ($P>0.05$, Student's t-test.).

This result illustrated again (compare Fig.4.4.) the variability in fluorescent emission between individual cercariae. However it also indicated, since there was no significant difference in the mean fluorescent emission values between the two aliquots ($P>0.05$ Student's t-test), that the sample size of 60 cercariae was representative of the cercarial population.

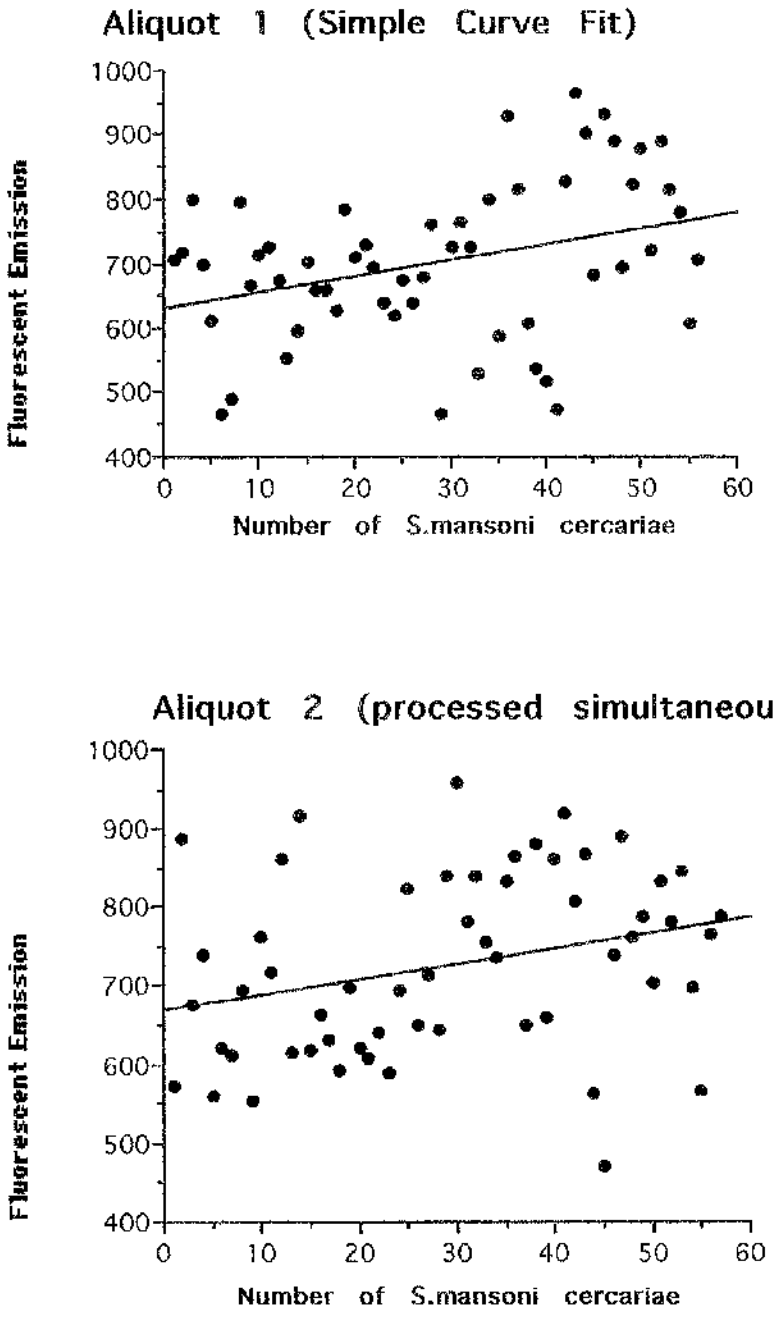
(ii) Aging of cercariae. To test the hypothesis that aged cercariae would have less calcium ions in the preacetabular glands than those newly shed, a batch of cercariae was divided into two aliquots. The first aliquot was processed immediately after collection for fluorescent emission. Meanwhile the second aliquot was stored in the light at room temperature for a period of 4 hrs 20 mins, before being processed.

The resulting fluorescent emissions (Fig. 4.7.), were significantly lower in the second aliquot ($P<0.001$ Student's t-test). This result of decreased fluorescent emission after a period of time was repeated in further investigations (Table 4.1.).

4.5. A study of four snails over a period of seven weeks.

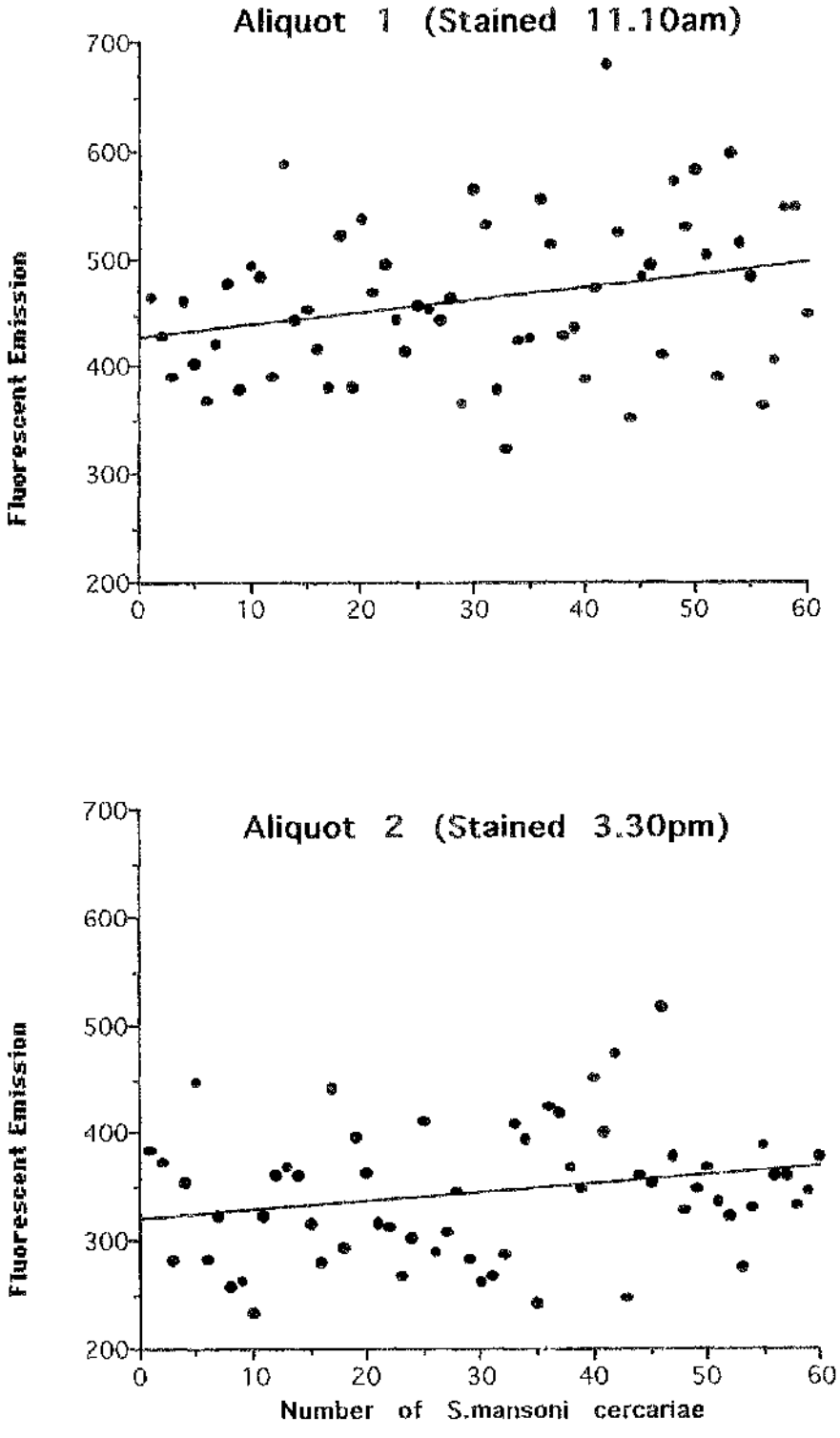
In this study the relationship between duration of patency of the infection in the snail and the calcium ion levels in the cercariae was considered. The

Fig. 4.6. **Fluorescent emission from two aliquots of a cercarial suspension.**



Note. The difference in fluorescent emission between Aliquot 1 and 2 is not significant. $P>0.05$.

Fig. 4.7. Fluorescent emission from two cercarial aliquots processed 4 hours apart.



Note. The difference in fluorescent emission between Aliquot 1 and 2 is significant. $P<0.001$.

Table 4.1 **Mean fluorescent emission from *S.mansoni* cercariae (± Standard deviation).**

	Time 0		Time 3hr 30 min	
	No. of cercariae	Fluorescence	No. of cercariae	Fluorescence
Study A	64	574 ± 106	72	512 ± 67
Study B	58	499 ± 72	64	418 ± 88

Note : Study A - snails were shed in dechlorinated water

Study B - snails were shed in distilled water

hypothesis that a more mature patency would result in cercariae with lower levels of calcium ions in the preacetabular glands was addressed.

To this end four snails were selected from two groups of snails which were at different stages of patency. The dates 19.5.95 and 23.8.95, are the projected dates of parasite maturation in snails post miracidium exposure and serve as a labelling system for infected snails.The first pair, identified as 19.5.95 snails, had been shedding cercariae for approximately thirteen weeks prior to selection. The other pair, designated 23.8.95 snails, were from a newly patent group having shed cercariae for only two weeks. Each pair of snails comprised a "healthy" red snail together with a pale one. Pale colour in a snail may be associated with either a very old non parasitised snail or a parasitised snail which is approaching death. Throughout the study the snails were maintained individually in a 12HL/12HD regime.

The protocol (Fig.4.5.), for quantitation of fluorescent emission from Alizarin Red S stained cercariae, was carried out on six different occasions. It should be emphasised that the microscope requires standardisation on each day of use. Although throughout this study, cercariae from snail 23.8.95 were used to standardise the microscope, direct comparisons could not be made between arbitrary data measured on different days.

To overcome this problem, the following adjustments were made which facilitated presentation of the results. As already mentioned cercariae from snail 23.8.95 were always used to standardise the microscope. The mean fluorescent value, calculated from observations of 60 cercariae from the 23.8.95 snail, was for presentation purposes taken as being 100%. Fluorescence from cercariae of the other three snails is represented relative to the 23.8.95 cercariae. Consequently the mean fluorescence of cercariae from snails 23.8.95 pale, 19.5.95 large and 19.5.95 pale is plotted as a percentage of the mean of 23.8.95 cercariae.

The results of three experiments are presented as column graphs in Fig. 4.8. Although performed on different days, and not directly comparable, they are selected because their fluorescent emission values fall within the same scale. They are included only to illustrate the standard deviation levels obtained throughout the present study

(i) Results The results of the seven week study of cercariae from four individual snails are presented as column graphs in Fig. 4.9.. The results indicated that at each investigation, the mean fluorescent emission was always highest in cercariae from the large 19.5.95 snail. The pale 19.5.95, on the three occasions recorded, produced cercariae which emitted a lower fluorescence than its contemporary healthier looking 19.5.95 snail.

The two newly patent 23.8.95 snails, produced cercariae which consistently emitted a lower fluorescence than the cercariae from the 19.5.95 large snail which had been shedding for a significantly longer period. There was no significant difference in the fluorescence emitted from cercariae shed from the two 23.8.94 snails throughout the study.

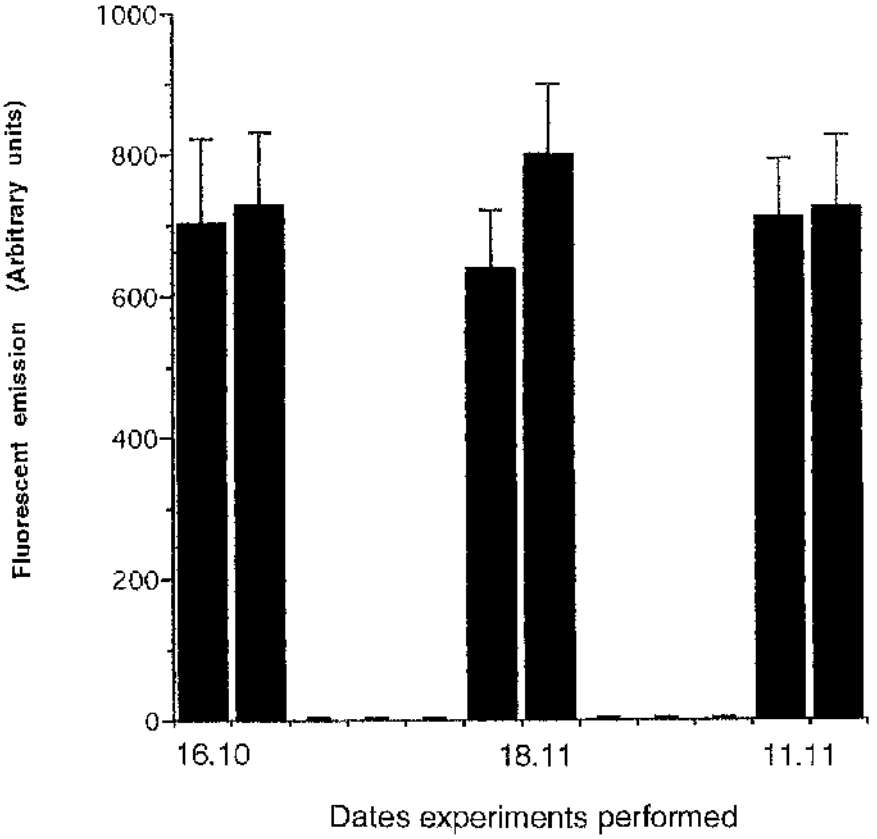
The pale snails from both pairs of snails died during the period of study.

4.6. Effect of distilled water on calcium in the preacetabular glands.

Distilled water has been recognised as an unsuitable exposure medium for successful parasite penetration (Stirewalt and Fregeau, 1965). The following study was undertaken to test the hypothesis that the calcium of the preacetabular glands was affected by cercarial exposure to distilled water.

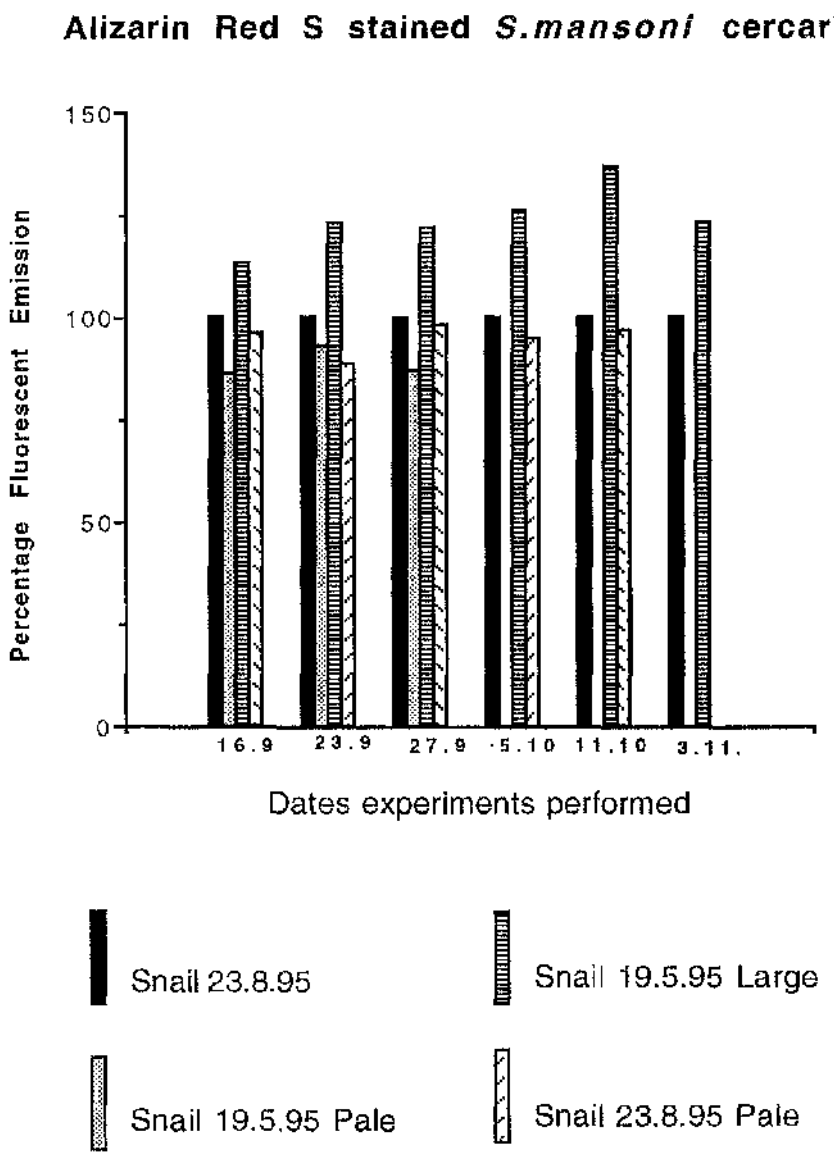
The two surviving snails from the above study were again monitored for fluorescent emission using the same quantitation protocol. In the five studies presented, snail 23.8.95 was always shed in dechlorinated tap water (DCTW). Concurrently snail 19.5.95 was only shed three times in DCTW. In the other

Fig. 4.8. Fluorescent emission from Alizarin Red S cercariae.



Key. Each column is the average of a minimum of 60 cercariae
T-bar ± Standard deviation

Fig. 4.9. A time course study of cercariae from four snails.



two studies it was shed in distilled water. Between experiments both snails were maintained individually in DCTW in a 12HL/12HD regime.

(i) Results. As seen in Fig. 4.10., on the two occasions that snail 19.5.95 was shed in distilled water the cercariae emitted a fluorescence which was similar to that of the 23.8.95 cercariae. In contrast when DCTW was the shedding medium, 19.5.95 cercariae were again, as in the previous study, emitting a mean fluorescence higher than that of 23.8.95 cercariae.

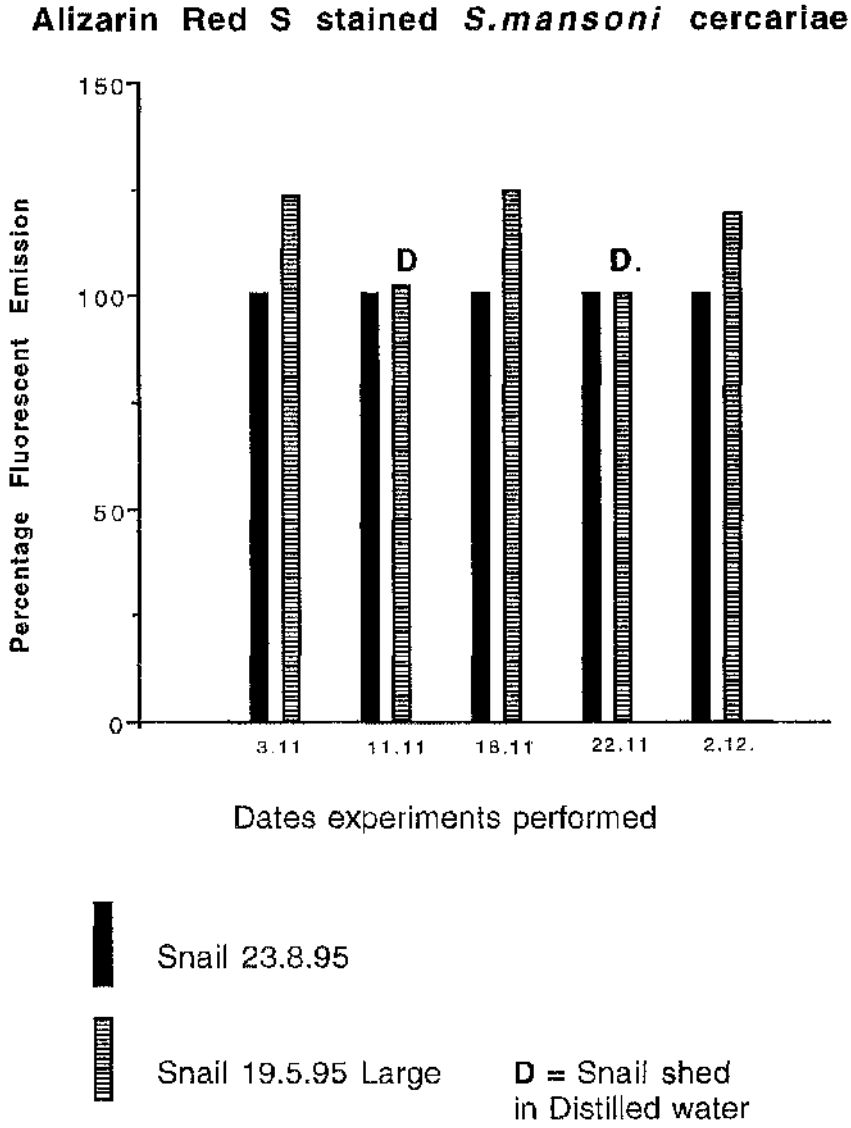
4.7. Discussion.

(I) Initial observations. This study has shown that Alizarin Red S, at a low concentration of 0.001%, is a suitable agent to quantify fluorescent emission from stained cercariae. In the preliminary studies, while developing the quantitation protocol, cercariae stained with ARS exhibited variability in the fluorescent emission from the preacetabular glands. This was interpreted as indicating varying amounts of calcium ions within each gland.

It is noteworthy that variability was also observed in some aspects of cercarial responses. For example when sedimenting cercariae on ice, some cercariae were capable of resisting the cold exposure and remained swimming for a longer period before becoming inactive and sinking to the bottom in the tube. Similarly when immobilising cercariae with carbachol, some cercariae resisted longer than others before eventually becoming motionless.

The possibility that these differences, in observed cercarial behaviour, could be related to cercarial age was considered. Snails maintained in a 12HL/12HD regime produce and shed cercariae on a daily rhythm. In laboratory maintained snails it is common practise, in order to maximise cercarial numbers, to subject parasitised snails to a period of prolonged darkness prior to shedding. That this method is universally employed implies

Fig. 4. 10. A study comparing cercariae harvested in distilled and dechlorinated tap water.



that some degree of "storing" of cercariae occurs in the snail. Cercariae shed from such snails may include a considerable range of cercarial ages. Cercariae collected under these circumstances could theoretically range from approximately three hours to several days of age depending on the time kept in the dark before shedding.

To overcome this possible age related effect, the snails in the studies 4.5. and 4.6. were maintained throughout the period of experimentation in a 12HL/12HD regime, and the cercariae were collected only over a period of two hours during the light period.

(ii) Aging of cercariae. The results (Fig. 4.7. and Table 4.1.) suggest that free swimming cercariae lose calcium ions from their preacetabular glands. The apparent loss of calcium ions was observed in cercariae from snails of both maintenance regimes, DCTW and distilled water. That the cercariae from 12HL/12HD snails, shed over a short two hour period, exhibited a loss of calcium ions suggests that "young" newly shed cercariae are losing calcium. Since the 12HL/12HD maintenance regime is similar to that of natural habitats, a possibility exists that this loss of calcium will occur in natural environments.

(iii) A study of four snails over a period of seven weeks. In this section comparisons are made firstly, between cercariae from a mature infection with those from a newly patent infection. Secondly, cercariae from a healthy snail are compared with cercariae from a pale snail. Thirdly, conjectural comparisons are made between cercariae from the same snail, but at different periods in patency.

Mature verses newly patent infection: The fluorescent emission results obtained over a seven week period (Fig.4.9.), indicated that the emission from

19.5.95 cercariae was always the highest. This is interpreted as evidence that the mean calcium ion levels in the preacetabular glands of these cercariae was consistently higher than that of cercariae from a more recent infection. This result is in conflict with the hypothesis that a more mature patency would result in cercariae with less calcium than cercariae shed from a recently patent snail.

Consider the history of the two pairs of snails. Snails 19.5.95 had been shedding for thirteen weeks prior to experimentation; snails 23.8.95 for only two weeks. The relevant factor here is that snail 19.5.95 had been providing both nutritional and calcium requirements for its parasite population for a considerably longer time. It has previously been shown that within six days of exposure to four miracidia of *S. mansoni*, the host snail had depleted calcium reserves (Shaw and Erasmus, 1987). Damage to Type A calcium storage cells, which are found in the connective tissue of the snail digestive gland, together with pitting of the nacreous layer of the inner shell was also observed at this period by these researchers. Clearly very soon after parasite exposure the metabolism of the host snail is affected with resulting adverse effects on calcium reserves. The physical trauma to host snail tissue cannot be discounted, sometimes cercarial suspensions can be coloured due to leakage of snail haemolymph during shedding (personal observations).

Despite these obvious disadvantages to the snail, the cercariae from one of the longest shedding snails, 19.5.95, appeared always to contain the highest mean levels of calcium ions in their preacetabular glands. This result could be interpreted as evidence that calcium availability is related to the individual snail only and is independent of the duration of patency.

It has been suggested above that the snail alone is responsible for the observed calcium ion levels in the test cercariae. It is a fact that the cercariae accumulate their calcium ions while still within the daughter sporocysts in the host tissue (Davies, 1983). However the cercariae, within the daughter

sporocyst, have to remove the calcium from the surrounding snail tissue and haemolymph, and store it in their glands. Thus, although the snail provides the supply of calcium, the cercariae is required to process it.

The possibility exists that 19.5.95 cercariae were better adapted to utilise the available source of calcium than the cercariae in the other three snails. Within the snail, parasite reproduction is asexual with cercariae being produced continuously by mitotic division only. Consequently, if cercariae were advantage in their ability to sequestrate calcium, then this characteristic could be a genetic characteristic exhibited consistently throughout patency.

Healthy verses pale snail: The hypothesis being tested here was that pale snails would produce cercariae which had less calcium ions than cercariae from healthy snails. As already mentioned, cercariae from the healthy 19.5.95 snail shed cercariae which had higher levels of calcium ions than cercariae from the pale 19.5.95 snail. This result supported the hypothesis. However a conflicting result is obtained when comparisons are made between the healthy and pale snail in the 23.8.95 pair. In the newly patent pair of snails, there was no difference in the calcium ion levels in the cercariae. It is therefore concluded that pale snails do not necessarily produce cercariae with significantly low levels of calcium ions in their preacetabular glands.

It is accepted that paleness may not be associated with reduced calcium ions in the snail, but it is relevant that the two pale snails died during this study. It supports the view that the pale snails were "unhealthy" and in fact dying. Nonetheless calcium ion levels did not differ in cercariae from the two 23.8.95 snails. This may be confirmation that calcium sequestration is parasite related as suggested above and further evidence of the parasite's ability to optimise available resources in its host.

Stage of patency: The hypothesis that cercariae produced in early infection will have more calcium ions in their preacetabular glands than cercariae shed at a later period of infection was now addressed. Because the microscope has to be standardised each day for fluorescent emission quantitation, a time course study of alizarin stained cercariae from one snail throughout patency was unachievable. An alternative approach to assess what was happening over a period of time was attempted as follows.

Focusing attention on the two healthy snails only, examination of the data (Fig.4.9.) suggests that the relative difference between fluorescent emission from 19.5.95 and 23.8.95 cercariae remained similar over the period studied. The apparent consistent relationship between calcium ion levels in cercariae from both snails can be interpreted in two ways.

It is speculated that either, (a) the levels of calcium ions in the glands of cercariae from both snails did not change dramatically over the period studied or, (b) the levels of calcium ions were changing, but at similar rates in the glands of cercariae from both snails over the period studied.

The suggestion that calcium ion levels did not dramatically change in the cercariae is considered the more likely explanation. The alternative scenario (b), that levels of calcium ions were changing, and it must be emphasised, at similar rates seems a less acceptable explanation as it would require a degree of synchronisation between parasites derived from different snails at such unmatched stages of patency.

From the available data, it seems more reasonable to suggest that calcium ion levels in the preacetabular glands of cercariae from an individual snail no not decline significantly as patency matures.

(iv) Effect of distilled water on calcium in the preacetabular glands. As detailed in the previous section, 19.5.95 cercariae had on six occasions exhibited the higher levels of calcium ions in their preacetabular

glands when compared with 23.8.95 cercariae (Fig.4.9.). However on two subsequent and intermittent occasions, when snail 19.5.95 was shed in distilled water, the results indicated (Fig.4.10.) that the calcium ion levels were reduced to levels similar to those of 23.8.95 cercariae which were harvested in dechlorinated tap water. The calcium ion content in 19.5.95 cercariae rose, above that of the 23.8.95 cercariae, when the 19.5.95 snail was again shed in dechlorinated tap water.

Fusco *et al* (1991) demonstrated that cercariae, preloaded with $^{45}Ca^{2+}$, exhibited a significant efflux of calcium after 20 mins in calcium free buffer. The result in the present study, of apparent decrease in acetabular calcium ions, may be evidence that an efflux of acetabular calcium is occurring in distilled water. The results appear to confirm the hypothesis that calcium ion levels are reduced in the preacetabular glands of cercariae harvested in distilled water.

The aim of the investigations described in this chapter was to develop a protocol which would allow comparisons between individual cercariae. The protocol described achieves this goal. The protocol indicated different levels of calcium ions in the preacetabular glands of cercariae stained for 10 mins with 0.001% ARS. However the protocol is limited in that the total levels of calcium ions in the preacetabular glands were not calculated and compared.

The results of this study are summarised on the following page.

4.8. Summary.

In summary the results of this study have suggested the following conclusions.

(i) Alizarin Red S stain is a suitable indicating agent in fluorescent quantitation studies.

(ii) The fluorescent quantitation protocol suggested different levels of calcium in the preacetabular glands of individual *S. mansoni* cercariae.

(iii) Cercariae harvested from a snail shed in distilled water exhibited decreased levels of calcium in their glands when compared with cercariae from the same snail shed in dechlorinated tap water.

(iv) Cercariae after aging appeared to exhibit a decrease in calcium levels in their glands.

(v) Calcium levels in the glands appeared to be snail related and independent of age of patency,

(vi) Healthy and pale snails appeared capable of producing cercariae with similar calcium levels in their glands.

The last three conclusions are inferred from observations in this study. If these suggestions are valid, they could have profound effect on cercarial infectivity sucess in the field and could contribute to variable infectivities observed in laboratory experiments. Further studies are required to confirm or refute these results.

CHAPTER 5

The relationship between calcium of the cercariae, the snail and the shedding water.

5.1. Introduction

The studies described in the previous chapter were concerned with comparisons between individual cercariae and groups of cercariae. Quantitative analysis showed differences in fluorescent emission from the preacetabular glands of *S. mansoni* cercariae stained with Alizarin Red S. Comparisons were relative and measurements of total cercarial calcium ions (Ca^{2+}) were not made. In contrast, in this chapter the total Ca^{2+} content of cercariae is determined together with calcium levels of the water into which cercariae were shed.

The flow diagram (Fig.5.1.) outlines the protocol followed to obtain cercarial pellets and shedding water supernatant. The details of experimentation are described in Chapter Two. In the first four studies the Ca^{2+} in pellets and water was determined using Plasma Emission Spectrometry (Ch. 2.). In the later studies snail shedding water was analysed by Atomic Absorption Spectrometry (Ch. 2.).

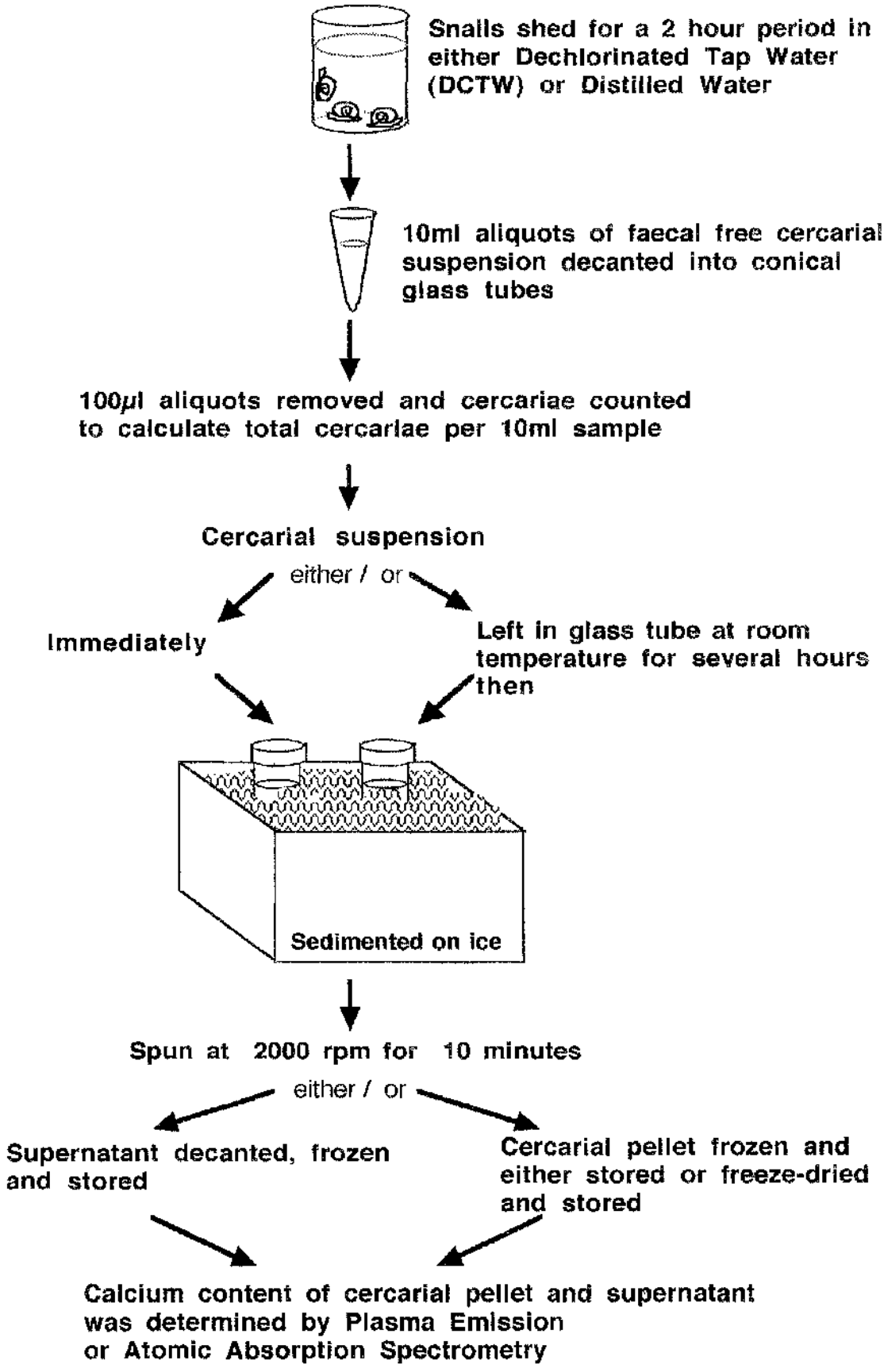
Finally an alternative approach, using the chelating agent EGTA in conjunction with the fluorescent probe PKH26, was employed to investigate the effect of aging (approximately 4 hours) on calcium ions in cercariae. The results are presented in this chapter.

5.2. Calcium content of *S. mansoni* cercariae.

Snails were shed in DCTW, the routine maintenance medium, to determine the calcium content of cercariae harvested under laboratory conditions. To investigate the effect of distilled water on cercarial calcium; snails were shed in distilled water and calcium was determined in both newly-shed cercariae and cercariae from the same pool which had been left swimming at room temperature for a period of approximately 4 hours.

(i) Results The pooled results from four studies, where calcium is

Fig 5.1. Protocol to calculate calcium in *S.mansoni* cercariae and in the snail shedding water.



expressed in µg/1,000 cercariae, are presented in Table 5.1. When snails were shed in DCTW the mean value of calcium in newly shed cercariae was 0.615 µg per 1,000 cercariae. This value is the mean of nine cercarial samples (each sample consisting of the pellet obtained from a 10ml cercarial suspension) and it should be noted that the values ranged from 0.54 -0.8µg/1,000 cercariae.

Newly harvested cercariae from snails shed in distilled water had a mean calcium value of 0.603 µg/1,000 cercariae. Cercariae from the same shedding, but having a swimming period of 4 hours at room temperature in their shedding water prior to analysis, had a mean value of 0.480 µg/1,000 cercariae. The difference, 0.123µg/1,000 cercariae, in calcium values between the newly harvested and swimming cercariae was significant ($P < 0.05$ Student's t-test).

5.3. Calcium content of snail water estimated using two methods of analysis.

The relationship between environmental water and snails is now considered. As discussed in detail earlier, parasitised snails need calcium not only for their own metabolism, but also for the rapidly multiplying cercariae in the host snail. Davies (1983), using X-ray probe microanalysis of parasitised snails, showed that cercariae, still within sporocysts, have preacetabular glands which are rich in calcium.

(i) Snail relationship. To determine if any calcium exchanges were occurring between environment and snails, calcium levels were calculated in DCTW and distilled water both before, and after, "contact" with non parasitised and parasitised snails. The background calcium content of water before snail contact could then be taken into account when results were being processed.

"Contact" consisted of parasitised snails being rinsed free of debris and exposed to 2,000 lux of light for a period of 2 hours in a beaker with a known

Table 5.1. Pooled data from four studies showing average calcium (μg) per 1,000 *S.mansoni* cercariae.

Group	Water snails shed in	Number of 10ml cercarial suspensions	Total number of cercariae	Calcium (μg) per 1000 cercariae ± standard deviation
A	DCTW	9	135,696	0.615 ± 0.14
B	Dist. W	3	60,400	0.603 ± 0.03 *
C	Dist. W	3	74,100	0.480 ± 0.05 *

Key : Group A - Newly harvested cercariae from snails shed in dechlorinated tap water.

Group B - Newly harvested cercariae from snails shed in distilled water.

Group C - Cercarial suspension left at room temperature after snails were shed in distilled water for a period of 4 hours.

* Significant difference ($P < 0.05$ Students t-test)

volume of water (the snails are then said to have been shed) . The non parasitised snails received the same treatment and were referred to as having been sham shed.

Results:Plasma Emission Spectrometry analysis. As can be seen in Fig. 5.2.(a), sham shed (non parasitised) snails lost calcium to the distilled water. In contrast, they removed calcium from their environment in DCTW.

Fig. 5.2.(b) shows that parasitised snails lost calcium when shed in distilled water. It is noteworthy that parasitised snails also lost calcium when shed in DCTW. The weight matched set of snails (12^3) lost more calcium in distilled water (247µg), compared with the loss (171.9µg) experienced in DCTW .

The observation that parasitised shedding snails lose calcium in DCTW is intriguing since DCTW is the maintenance regime for both parasitised and non parasitised laboratory snails. Further supportive evidence of this loss was obtained when Alizarin Red S was introduced to the DCTW supernatant from a sedimented cercarial suspension. A pink colour resulted which was visibly deeper than the very pale pink observed in the control DCTW. This again suggested that calcium was being lost to the DCTW by the shedding snail or the cercariae or by both.

(ii) Cercarial relationship. To confirm the observation that parasitised snails were losing calcium when shed in DCTW, a study was undertaken using two groups of snails which were patent on different dates. In an attempt to ascertain if cercariae were losing calcium to the water, the cercarial suspensions from both sheddings were divided into two equal 10ml samples. One 10ml sample, from each group of patent snails, was sedimented immediately. The remaining 10ml samples from each group were left at room temperature for 3hrs 30 mins before sedimentation. Supernatants were filtered

Key  Snails shed in Distilled Water.

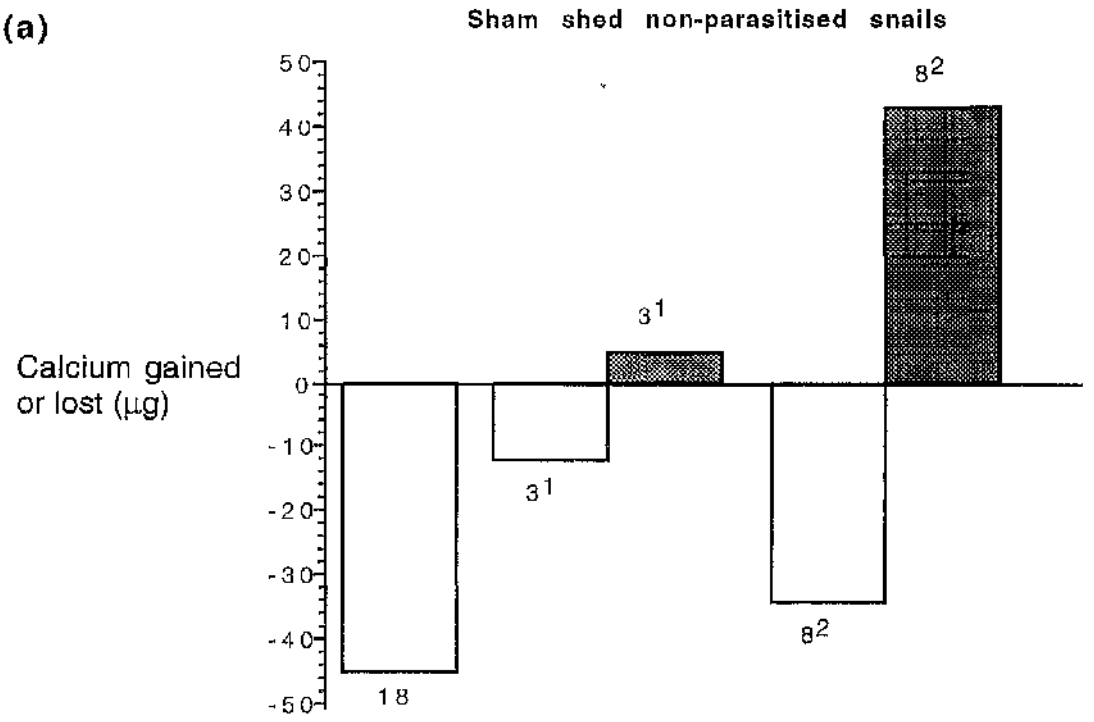
 Snails shed in Dechlorinated Tap water.

Where known, the number of snails exposed is indicated at the columns.

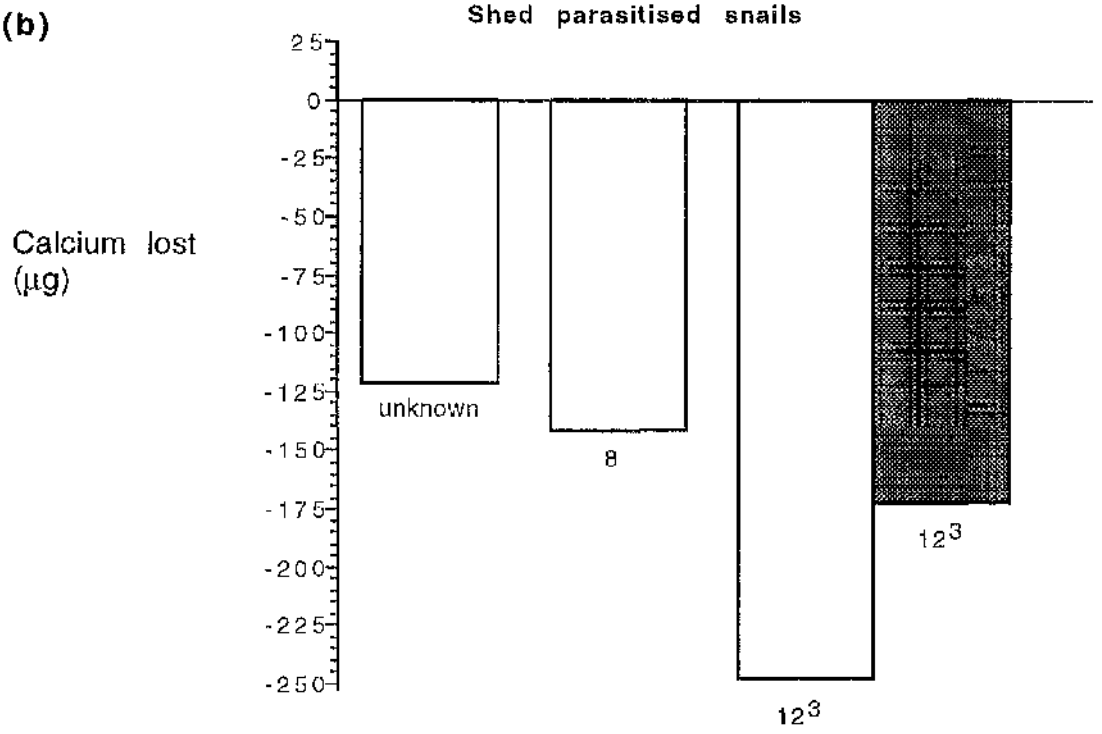
3^1, 8^2, 12^3 are the number of snails in size and weight matched sets.

Fig. 5.2 **Calcium lost or gained by *B.glabrata* snails over a 2 hour shedding period.**

5.2(a)



5.2(b)



before freezing for storage. In this study water was analysed by Atomic Absorption Spectrometry.

Results: Atomic Absorption Spectrometry analysis. The results of this investigation (Table 5.2. samples 1) showed that both sets of parasitised snails lost calcium (84.7 and 142.2µg respectively) to the DCTW during the 2 hour shedding period. After maintenance of cercarial suspensions at room temperature for 3 hour 30 mins, calcium values of 92.2 and 146.5µg were obtained (Table 5.2. samples 2). This represents a small increase of calcium in the shedding water, of 7.5 and 4.3µg respectively.

(Iii) Time course study. To investigate further the possible loss of calcium from cercariae, a short time course study was carried out. The cercarial suspension, while being gently stirred with a magnetic stirrer, was divided into 9ml aliquots. The first aliquot (T_{oh}), was placed on ice for a period of 2 hours to sediment cercariae. The remaining aliquots were stored in the light at room temperature. Thereafter at one, two and four hours aliquots (T_{1h}, T_{2h} and T_{4h}) were similarly sedimented on ice.

The calcium content of the supernatants was determined by Atomic Absorption Spectrometry.

Results: Time course study. The results (Table 5.3) indicate very small increases of 5.5, 3.0 and 3.5 µg of calcium in the DCTW supernatants from swimming cercariae. The calcium loss, although observed at each time point, does not increase progressively with time.

5.4. Calcium and snail relationship.

From the studies concerning snails and shedding water [Fig. 5.2.b) and Table 5.2.], it appears that the snail is not only providing calcium for the cercariae, but is apparently losing it to the shedding water as well. The relationship between total calcium loss and snail mass was considered in an

Table 5.2. Calcium (μg) lost to dechlorinated tap water from *B.glabrata* snails after a 2 hour shedding period.

Number of snails and Date snails patent	Calcium (μg) per 10ml sample	
	Sample 1	Sample 2
7 on 1 / 5 / 97	84.7	92.2
12 on 21 / 5 / 97	142.2	146.5

Note : After a 2 hour shedding period, two batches of cercarial suspensions were each divided into two 10 ml samples. Samples 1 were processed immediately and Samples 2 were processed after 3 hr 30 min, at room temperature.

Table 5.3. Calcium content of dechlorinated tap shedding water at 0, 1 2 and 4 hours after shedding period.

Time post 2 hour shedding period	Calcium (µg) per 10ml sample	
	Total	Increment after time
T 0hr	70.0	
T 1hr	75.5	5.5
T 2hr	73.0	3.0
T 4hr	73.5	3.5

Note : After a 2 hour shedding period, a batch of cercarial shedding water was divided into four samples of equal volume. T 0hr was processed immediately after shedding. T 1hr, T 2hr and T 4hr were processed after the cercarial suspensions were left at room temperature for 1, 2 and 4 hours respectively.

experiment where calcium content of both cercarial pellet and respective supernatant shedding water was monitored from weighed snails. The amount of calcium lost is expressed as a percentage of the weight of shedding snails.

(i) Results: Table 5.4. shows that total calcium lost when snails were shed in DCTW comprised 0.008% of the total weight of the snails. When shed in distilled water, calcium lost from snails was 0.01% total weight of snails.

5.5. Cercarial incubation in EGTA.

An alternative approach was undertaken to study calcium ions of cercariae. Cercariae were incubated with the calcium chelating agent EGTA (ethylene glycolbis tetraacetic acid) and subsequently stained with the fluorescent lipid probe PKH26.

(i) A newly shed cercarial suspension was divided into three aliquots. The first aliquot was stained with PKH26 (1µl probe in 100µl diluent C), washed and examined microscopically. The second aliquot was exposed to 10mM EGTA (in PBS) for 10 mins and after washing the cercariae were left for one hour in water prior to staining with PKH26. The third aliquot was similarly treated, replacing EGTA with PBS alone.

Results. The staining of newly shed cercariae with the fluorescent probe PKH26 clearly highlighted the nervous system of both body and tail of the parasite. The longitudinal nerve cords and cell bodies of the cercariae stained fluorescent red and were clearly defined (Fig. 5.3.). In the tail, branching of the longitudinal nerve into the hair-like sensory extensions of the tail surface was also detected.

There was no obvious difference between the staining patterns of cercariae which had been incubated either with or without EGTA. When compared with newly shed cercariae however, although fluorescence was observed, both treated and non-treated cercariae exhibited a more diffused staining. The longitudinal nerve cords and cell bodies of the cercariae were

Table 5.4. Total weight of calcium (μg) lost, expressed as a percentage of total snail group weight (g).

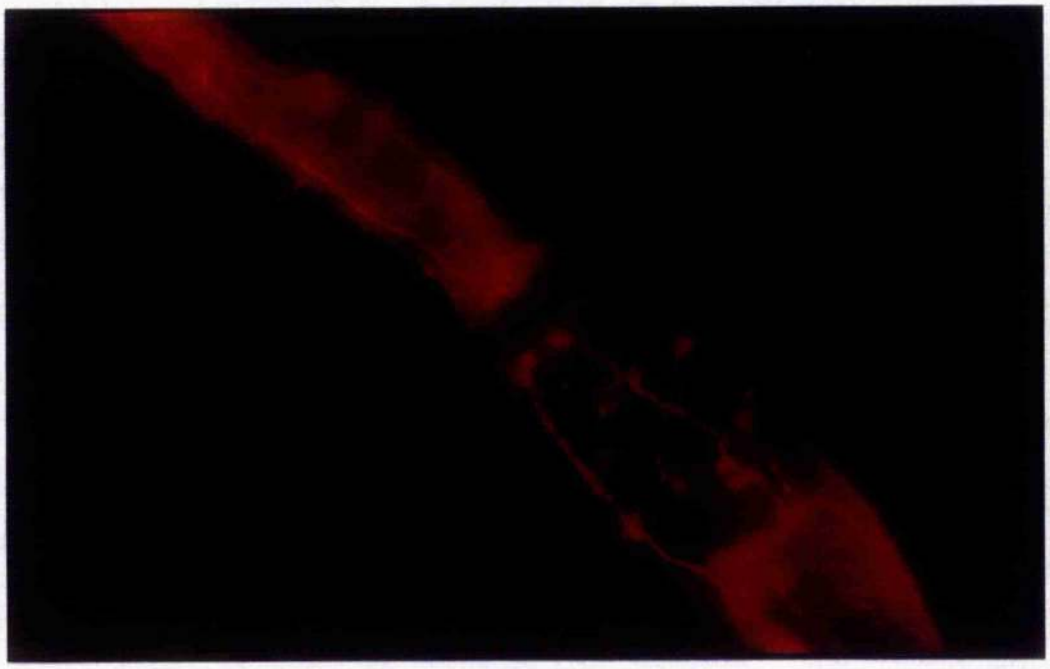
Number of snails and Date snails patent	Total weight (g) of snail group	* Total weight (μg) of calcium lost in :		Calcium loss as % weight of snails
		Distilled water	DCTW	
8 on 2 / 4 / 97	1.344	152.57		0.01
12^1 on 1 / 6 / 97	2.323	258.3		0.01
12^1 on 1 / 6 / 97	2.303		191.71	0.008

Key : Groups 12^1 , 12^1 are size and weight matched snails.

* Total weight of calcium lost comprises calcium in cercarial pellet together with additional calcium in shedding water supernatant.

Fig. 5.3. *S.mansoni* cercaria labelled with the lipid probe PKH26.

Dark field exposure. (—— Represents 15.6 μm).



not now clearly defined and only limited staining was detectable in some nerves

After a further period of 30 mins profound changes were detected, under bright field examination, in the areas proximate to the cercariae. The untreated cercariae demonstrated aggregated material around the body and tail junctions. The aggregations appeared spherical. Exudate at the head of the body and extending down the body length was observed in these cercariae. In contrast the EGTA treated cercariae had no exudate associated with the body and no aggregations at the junction of the head and tail.

(ii) A further investigation, using the protocol of the previous section [5.5. (i)], was undertaken to study further the effects of EGTA (in distilled water) exposure on cercariae. The incubation period was extended to four hours and, since the stock EGTA was in distilled water, the control aliquot had EGTA replaced with distilled water.

Results. The PKH26 stained newly hatched cercariae again exhibited clearly labelled cell bodies and nerves in the parasite bodies and tails. A pattern of discrete spots was observed on cercarial tails. They appeared to be on the surface. It is noteworthy that, when carbachol was added (to immobilise the cercariae), the spots immediately disappeared.

Variation in staining patterns was observed in the parasites. Some of the cercariae were uniformly stained, both bodies and tails, with a very bright yellow fluorescence. The nerve cords could be identified by slowly focussing up and down. Several cercarial preparations were examined and all exhibited the two different staining patterns.

Both the EGTA treated and control cercariae were equally clearly stained. On this occasion they exhibited the same patterns of staining as the newly hatched cercariae. The clearly defined nerve cords and cell bodies were stained red while some cercariae had uniform surface staining.

Initially there was no exudate from the slide mounted cercariae. After 30 mins however, exudate was present at the tip of the body, and at the body and tail junction. It is noteworthy that the exudate was present in the EGTA treated cercariae as well as the control cercariae. This result is in contrast to the previous study [5.5. (i)], when exudate was only observed in the control cercariae which had not been EGTA treated. The exudate at the cercarial body/tail junction is clearly seen in the photograph in Fig.5. 4.(a). It is relevant that exudate at the body/tail junction is fluorescent [Fig.5.4.(b)], indicating that calcium is present. The photograph (Fig. 5.5.), is included because it clearly shows calcium at the body/tail junction. This photograph was taken whilst developing the ARS protocol in the previous chapter. It is noteworthy that immunogold electron microscopy showed calcium binding protein (CaBP) only in selected regions of cercariae, the body/tail junction region being one (Ram *et al*, 1994).

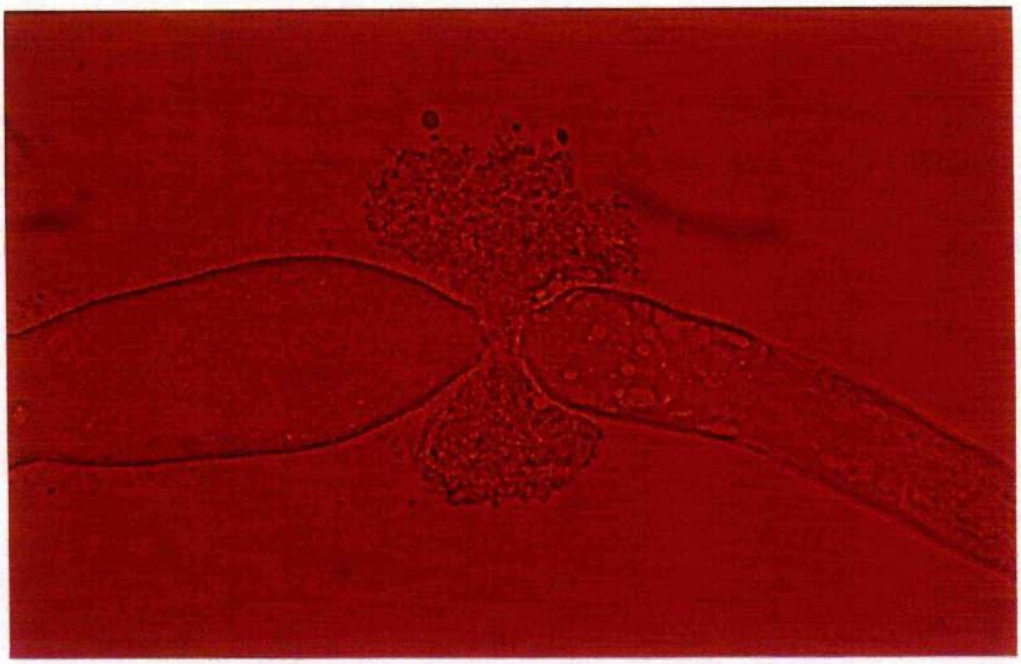
(iii) The effect of EGTA incubation on calcium ions in the preacetabular glands was investigated. Aliquots of cercarial suspensions were incubated for 2 hours, in 20mM EGTA, 10mM calcium and DCTW respectively. Thereafter 1ml aliquots were stained, for 1 hour, with Alizarin Red S (ARS) and washed three times *prior* to examination.

The remaining cercarial suspensions, in 20mM EGTA, 10mM calcium and DCTW respectively, did not receive ARS exposure. They were left on the bench, in round bottomed glass tubes, at room temperature for a period of 72 hours.

Results. No differences were observed in the ARS staining of cercarial glands between all three incubation treatments. Very clear staining of the preacetabular glands and tubules were observed. All three groups exhibited very bright red fluorescence. The black and white photograph (Fig. 5.6.), represents the very discrete labelling exhibited in the cercariae.

Fig. 5.4. Exudate of *S.mansoni* cercaria at body/tail junction.

(a) Bright field exposure. (—— Represents 18.8 μm).



(b) Alizarin Red S stained exudate. Dark field exposure.

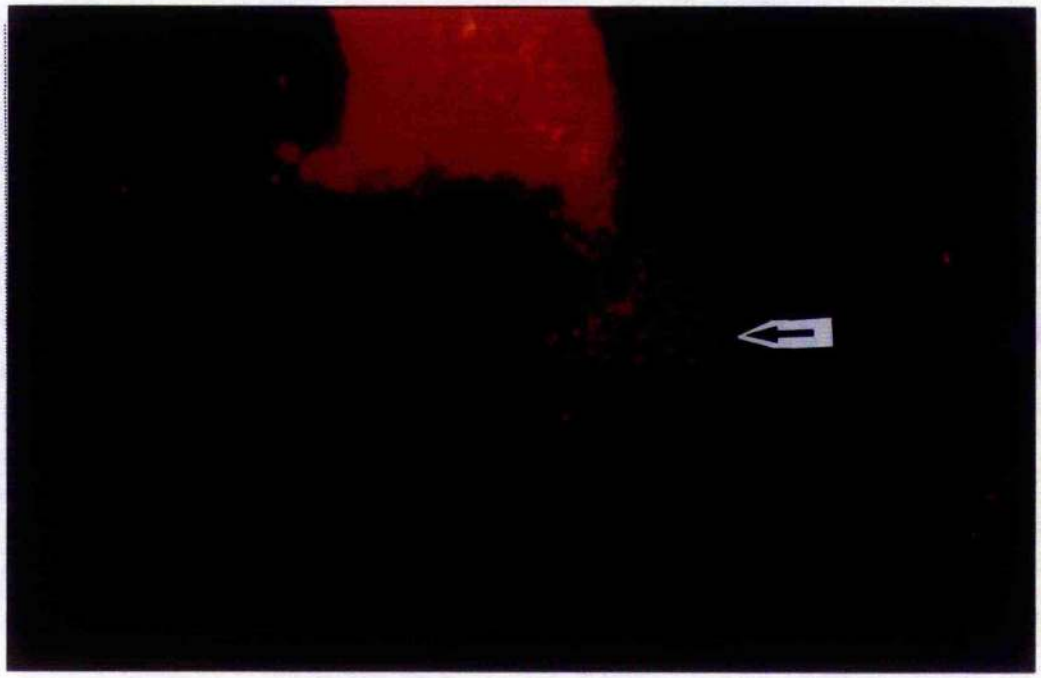


Fig. 5.5. ARS stained cercaria showing calcium at body/tail junction.

Calcium at body/tail junction. (—— Represents 13.4 μm).

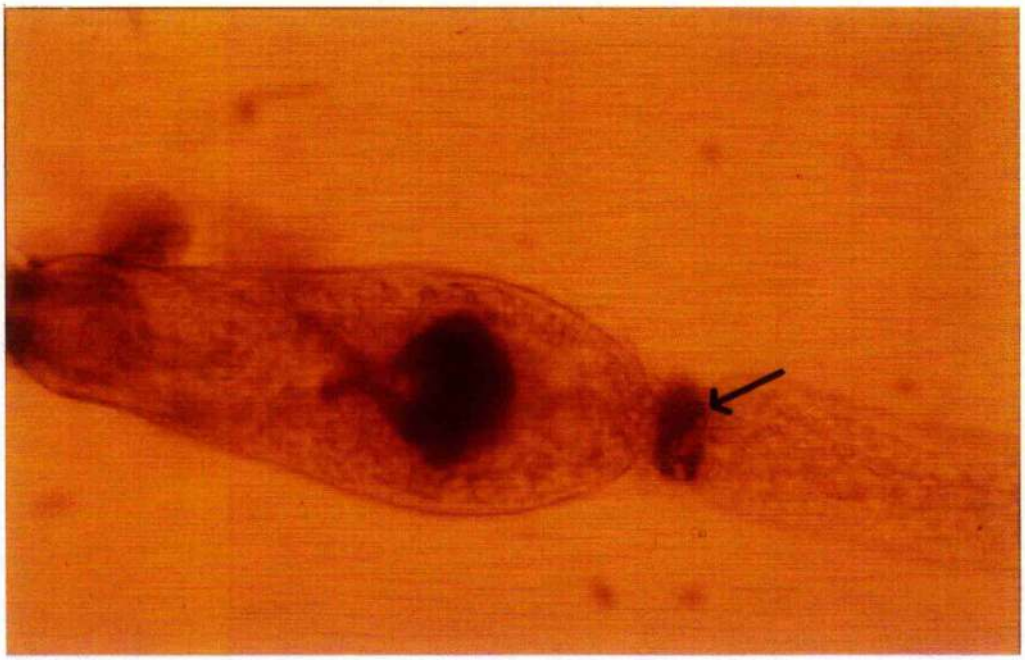
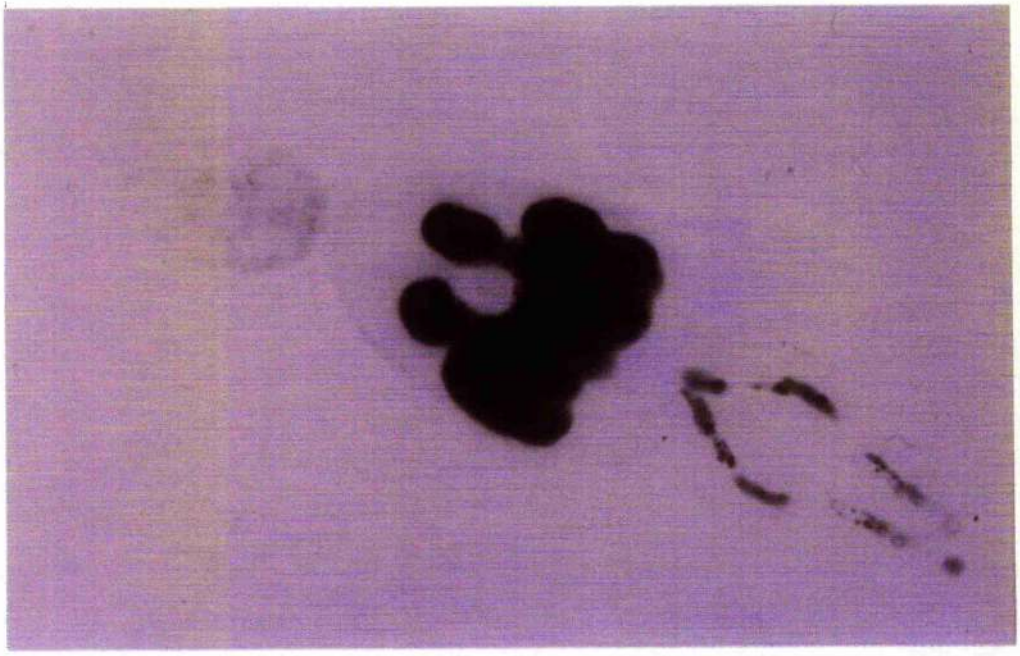


Fig. 5.6. ARS stained cercaria photographed using black and white film.

Stained preacetabular glands. (—— Represents 13.4 μm).



The unstained samples (stored 72 hours), exhibited marked differences when examined microscopically. The cercariae incubated in 20mM EGTA, still had attached tails and appeared undamaged, having no apparent detachment of the membrane (Fig. 5.7.). Subsequent studies confirmed this observation, with EGTA incubated cercariae retaining a high percentage of their tails even over a 96 hour period (Table 5.5.). By 72 hours some membrane detachment was observed in isolated cercariae.

In contrast the other cercarial suspensions, in DCTW only or with the addition of Ca^{2+}, had no tails and were severely damaged, exhibiting numerous vesicles on their bodies and tails. The damaged cercariae were surrounded by actively swimming bacteria (Fig. 5.8.).

It is noteworthy that the EGTA cercarial suspension (after 72hours), when compared with the calcium and DCTW cercarial suspensions, had very few bacteria. The difference was very obvious, with the non treated cercarial preparations having a dense population of actively swimming organisms. The contaminated suspensions had many "rod" shaped organisms which were not present in the EGTA treated samples.

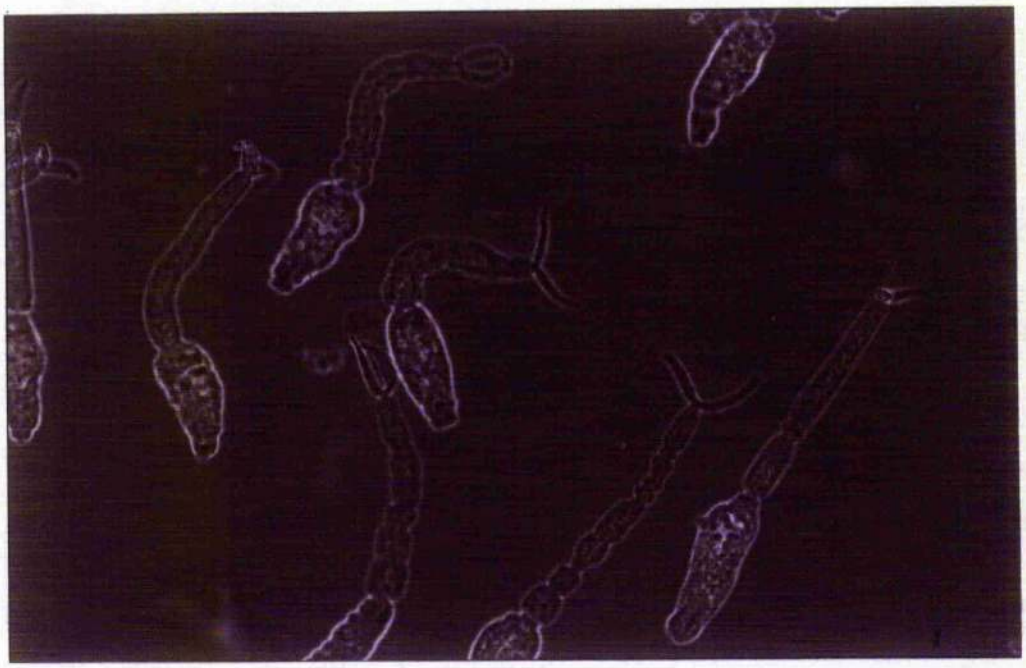
5.6. Discussion.

(i) Total calcium content of cercariae. The average value of 0.615µg per 1,000 cercariae (the equivalent of 0.615ng per cercaria), is much lower than the approximate 10-15ng that Dresden and Asch (1977) calculated a single cercaria to contain. Our results, although varying slightly between different batches of cercariae, never exceeded the value of 0.8µg per 1,000 cercariae.

A possible explanation may be found in the calcium content of the snail maintenance water. Analysis of the maintenance DCTW (both by Plasma Emission and Atomic Absorption Spectrometry) revealed on different occasions values ranging from 3.5-4.7ppm (equivalent to 3.5-4.7mg/litre). It

Fig. 5.7. Cercariae incubated 72 hours in 20mM EGTA.

(a) Tails attached. (—— Represents 107 μm).



(b) Membrane undamaged. (—— Represents 16 μm).

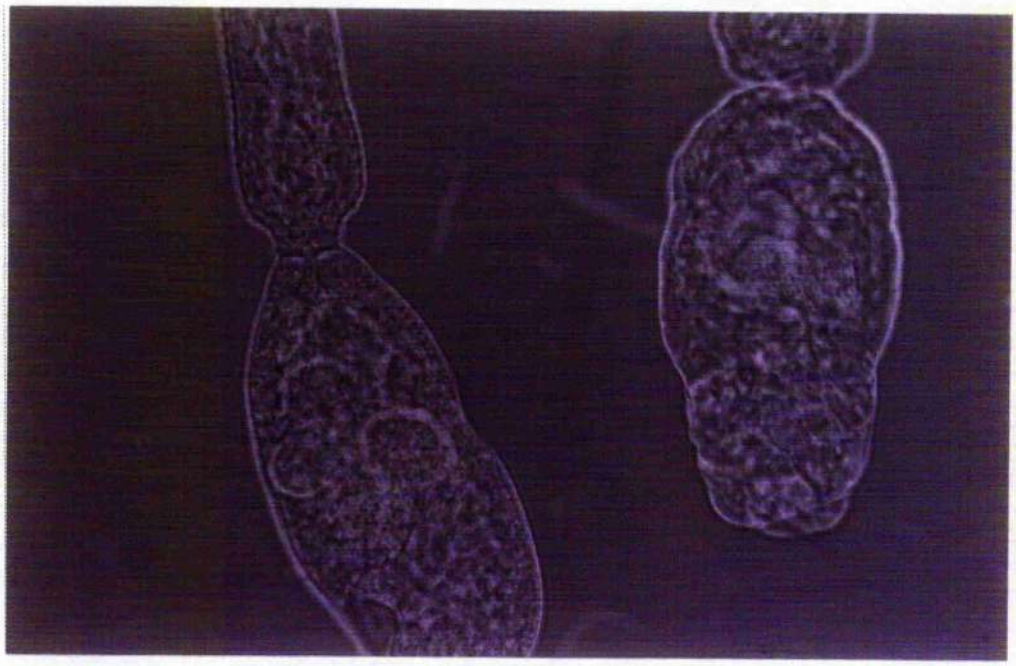


Table 5.5. The role of calcium ions in cercarial tail loss.

Treatment (Time in hours)	Bodies only	Intact cercariae	Total number of cercariae	% tails detached
20mM EGTA (72)	39	87	126	31
10mM Ca^{2+} (72)	92	0	92	100
DCTW (24)	138	0	138	100
10mM EGTA (24)	39	55	94	42
10mM Ca^{2+} (24)	187	0	187	100
DCTW (48)	264	14	278	95
10mM EGTA (48)	82	221	303	27

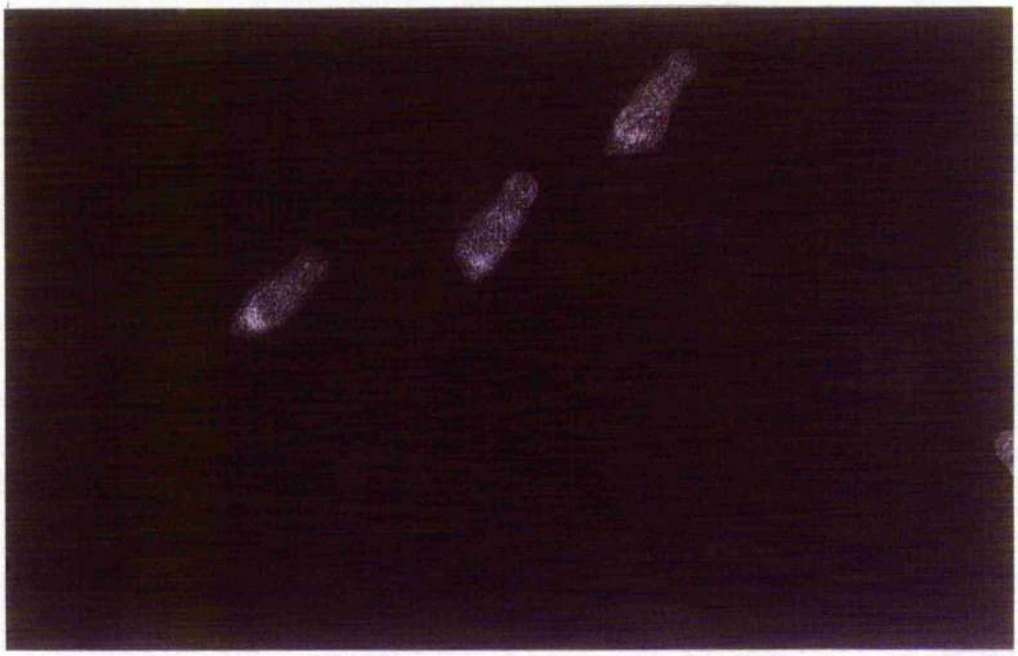
Key : EGTA ethylene glycolbis tetraacetic acid (a calcium chelating agent).

Ca^{2+} calcium chloride solution

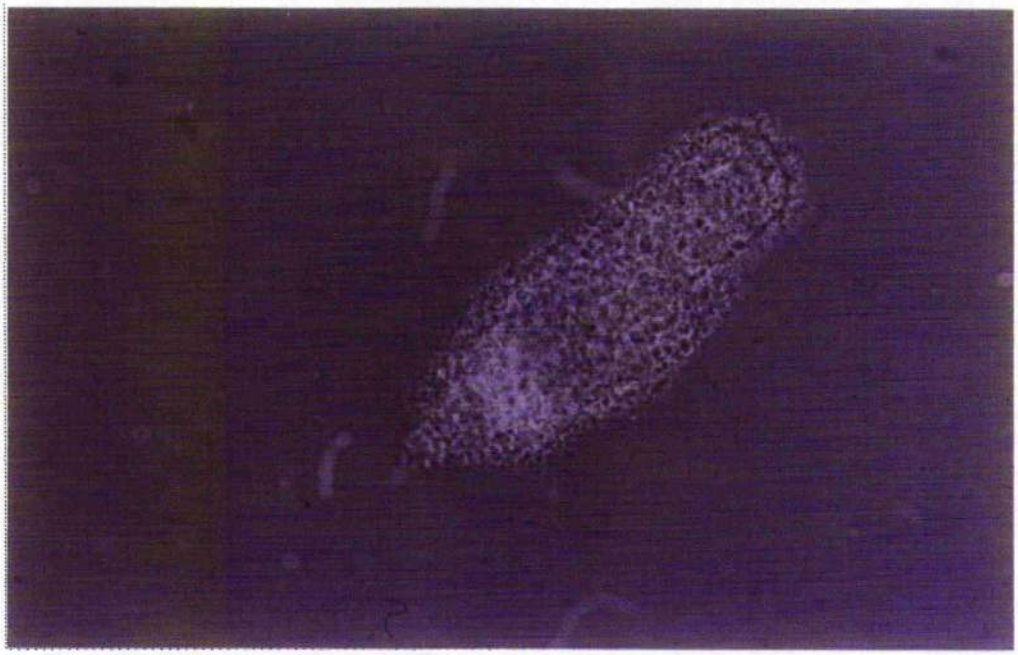
The results of the three experiments presented suggest that calcium is necessary for cercarial tail loss.

Fig. 5.8. Cercariae incubated 72 hours in 10mM $CaCl_2$.

(a) No tails attached. (—— Represents 142 μm).



(b) Membrane damaged. (—— Represents 18.8 μm).



has been suggested that very soft water (0-10ppm calcium) can be a limiting factor for *Biomphalaria* in the field (Schutte and Frank, 1964).

Clearly the calcium ion concentration in the maintenance DCTW falls below the optimal level. It is suggested that the low levels of calcium in the DCTW of the present study, may cause some metabolic stress to the snail and contribute to the lower calcium values of the cercariae they produce.

The significantly lower level of calcium in cercariae after maintenance in distilled water may be evidence of calcium efflux into an environment having ions of lower concentrations. Although the efflux of ^{45}Ca labelled calcium from *S. mansoni* cercariae into calcium-free medium has previously been observed (Fusco *et al* 1991), the specific source of the released calcium ions from within the cercariae was not established. The possibility that some may come from the cercarial preacetabular glands must be considered. The results of the fluorescent quantitation studies of the previous chapter suggested that calcium was lost, from preacetabular glands of cercariae, in distilled water.

(ii) Calcium content of shedding water. Snail relationship. The observed loss of calcium, from both parasitised and non parasitised snails, to distilled water is perhaps to be expected. The calcium level of distilled water ranges from 0.01-0.1ppm, consequently the snail may be physiologically stressed in this medium, and unable to prevent the loss of calcium into this hypotonic environment. Conversely uptake of calcium, by sham shed non-parasitised snails, in DCTW is confirmation that snails obtain calcium from the environment. Greenaway (1971) using ^{45}Ca demonstrated that *Lymnaea stagnalis* absorbed calcium directly from the external medium.

The loss of calcium in DCTW by parasitised snails is interesting (Fig. 5.2. and Table 5.2.). Calcium reserves of the parasitised snail are are severely depleted even in the prepatent period [Chapter 1.3.(ii)]. It is therefore suggested that calcium release in DCTW is not an active process by the host, but a leakage resulting from the physical process during cercarial shedding.

Parasitism may cause changes to both, internal and external, membranes and teguments. Consequently, they may be more permeable to essential ions and more susceptible to physical damage. Physiological changes resulting from parasitism may reduce the host snails repair responses, exacerbating any physical damage.

Calcium content of shedding water. Cercarial relationship. The evidence indicating that cercariae, after a period of 3-4 hours in shedding water at room temperature, lose calcium to DCTW (Tables 5.2.and 3.) may initially appear tenuous. The amount of calcium ions lost ranges from only 3-7.5 µg in a 10ml aliquot of cercarial suspension. This weight, against a background of up to 146 µg calcium lost by the shedding snails, initially appears too small to be of any significance, either statistically or physiologically. However, previous studies indicated that, a 10ml cercarial suspension may contain 24,600 cercariae which have a calcium component of 19.8 µg (part of the data included in Group A, Table 5.1.). If calcium loss (average 4µg) to DCTW from swimming cercariae is compared to calcium of cercarial pellet (19.8µg), then the loss would constitute 20% of total cercarial calcium. The loss now appears relatively significant and it is conceivable that such a loss could have physiological consequences for the cercariae.

(III) Calcium and snail relationship. The results in Table 5.4. indicate, as expected from unprocessed data, that snails lose a higher percentage calcium, compared to total snail weight, when shed in distilled water compared with loss in DCTW. The low percentage values of 0.01% in distilled water and 0.008% in DCTW, are perhaps misleading. A more meaningful comparison would be to sacrifice the snails and compare calcium loss with total calcium content of the shedding snails.

(iv) Cercarial incubation in EGTA. The fluorescent probe PKH26 was used as an indicator to highlight any differences between newly shed cercariae and cercariae which had been incubated, either with, or without

EGTA. It should be noted that the two latter groups when exposed to PKH26, were approximately 3 hours older than the newly shed cercariae. The two important observations that emerged from this investigation will be discussed separately.

(iv,i) Cercarial nervous system. The staining of newly shed cercariae with PKH26 resulted in a clearly defined fluorescently labelled nervous system of living cercariae. After several trials, variation in staining patterns were observed. In the same preparation, some cercariae displayed clear bright red nerve chords and cell bodies, whilst others exhibited bright yellow total surface staining. In the latter group, detail of nerve staining could be visualised by slowly focussing up and down. The nerves fluoresced a sharper bright yellow. Both newly shed cercariae and cercariae which had been incubated, either with or without EGTA, exhibited the variety of staining patterns.

This variation in staining suggests selective staining of damaged cercariae or cercariae of different maturity. It should be appreciated that even newly shed cercariae, as a consequence of dark maintenance of parasitised snails, will most probably comprise a population of mixed aged individuals. It is noteworthy that dead cercariae did not label, suggesting that the PKH26 probe is selective for live parasites.

The observation that carbachol treatment resulted in the disappearance, of surface "spots" on the cercarial tail, suggests that they could be nerve endings or clusters. Carbachol affects the nervous system via the acetylcholine neurotransmitter ; consequently the disappearance of these "spots" may indicate retraction of muscle associated with these nerve endings or clusters. Further investigations are required to elucidate this observation.

Haas *et al* (1995), described the role of host chemical cues on stimulation of cercarial behaviour. The "spots", together with the labelled nerve branches in the hair-like extensions of the tail, are perhaps indicative of the

sensory properties of the tail. The cercariae swim tail first, consequently sensory receptors associated with the tail are clearly an advantage and may act in conjunction with papillae of the body.

This technique of nerve staining has several advantages over others in current use. Not only is it is a simple method of preparation but, most significantly, unlike other methods, the cercaria is still alive after the treatment. That stained swimming cercariae can be observed microscopically could be potentially very useful in neurological studies.

(iv,ii) Cercarial exudate and aggregations. Although the initial experiment indicated differences, between the EGTA treated and non-treated cercariae, concerning the exudate and aggregations, further experimentation did not confirm this observation. Repeated experiments revealed that EGTA incubation did not prevent exudate and aggregation formation.

When viewed immediately after mounting on a slide, there was no exudate from cercariae, in either of the two incubation treatments. However after a period of 30 minutes, exudate and aggregations were observed in both preparations. The time dependent relationship, before exudate appearance, is thought to be associated with cercarial penetration behaviour. The searching, testing and deposition of acetabular gland content by cercariae, on even artificial surfaces, has been well documented (Linder, 1986). The study in this thesis has indicated that incubation, for a period of 2 hours in EGTA, followed by further incubation of 1 hour in ARS, did not inhibit this behavioural pattern. It appears that contact, with either the slide or coverslip, stimulated the response within a 30 mins time period. The negatively charged glass surface may stimulate a response similar to that of the negatively charged endothelium, which a penetrating cercariae would naturally encounter.

Similarly, both the EGTA and DCTW cercarial suspensions which had been left in round bottomed glass tubes for 72 hours, had exudate present at body/tail junction area.

The EGTA incubation period, in the concentrations described, did not prevent ARS staining of the preacetabular glands. Cercariae after treatment exhibited both, clearly stained glands and tubules, and were able to swim actively. The possibility that more concentrated EGTA may have different consequences is discussed in the next chapter.

These preliminary studies using PKH26, did not highlight any significant differences between newly shed cercariae and EGTA treated, or untreated cercariae. However characteristic of each group was the variability of labelling with PKH26.

The results of this study are summarised on the following page.

5.7. Summary.

In summary the results of this study have shown the following.

(i) The mean value of total calcium in newly shed cercariae was 0.615µg per 1,000 cercariae.

(ii) Maintenance of cercariae in distilled water for a period of 4 hours significantly reduced calcium content of cercariae.

(iii) Preliminary results suggest that cercariae in dechlorinated tap water (DCTW), for a period of 4 hours, also lose calcium to their environment.

(iv) Parasitised snails lost calcium, to the shedding water, during a 2 hour shedding period in DCTW.

(v) Incubation of cercariae in 20mM EGTA (ethylene glycolbis tetraacetic acid) for a period of 4 hours did not,

(i) adversely affect vitality of cercariae.

(ii) inhibit exudate and aggregation formation at body-tail junction.

(iii) inhibit Alizarin Red S staining of preacetabular glands.

(vi) Incubation of cercariae in 20mM EGTA for a period of 72 hours,

(i) prevented tail loss in cercariae.

(ii) inhibited membrane damage.

(iii) affected bacterial contamination.

(vii) Labelling cercariae with the fluorescent PKH26 lipid label,

(i) highlighted the nervous system of both body and tail.

(ii) revealed variation in uptake of this label by cercariae.

CHAPTER 6

Discussion

6. Discussion.

This study has investigated cercarial production in snails maintained in different light regimes. The contributing effect of calcium on cercarial production is considered in this chapter.

A protocol, applying quantitative fluorescent emission microscopy, was developed which facilitated comparisons of preacetabular calcium in individual cercariae. Thereafter the total calcium ion content in cercariae was examined together with that of the snail maintenance water.

This discussion seeks to focus the results of this study, indicating their significance with current knowledge.

6.1. Cercarial production

The initial study in this thesis investigated cercarial production from snails maintained in different light regimes. The results indicated that parasitised snails maintained in the dark produced fewer cercariae than snails maintained in a 12Hour Light/12Hour Dark (12HL/12HD) regime. Of significance, was the similarity in the pattern of average daily cercarial emission between the two groups of snails (Fig. 3.5). This result supports the suggestion that cercarial production is not solely light regulated, but the result of the dynamics of the parasite larval stages within the snail (Théron and Moné, 1984). Any unknown factor which may have influenced cercarial production or emission was effective in both maintenance regimes.

The low light intensity, of 543 lux in the 12HL/12HD maintained snails, together with the small volume of 50 ml maintenance water, were considered to be contributing factors for the relatively low average daily yield of 90 cercariae per snail [calculated from data comprising Table 3.1 a) and b)], when compared to the higher 160 per snail observed by Théron (1981). It is noteworthy that momentary exposure to 41 lux light, when water was being removed, appears sufficient to stimulate cercarial emission from dark

maintained snails. That cercariae emerged in such low light may be of relevance in the field. This indicates that snails inhabiting murky, shaded pools will be efficient transmitters of the parasite. The role of both light intensity and volume of maintenance water, on cercarial production is discussed in detail in Chapter 3.

Further information relevant to cercarial production became apparent in later investigations. In the studies described in Chapter 5, the calcium content of the dechlorinated tap snail maintenance water was determined. Analysis of the maintenance DCTW (both by Plasma Emission and Atomic Absorption Spectrometry) revealed on different occasions values ranging from 3.5-4.7ppm. This water value would have been categorised as "very soft" by Schutte and Frank (1964) who described this category of 0 - 10 ppm, to be a limiting factor for the presence of *Biomphalaria* in the field.

Cercarial emergence was seen to increase in a linear fashion from snails maintained in water with calcium levels between 1.5mg/l and 30mg/l (Mishkin and Jokinen 1986). Maximum cercarial emergence occurred in water with calcium concentration of 30mg/l. These researchers suggested that environmental calcium not only determined the distribution of the snail hosts, but also, affected the productivity of intramolluscan schistosome infection. It is suggested that the maintenance water, with low calcium level, may contribute to the relatively lower numbers of emerging cercariae from snails in this study (Chapter 3.2.).

The increased emergence of cercariae from snails maintained individually in 2 litres of dechlorinated tap water may be explained by studies of Thomas and Lough (1974). They observed that *B. glabrata*, in a closed system, increased the net uptake of calcium when calcium concentration was fixed, but volume of water was increased. The snails in the present study which were maintained individually in a closed system of 2 litres of water (Chapter 3.3.), may consequently have had more available calcium than the

experimental snails maintained in 50ml of water. The extra calcium may have contributed to the increased number of shed cercariae. This interpretation would support the hypothesis of Mishkin and Jokinen (1986), that calcium levels of the water environment determine the cercarial production.

It is concluded that light intensity of 543 lux and calcium ion concentrations of 3.5 - 4.7 ppm in the dechlorinated tap maintenance water, may have contributed to the cercarial production observed in this study.

6.2. Calcium content of Cercariae.

The total calcium ion (Ca^{2+}) of 0.615µg per 1,000 cercariae, is lower than the estimate of 10-15µg previously reported by Dresden and Ash (1977). A possible explanation for this may be attributed to the soft water that the snails are maintained in.

In Puerto Rico, natural habitats of *B. glabrata* were found to have at least 16mg/litre of calcium (Pimentel and White, 1959). Clearly the calcium ion concentration in the maintenance DCTW falls below the optimal level, and calcium uptake is consequently by an active, energy-requiring transport system (Review by McMahon, 1983). *B. glabrata* maintained in less than 2mg/litre, had relatively small, extremely fragile shells which were pitted internally (Mishkin and Jokinen, 1986). Growth rate, survivorship and fecundity rates all decline with reduction in calcium concentrations below optimal environmental values (Harrison *et al*, 1970; Thomas *et al*, 1974). As already referred to, low levels of environmental calcium reduces numbers of emerging cercariae (Mishkin and Jokinen 1986). It therefore seems reasonable to suggest that the low levels of calcium in DCTW in the present study, may contribute to the lower calcium values of the cercariae produced by the snails.

Snails can also obtain calcium from their food. Using ^{45}Ca as a radioactive tracer, van der Borght and van Puymbroeck (1966), demonstrated that a high proportion of shell calcium may come from ingested food. These

workers observed very efficient calcium absorption in the gut epithelium of *L. stagnalis* . Even if an equally efficient mechanism of calcium uptake from food occurs in *B. glabrata*, the possibility that parasitism, with the very high number of dividing sporocysts and migrating cercariae, may adversely affect the epithelium absorption capabilities of the host digestive gland must be considered.

When calcium ion content of DCTW, before and after cercarial contact, was determined, it became apparent that calcium was being lost from the cercariae. Although amounts measured (ranging from 3-7μg) were small, several different experimental approaches indicated that cercariae left swimming for 3-4 hours, lost calcium during that period. Results (Table 5.1, B and C and Table 5.2, samples 2 and Table 5.3) inferred that the calcium lost, comprising an estimated 20% of total cercarial calcium, could be considered a biologically significant amount [Chapter 5.6. (ii)].

The question now addressed is, from where within the cercariae is the source of lost calcium? Fluorescent quantitation studies suggested that the calcium lost could be from the preacetabular glands of the cercariae [Chapter4.4. (ii)]. Using electron probe analysis and atomic absorption spectroscopy, Dresden and Edlin (1975) determined levels of calcium, equivalent of, up to 10 M in cercarial glands. Surprisingly the calcium present in the glands was 1,000 to 2,000 times more abundant than in the tail. They demonstrated that *in vitro*, these high levels of calcium inhibited protease activity reversibly and suggested that calcium kept the preacetabular proteases in an inactive state *in situ*. Activation of the proteases was possible after secretion of the gland contents when, it was suggested, calcium ion concentration would be diluted to the levels necessary for the enzyme activity. They also reported evidence that calcium granules were present round the outside of the cercarial body.

The suggestion that cercariae while aging lose calcium ions to their

environment has not, to our knowledge, been reported. However indirect verification of this characteristic is reported by Samuelson *et al* (1984). Using a time-lapse video system they present evidence that cercariae while swimming in artificial pond water, released glandular contents to the exterior- "cercariae inverted and everted their oral suckers at a frequency of 0.5-1 Hertz, and secreted granular acetabular contents" (see Fig 4. and 5. Samuelson *et al*, 1984).

Three factors, fluorescent quantitation results (Table 4.1. and Fig 4.7.), the proportionately high preacetabular gland content (Dresden and Edlin, 1975) and the time-lapse video studies (Samuelson *et al*, 1984), support the hypothesis that calcium ions, detected in a cercarial suspension of shedding water after a period of 3-4 hours at room temperature, are from the cercarial preacetabular glands.

The observed calcium losses in free swimming cercariae, as detected in this study, may be of relevance if they are occurring in the field. Although a variety of strategies exist which increase the chances of successful vertebrate host location by the parasite (detailed in Chapter 1), it is reasonable to assume that the cercariae will spend some time swimming freely. If calcium is reduced in the glands while locating a host, the proteases may be activated more readily when they are eventually excreted, conferring penetration/maturation advantage to the cercariae. The period between natural shedding and host location may therefore result in the parasite being more suitably equipped for penetration. However the converse would apply if, after a prolonged host searching period, insufficient calcium was present in the glands. Hypothetically in such circumstances proteases could be prematurely activated, causing damage to the cercariae with resultant adverse effect on infectivity.

These suggestions, although speculative, are worth consideration. Clearly the very high level of calcium in the preacetabular glands serves a

purpose. It is not unreasonable therefore to speculate that varying this critical concentration could have consequences for cercarial viability and infectivity. Those parasites which on emission have high calcium ion concentrations in their preacetabular glands may be selectively advantaged.

6.3. Calcium and snail relationship.

The observation that snails lost Ca^{2+} in DCTW water during cercarial emission was initially surprising. We were unaware of any reference to this phenomenon in the literature. Short-term decreases in external calcium concentrations are compensated for by the buffering action of shell calcium in *L. stagnalis* (Greenaway, 1971). Similar mechanisms appear to compensate for depletion of internal calcium in *B. glabrata* soon after parasitism by *S. mansoni* (Davies and Erasmus, 1984, Shaw and Erasmus, 1987).

The suggestion that parasitised snails are not only providing calcium for the developing cercariae, but also losing it during cercarial emergence is most intriguing. At first consideration it appears a waste of calcium. After all the host snail, whether self-imposed, or parasite-induced, has reduced fecundity, stunted growth and a much thinner fragile shell as a result of parasitism. It is generally accepted that these responses are associated with host-parasite competition for energy and calcium reserves. The additional loss during cercarial emission will deplete further the calcium of haemolymph and tissues. This loss will be balanced by the buffering mechanisms of the shell calcium. It is not surprising that parasitised snails should have such fragile shells.

However, it is conceivable that snails could lose calcium by leakage while cercariae are emerging into the water. Microscopic examination reveals individual cercariae vigorously thrashing their bodies to free a tail, still seemingly strongly attached to host tissue. Often groups of cercariae escape in a clear gelatinous mass. Tissue damage, even slight, to the snail could result in body fluids escaping together with haemolymph components into the shedding medium. Although calcium is the main concern in this study, other

ions such as sodium and magnesium may also be lost to the water. In some instances, water is tinged after the shedding period supporting the view that body fluid is lost from the snail during shedding because of physical tissue damage.

Could this release of calcium from the snail during cercarial emergence, therefore, serve some necessary function and not merely be a waste of a valuable resource? Calcium influx is necessary for stimulation of cercarial tail loss and protease release (Hara, *et al.* 1993 and Fusco, *et al*. 1991 respectively). It is suggested that, in the present conditions of soft water, calcium from the snail may contribute to the pool of ions required by the cercariae for successful penetration.

There is no evidence to suggest that calcium ion channels of the snail are adversely affected by schistosome parasites; however this possibility is not ruled out. It would be informative to undertake a time course study of parasitised snails throughout patency. The water could be monitored weekly for calcium during a 2 hour shedding period. Sham shedding prepatent snails will reveal when infected snails first start to lose calcium to their environment.

To finally elucidate interrelationships of calcium, snail, shedding water and cercariae an experiment has been designed employing radioactive tracer studies, the principles of which are outlined in a flow diagram in Fig.6.1. This protocol, using ^{45}Ca and a scintillation counter would assist analysis of three important proposed calcium exchanges. The study could confirm that both shedding snails and swimming cercariae lose calcium to the water. It would also test the hypothesis that any calcium lost from shedding snails contributes to the influx associated with protease release and tail loss during linoleate stimulation.

Whether the release of calcium during cercarial shedding occurs in the field is speculative. The phenomenon may be a consequence of laboratory maintenance. The routine practice of maintaining snails in the dark for a

Fig 6.1

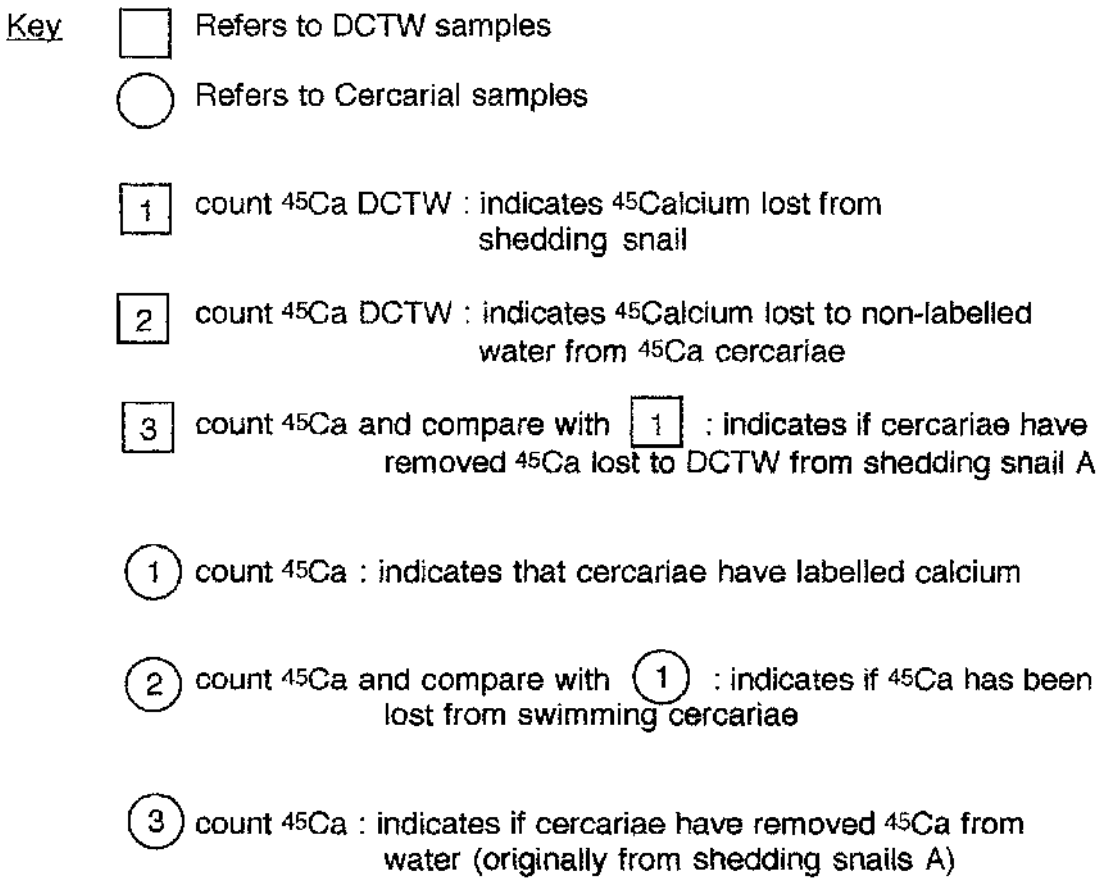
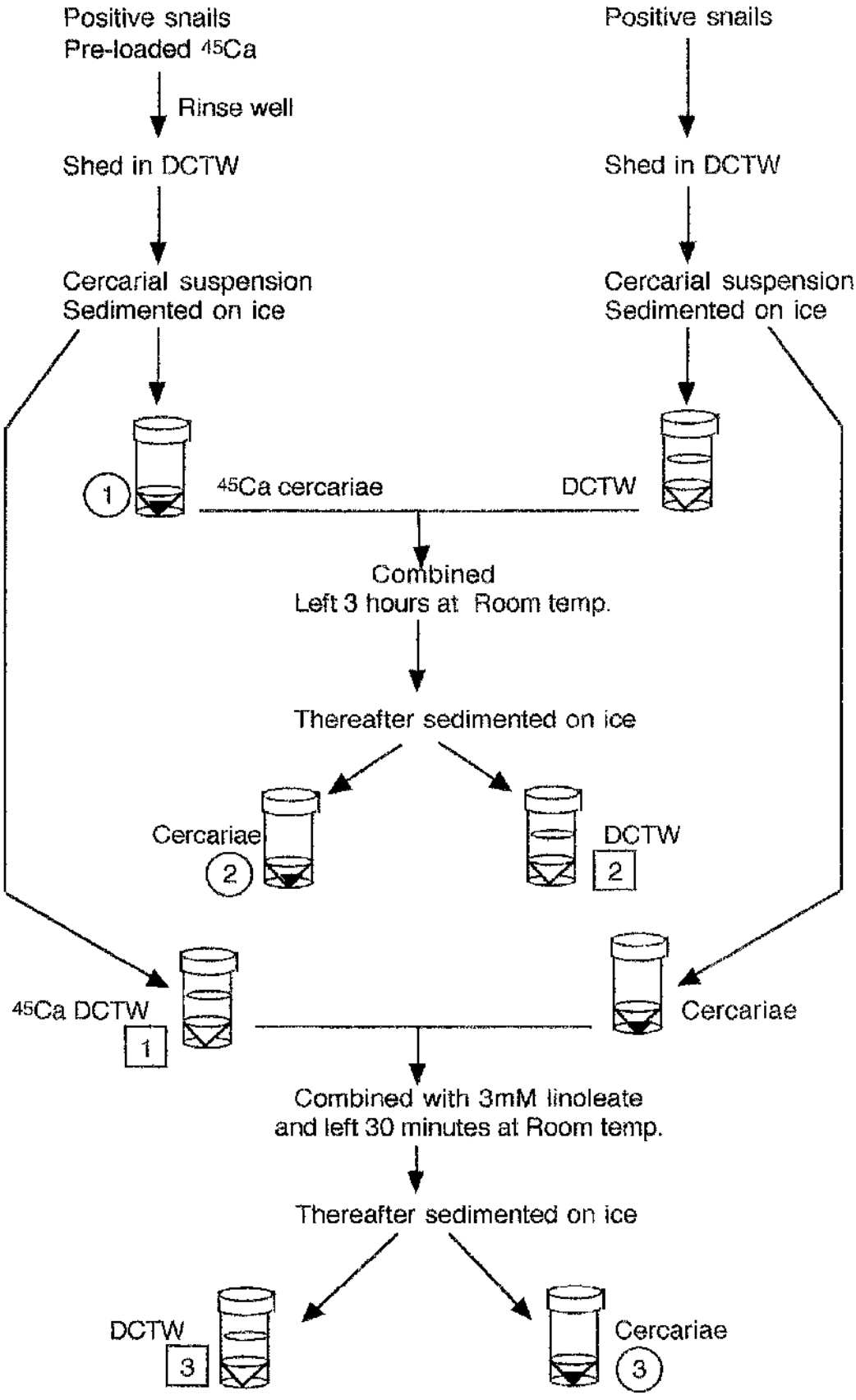

Key
Refers to DCTW samples
Refers to Cercarial samples
1
count 45Ca DCTW : indicates 45Calcium lost from shedding snail
2
count 45Ca DCTW : indicates 45Calcium lost to non-labelled water from 45Ca cercariae
3
count 45Ca and compare with 1 : indicates if cercariae have removed 45Ca lost to DCTW from shedding snail A
1
count 45Ca : indicates that cercariae have labelled calcium
2
count 45Ca and compare with 1 : indicates if 45Ca has been lost from swimming cercariae
3
count 45Ca : indicates if cercariae have removed 45Ca from water (originally from shedding snails A)

Fig 6.1. Principles of experiment to trace calcium flux between water, snail and cercariae.



period prior to shedding, and then exposing them to high light intensity of 2,000 lux, may result in excess numbers of emerging cercariae causing abnormal tissue damage and haemolymph leakage. The field situation, with gradual light fluctuations may stimulate a more natural steady emergence of cercariae with minimal tissue damage.

6.4. EGTA incubation.

(i) PKH26. Cercariae incubated for a 2 hour period in 20 mM EGTA, did not differ significantly from non-treated control cercariae when subsequently exposed to PKH26. Both groups had parasites exhibiting clearly labelled, fluorescent red nerve bodies and cords. In addition some cercariae had overall surface labelling contrasting with those specimens which had no labelling at all. The probe appeared to be selective and further experimentation may indicate its potential as a qualitative label, permitting quick scanning of cercarial populations. To our knowledge this is a novel method of fluorescently labelling the nervous system of actively swimming cercariae.

(ii) ARS. Cercariae incubated for a 2 hour period in 20 mM EGTA, did not differ significantly from non-treated control cercariae when subsequently exposed to ARS. Both groups of cercariae exhibited discrete fluorescent staining of the preacetabular glands indicating the presence of calcium ions. Quantitation studies were not carried out. Lewert *et al* (1966) did not witness inhibition of ARS staining in EDTA treated cercariae until concentrations were such that cercarial vitality was adversely affected. The concentration required was greater than 12mM EDTA, but it was not specified by them.

It would be informative to expose cercariae to a range of EGTA concentrations and, after ARS staining, quantitate the fluorescent emission in the PAG. This would perhaps indicate any calcium ion changes in the glands and elucidate the effect of increasing EGTA concentrations.

(iii) Tail loss. Previously it has been shown that cercarial tail loss, induced by 0.3mM linoleate, was significantly suppressed when cercariae were incubated in 20mM EGTA (Hara *et al* , 1993). It is noteworthy that in their study cercarial swimming behaviour was unaffected by 20mM EGTA incubation, indicating that cercariae appeared otherwise unaffected by the treatment. Both these observations were confirmed in the present study which also indicated that suppression of tail loss was still apparent after 72 hours.

Morphological and biochemical studies (Samuelson and Caulfield, 1985), suggested that the glycocalyx was composed of acidic high molecular weight fibrils which were antigenic. Transformation in medium resulted in more than 60% of the glycocalyx being released. Marikovsky *et al*, (1988b), purified and partially characterised two serine proteases which are secreted by viable transforming schistosomula. They suggested (Marikovsky *et al*, 1988a), that these 28-kDa and 60-kDa proteases were involved in releasing the immunogenic glycocalyx from transforming cercariae, rendering the schistosomulum more refractive to complement killing.

Recent studies, using *in situ* hybridisation with a cDNA probe, localised mRNA for the serine protease to the acetabular gland cells, the first morphologically distinguishable parasite cells that differentiate from embryonic cell masses present in the snail host (Fishelson *et al*, 1992). They also showed, using antiprotease antibodies, that the protease was packaged in vesicles of three morphological types. During penetration they found ruptured vesicles, not only adjacent to degraded epidermal cells and dermal-epidermal basement membrane of the host, but also along the surface of the penetrating larvae. This work confirmed the proposed dual role for the protease in aiding invasion of host skin and helping to remove the glycocalyx . Significantly, Fishelson *et al*, established that the protease, previously considered to be exclusively in the preacetabular glands, was also present in the postacetabular glands. This is considered to be a most intriguing

observation. Secreted postacetabular cell mucins form a film around the cercarial bodies (Stirewalt and Walters, 1973). Exudate, flowing backwards in close proximity to the glycocalyx of swimming cercariae has been recorded on video.

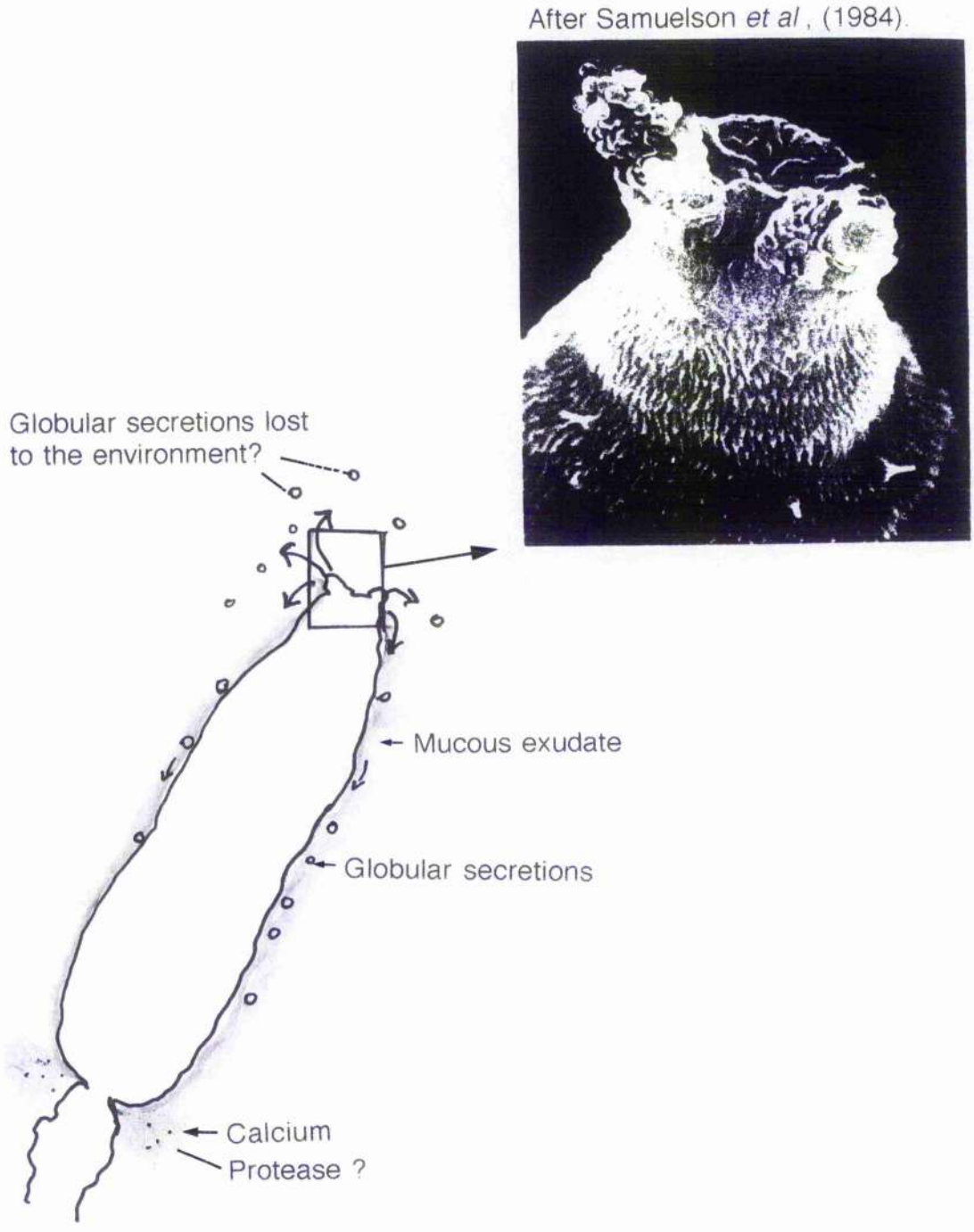
It is now apparent that the initial exudate, which was previously considered to have adhesive, lubricating, protective and enzyme-directive functions only (Stirewalt and Krudenier, 1961), may contain the enzymes. Equally it is suggested that the exudate, seen round the cercarial body after a period of incubation in DCTW in this study, and significantly at the body/tail junction may also contain proteases. This could be verified immunologically with antiprotease antibody. A model of a swimming cercaria is presented in Fig. 6.2.

These observations on the components of the mucin surrounding cercariae, together with the observed calcium in aggregations at the body-tail junction in the present study, may indicate a further protease role. It is postulated that acetabular proteases, necessary for penetration and glycocalyx removal, are also associated with cercarial tail loss. This hypothesis could be tested by studying the effect of protease inhibitors on tail loss.

(iv) Cercarial infectivity. The results of this study suggest that aging cercariae lose calcium to the water. Could this loss of calcium contribute to the variability of infectiveness which is characteristic of cercarial populations? Lewert *et al* (1966), demonstrated that EDTA chelation of *S. mansoni* cercarial calcium significantly depressed both penetration, (21% compared to 89% in controls) and maturation of those cercariae which successfully penetrated (0 compared to 41%in controls).

From this study it is estimated that cercariae swimming for a period of 3-4 hours may lose 20% of their calcium. As a consequence they may only have sufficient calcium to permit penetration (the 21% of Lewert *et al* 1966).

Fig. 6.2. Model of a swimming cercaria with exudate.

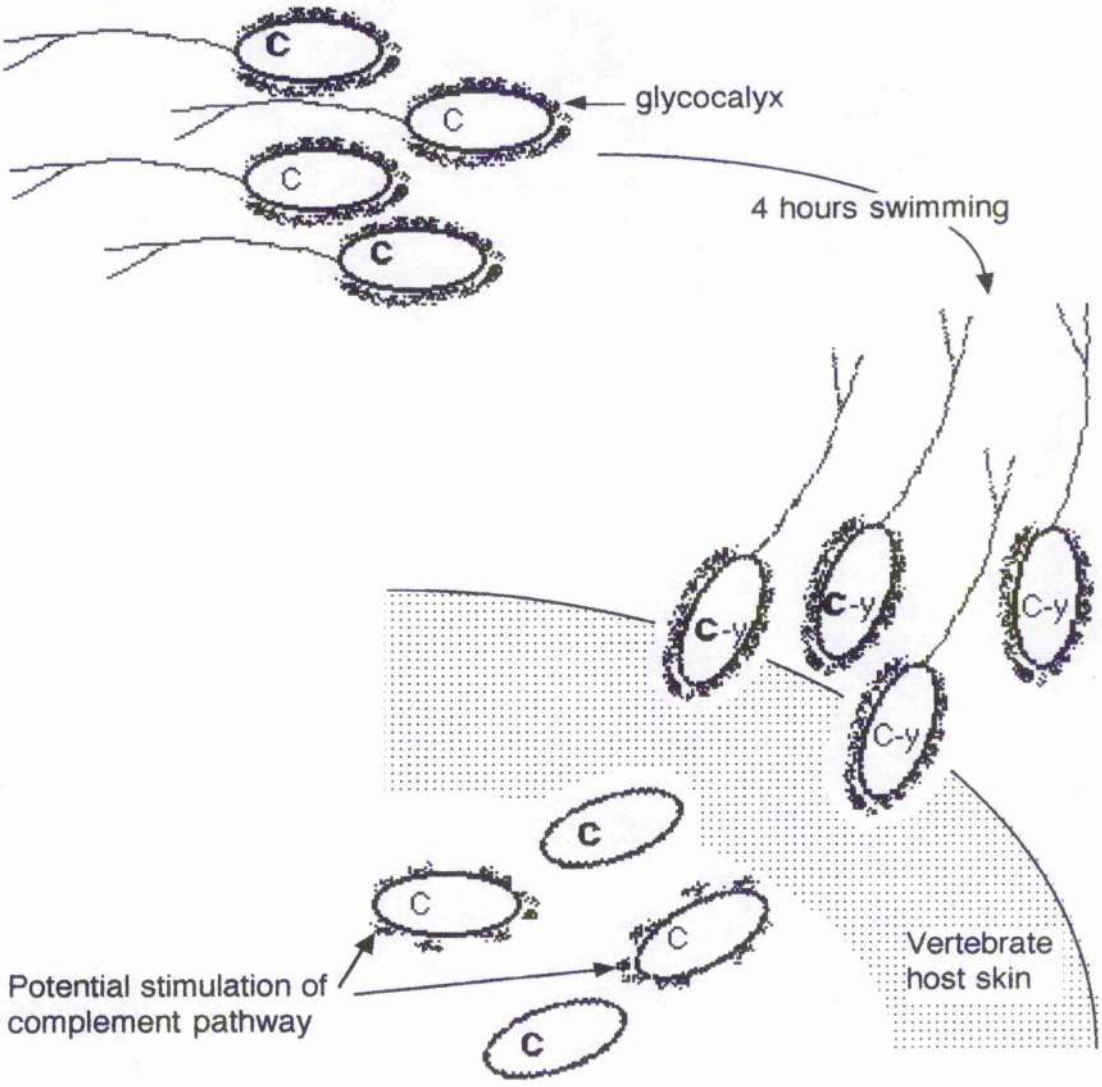


Remembering that proteases and calcium are required for glycocalyx removal, it is now suggested that the cercariae with reduced calcium may not be capable of removing all of the glycocalyx. These cercariae on penetration will stimulate an immune killing response via the complement pathway, and could account for the 0% maturation witnessed by Lewert *et al* (1966) when cercariae were EDTA treated. A diagram summerising this hypothesis is presented in Fig.6.3.

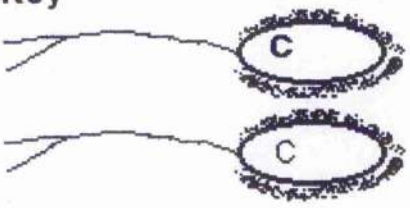
Although calcium was being monitored in this study, it is also possible that the proteases of the preacetabular glands may also be lost to the water during the aging period studied. The loss of two components could have a synergistic effect, and diminish the removal of the glycocalyx, rendering the cercariae more susceptible to immune killing. An experiment designed to determine if aged cercariae, when compared with newly shed cercariae, would have significantly more glycocalyx adhering after transformation is outlined in Fig.6.4. Subsequent infectivity studies, using schistosomula derived from the two skin preparations (Fig.6.4.), could ascertain the significance of adherent glycocalyx in stimulating an immune response in the host with eventual unsuccessful parasite maturation.

In conclusion this study has highlighted the complex relationship between environment, host and parasite production. The novel hypothesis that calcium content of the preacetabular glands could contribute to variability in infectivity has been addressed. The data, has indicated that individual cercariae have variable amounts of calcium in their preacetabular glands (PAG). It has also revealed that both shedding snails and swimming cercariae lose calcium ions to their environment. Given the accepted important role of PAG calcium in sucessful host penetration, it is suggested that this loss of PAG calcium may contribute to variability in parasite infectivity. Future experimentation has been described to elucidate the role of calcium in successful maturation in the vertebrate host.

Fig. 6.3. Model of cercarial penetration.



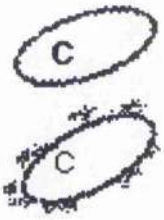
Key



Cercariae with maximal Calcium on emission from snail.

Cercariae with minimal Calcium on emission from snail.

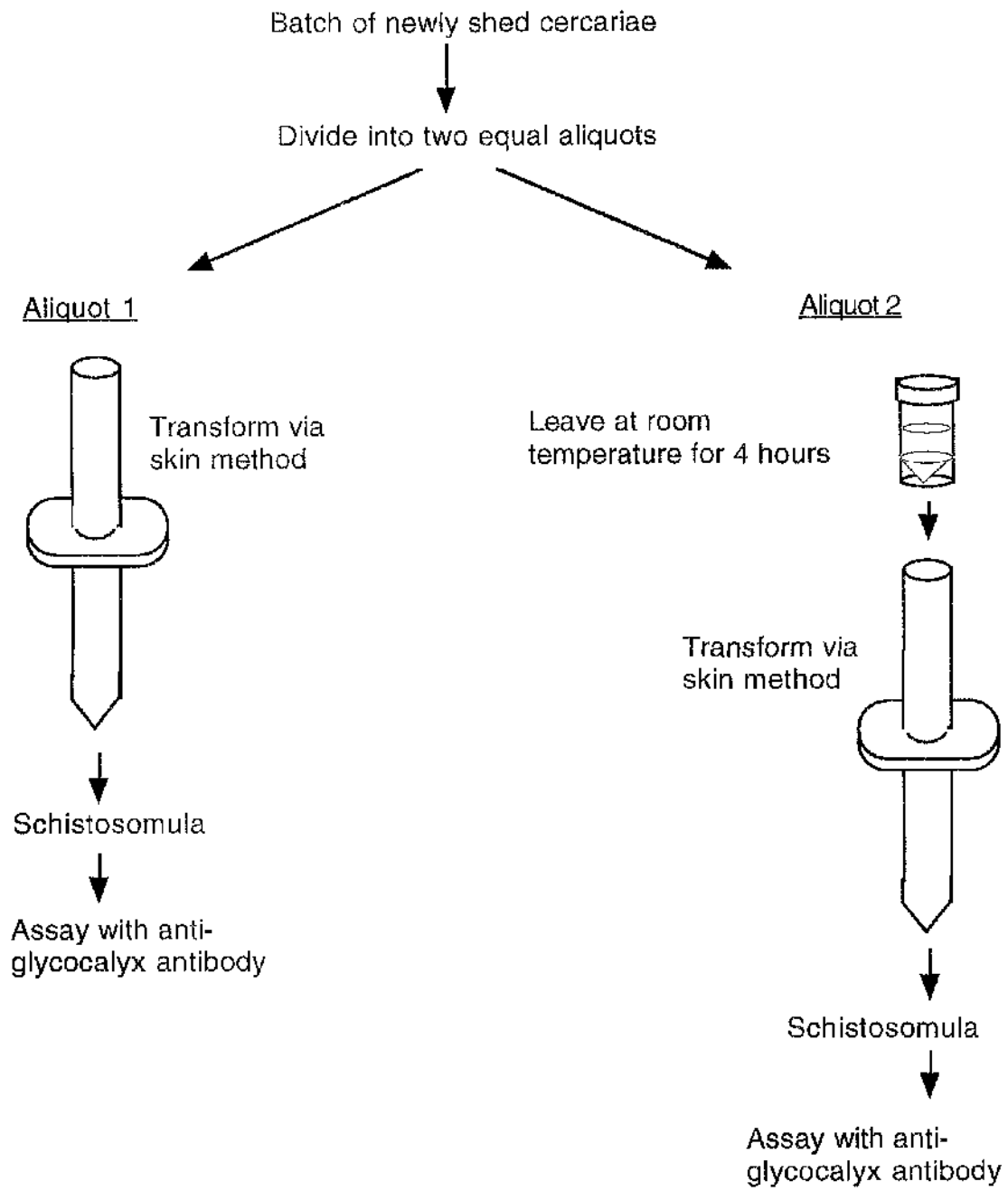
y Calcium lost during 4 hour swimming period.



Schistosomulum with glycocalyx removed.

Schistosomulum with remnants of immunogenic glycocalyx attached.

Fig. 6.4 Experiment to test the hypothesis that aged cercariae will have glycocalyx after penetration.



Compare the two preparations to determine if more glycocalyx is present in schistosomula derived from aged cercariae.

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