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STUDIES ON THE EFFECTS OF ENVIRONMENTAL FACTORS AND SELECTED PATHOGENS ON MORBIDITY AND MORTALITY OF HATCHERY REARED *OREOCHROMIS MOSSAMBICUS* (PETERS) EGGS AND FRY

A thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy

by

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May 1986

5/87

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M A L I

A N D

R U W A N ____

DECLARATION

I hereby declare that this thesis has been compiled by myself and is the result of my own investigations. It has neither been accepted, nor is being submitted for any other degrees. All the sources of information have been duly acknowledged.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Christina Sommerville for her invaluable advice, guidance and support throughout this project and for her critical review of the manuscript. I am extremely grateful to her.

I wish to thank Dr. G.N.Frerichs for his advice and help on microbiology and Dr. Mary Tatner for her support on immunology.

Thanks are also due to Dr. N.J.Dix for his guidance on mycological techniques and fungal identifications. I acknowledge Drs. R.H.Richards, J.F.Muir and D.J.Alderman for their support in related fields.

I received technical and secretarial assistance from a number of people and thank Iain MacGowan, Keith Ranson, William Hamilton, Stuart Millar, Mary Alexander, Paul Thomson, William Struthers, Elizabeth Stenhouse, Ian MacRae, Sheila MacEwan, Beatrice Dale and Laura Cumming.

My sincere thanks go to Krishan J. Rana for his help on various aspects of the project. I would also like to acknowledge the help of Patrick Chipungu. Furthermore, I thank all my friends and colleagues for their support and encouragement throughout.

I gratefully acknowledge the British

Council for their sponsorship and thank its staff, both in Edinburgh and London.

Finally I wish to thank the head of the Depart ment of Zoology, University of Colombo and my colleagues for their support during my absence.

ABSTRACT

The dynamics of the physical, chemical and microbiological parameters of hatchery water and their effects on the hatchability and survival of artificially incubated eggs and fry of O. mossambicus in two different recirculatory systems was studied. The tolerance levels of fry stages to important physical and chemical parameters of hatchery water were also estimated. The susceptibility of yolksac fry appeared to increase as they developed and utilised yolk.

The egg mortalities were found to be initiated by mechanical injuries to the chorionic membrane which subsequently attracted bacterial colonisation; the primary cause of death. Fungal infections appeared to have only a secondary role. This mechanism was elucidated using microbiological and SEM techniques.

Chemical disinfectants and ultraviolet sterilisation of hatchery water were tested in controlled experiments and an assesment of their relative efficiency in improving hatchability was made.

Anti-bacterial activity of the buccal and skin mucous of brooding O. mossambicus females was investigated with the view to understanding the protection offered to the progeny whilst brooding. No antibacterial activity could be demonstrated.

Experimental infections of O.mossambicus with I.multifiliis were carried out and lethal and sublethal levels of infections for various age groups of fish were estimated. The sequential pathology of sublethal infections of I.multifiliis on O.mossambicus fry was studied.

Adult O.mossambicus females were effectively immunised against I.multifiliis by controlled infections. Antiparasitic factors were demonstrated in both serum and mucous only in immunized fish. Evidence for a specific acquired immune response of humoral origin was found and its implications for aquaculture were discussed.

A protection offered to the fry by the immunized females against I.multifiliis infections whilst brooding was demonstrated. This appeared not to operate through a transfer of passive immunity from mother to fry. Alternative mechanisms are suggested.

CHAPTER 1.

GENERAL INTRODUCTION.

According to Fryer and Iles (1972) the culture of tilapia is believed to have originated some 4,000 years ago, 1,000 years before carp culture was first initiated in China. However, the first recorded work on scientifically orientated tilapia culture was conducted in Kenya as early as 1924 (Meschkat 1967). The tilapias were later introduced and became established as potential farm species in the Far East (Ling 1977) and North America (Iverson 1976) during the 1940s and 1950s respectively. In the late 1950s this "miracle fish", tilapia, was found to be widely distributed around the tropics. Since then, to meet the rising demand for fish protein, tilapia farming has become an expanding and developing interest in many tropical and subtropical countries of Central and South America, Africa, the Middle East and South and South East Asia. Recently tilapia have even been successfully produced in Europe and North America in geothermally heated waters (Lauenstein 1978 and Ray 1978). According to FAO (1978), the total world production of tilapia reached 197,000 tons in the year 1977 and the current production is estimated as high as 60,000 tons per year in the South East Asia alone (Macintosh pers. com.).

Tilapias are not only considered to be

important for their food value but have also been used as efficient biological agents in weed and mosquito control, play a minor role as a bait fish, as a sport fish in angling dams and as an occasional addition to tropical aquaria as a pet fish (Balarin and Haller 1982).

The fish grouped as tilapias within the family cichlidae were initially placed in the genus _Tilapia_ A.Smith, but since the 1920s there have been attempts by taxonomists to divide them into a number of smaller groups. More recently, as the information about the fishes' biology became known, Trewavas (1982) divided the tilapias into three genera; _Tilapia_ A.Smith, _Sarotherodon_ Ruppell and _Oreochromis_ Gunther based on their differences in breeding and brooding behaviour. The genus _Tilapia_ is comprised of substrate spawners, _Sarotherodon_ includes paternal and biparental mouth brooders and _Oreochromis_ is confined to maternal mouth brooders. This new definition is followed throughout in this manuscript.

The number of tilapia species cultured is quite large. Balarin and Hatton (1979) listed 23 species but only two _Tilapia_ species and three _Oreochromis_ species have been widely used in aquaculture (Hepher and Pruginin 1982). These are _Tilapia_ _rendalii_ (Boulenger), _Tilapia_ _zillii_ (Gervais), _Oreochromis_ _mossambicus_ (Peters), _Oreochromis_ _niloticus_ (Linnaeus) and _Oreochromis_ _aureus_ (Steindachner).

Tilapias, though they are worthy food fishes, confound an important dilemma in aquaculture. The characteristic early maturation and prolific breeding produces large numbers of fry, causing "stunting" of the entire population (Lowe-McConnell 1982; Hepher and Pruginin 1982). This single phenomenon under tropical pond conditions is perhaps the greatest disadvantage in the use of indigenous cichlid species for aquaculture in many parts of the world (Okorie 1975 and Pillay 1979).

Intensive or semi-intensive culture requires a regular supply of large numbers of quality fry. Though tilapias have a reputation as prolific breeders, the controlled production of fry is still in its infancy (Balarin and Haller 1982). This inadequate supply of fry has often resulted in the slow expansion of intensive and semi-intensive culture of tilapias. Thus the fry production itself has become an important event in tilapia culture.

Recently it was considered that the design of systems for mass fry and fingerling production is the single most important requirment for the tilapia culture industry (Pullin and Lowe-McConnell 1982). In many countries tilapia farmers still rely on natural pond spawning for their fry supply. The disturbance of spawning activity of the brooding females by regular seining of ponds for fry harvesting, together with high predation of fry by fingerlings and brood-stock often results in low fry

production (Rana 1986a).

Hatcheries can provide exact quantities of tilapia fry of the species, age and the size most useful to the fish farmer. Under controlled hatchery conditions the genetic quality of the fry produced can be assured and the genetic selection for the desired qualities, ie. fast growth, better flesh quality, disease resistance etc. is permitted. Further, the synchronised breeding of females and selective feeding of fry are possible, the production of fry of uniform size and age can be guaranteed. Also the controlled breeding and rearing allows for hybrid crossing to provide all-male offspring and hormonal or similar treatment of fry to induce sex reversal or sterilisation to produce monosex fry. However, as tilapias have a relatively low fecundity, to produce the desired number of fry to meet the demand needs management of large numbers of brood-fish.

Various hatchery systems have been used for tilapia fry production. Simple net breeding cages "hapas" have been used in different countries such as the Philippines (Guerrero and Garcia 1983), Kenya (Dadzie 1980) and the United States (Hughes and Behrends 1983) with varying success. The use of different sized tanks has also been tested (Uchida and King 1962; Shelton, Hopkins and Jensen 1978). However, all these operations have disadvantages such as extensive brood-stock and fry handling, cannibalism (Silvera 1978; Berrios-Hernandez and

Snow 1983) and lack of complete control over the fry from their very early life.

Haller and Parker (1981) described an advanced circular arena system for tilapia fry production. The design was meant to provide an environment for the brood stock to behave as naturally as possible. It was also expected to optimise fry production and minimise fry handling. However, according to a recent report by Balarin and Haller (1982) fry production still remains low.

In nature Oreochromis females brood developing eggs and early fry in the buccal cavity. The final release of fry by the females depends mostly on social factors such as securing a territory which is often difficult to control under artificial hatchery rearing conditions. This leads to problems such as starvation of fry within the buccal cavity and increased inter-spawning intervals. In contrast, if the eggs and/or fry are removed from the buccal cavity of the brooding female, the spawning frequency can increase without affecting the subsequent clutch size (Lee 1979; Rana 1986b). Though this itself is very significant in the mass production of tilapia fry, the removed eggs and/or fry need to be artificially incubated and reared.

Oreochromis females exhibit a long period of parental care during which they protect and aerate the eggs, and later the young, within their mouth. Throughout

the brooding period the contents of the mouth are well aerated by the normal respiratory current of the female, which inevitably bathes the eggs or young as it passes through the mouth. The eggs are also moved around the mouth by a "churning" movement which help to keep their surfaces clean. Eggs removed from the mother's mouth can be reared artificially only if they are provided with a well-aerated environment and are kept in continuous motion (Fryer and Iles, 1972). Thus artificially reared eggs and fry need special systems to simulate this activity during the incubation.

Shaw and Aronson (1954) were the first to investigate the possibility of artificial incubation of *Oreochromis* eggs and the associated problems. Recently various workers such as Valenti (1975); Rothbard and Pruginin (1975); Lee (1979); Rothbard and Hulata (1980); Mires (1982); Snow, Berrios-Hernandez and Ye (1983) and Rana (1986a, 1986b) utilised various systems comprising trays, shaking tables, standard fish egg incubators such as "sugar-jars", McDonald jars and plastic cylindrical incubators to incubate *Oreochromis* eggs artificially.

However, the success reported was inconsistent. If the success and the efficiency could be improved it is most desirable to use artificial incubation together with aquaria or tank based hatchery systems, as it provides complete control over the fry from their very

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However, the success reported was inconsistent. If the success and the efficiency could be improved it is most desirable to use artificial incubation together with aquaria or tank based hatchery systems, as it provides complete control over the fry from their very

early life. This is especially important in the hormone treatment and similar operations to obtain all-male or sterile offspring, where it is essential to acquire control over the fry before their first feeding. The major drawback of carrying out such intensive hatchery operations is the considerable lack of knowledge of the host-pathogen-environment interactions which cause morbidity and mortality of eggs and fry under such conditions.

According to Pullin and Lowe-McConnell (1982) "...... Sustained research on the parasites and diseases of tilapias and their pathology is essential as the culture industry continues to expand. Acclimatisation and stress reactions of fish in culture systems are poorly understood and have implications for growth, survival, reproductive performance and susceptibility to pathogens. Studies in both areas are needed ....".

Though the intensive farming of tilapias is still in its infancy and much of their biology and culture is yet to be understood, the salmonid culturists were aware of the salient problems associated with artificial rearing, well over a half a century ago. Agersborg (1933) reporting on these salient problems in the artificial rearing of salmonids stated "....... It is necessary for the fish culturists to realise that all fish have a long racial history during which they have become adapted to certain physical and chemical conditions of their environment. This

characteristic is directly reflected in their physiological make up. When, therefore, fish are being reared artificially the culturist must realise that if he is to rear succesfully his quota of fish, he must not only eliminate any natural enemies, but see to it that the artificial environment is as good as the natural environment ....".

In *Oreochromis* hatchery operations which involve artificial incubation of eggs and rearing of fry, the artificial environment has to be as natural as possible to obtain a reasonable success, as the incubation is unusual in this case since it is carried out by the female parent. The artificial incubation is generally carried out in recirculatory water systems. Thus a better understanding on the dynamics of the physical, chemical and microbiological qualities of the hatchery water, together with the interactions between the environment, pathogens and the eggs and larvae is indispensable.

The effects of water reuse in fish hatcheries has been fairly well documented (Burrows 1964; Larmoyeux and Piper 1973; Mayer and Kramer 1973). Larmoyeux and Piper, (1973) demonstrated that the reduction in quality of hatchery water by reuse was detrimental to fish. Many hatcheries are designed to reuse water, and during this process, metabolic products of the fish are being added to the water and continue to accumulate. These metabolic

products, especially nitrogenous compounds, should not be allowed to build up in recirculatory systems. Proper system design and maintenance can reduce this accumulation of organic matter which may decrease oxygen and form a substrate for the potentially pathogenic bacteria and fungi. The depletion of oxygen and production of carbon dioxide can be overcome by aeration but a potential problem arises from the excretion of nitrogenous waste. Ammonia is the common form of nitrogen excretion in fish. Normally ammonia is oxidised to nitrite and then to nitrate via nitrification; nitrate is relatively nontoxic whilst ammonia and nitrite are proven fish toxicants.

The effects of ammonia and nitrite on the fish under artificial rearing conditions has been well documented (Russo and Thurston 1977; Alderson 1979; Sadler 1981; Colt and Armstrong 1981; Haywood 1983; Thurston, Russo, Luedtke, Smith, Meyn, Chakoumakos, Wang and Brown 1984; Soderberg, 1985). However, the understanding of the effects of these substances on the hatchery reared tilapias is surprisingly limited. There seems to be no literature available on the effects of ammonia and nitrite on the eggs and very early fry of Oreochromis species.

The microbiological quality of the hatchery water also plays an important role in survival of fish. Numerous potential fish pathogens, for example Pseudomonads, Aeromonads and myxobacteria are present in

nutrient rich water (Snieszko 1974). In recirculatory water systems where high organic input is evident, the increase in nitrogenous compounds causes chemical eutrophication which results in an increase in the abundance of the microbial flora. The understanding of the effects of such changes in microbial flora on the hatchery reared eggs and fry is vital, as they are obviously under stress due to exposure to the artificial environment. Further, the dynamics of such microbial populations, which include potential pathogens, in relation to the physical and chemical qualities of the water is not well understood in recirculatory hatchery systems. As the fish eggs and larvae are prone to fungal and bacterial infections, research into such occurrences is essential.

Not only are the environmental aspects of tilapias little understood but the diseases of tilapias have been less well studied than those of many other groups of cultured fish (Roberts and Sommerville 1982). Although a vast array of parasites have been recorded from tilapias and other cichlids, most of them are from wild fish and no evidence of clinical effects is given (Roberts and Sommerville 1982). Out of this vast array of recorded parasites, Ichthyophthirius multifiliis (Fouquet) is one of the most important parasites of cultured tilapias (C.Sommerville pers. com.). It is a significant problem for fry since they are particularly susceptible to infection.

According to a revised classification of protozoa (Levine 1980), Ichthyophthirius can be classified as follows.

Class	Olygohymenophorea
Sub Class	Hymenostomatia
Order	Hymenostomatidia
Sub Order	Ophryoglenina
Genus	Ichthyophthirius
Species	multifiliis

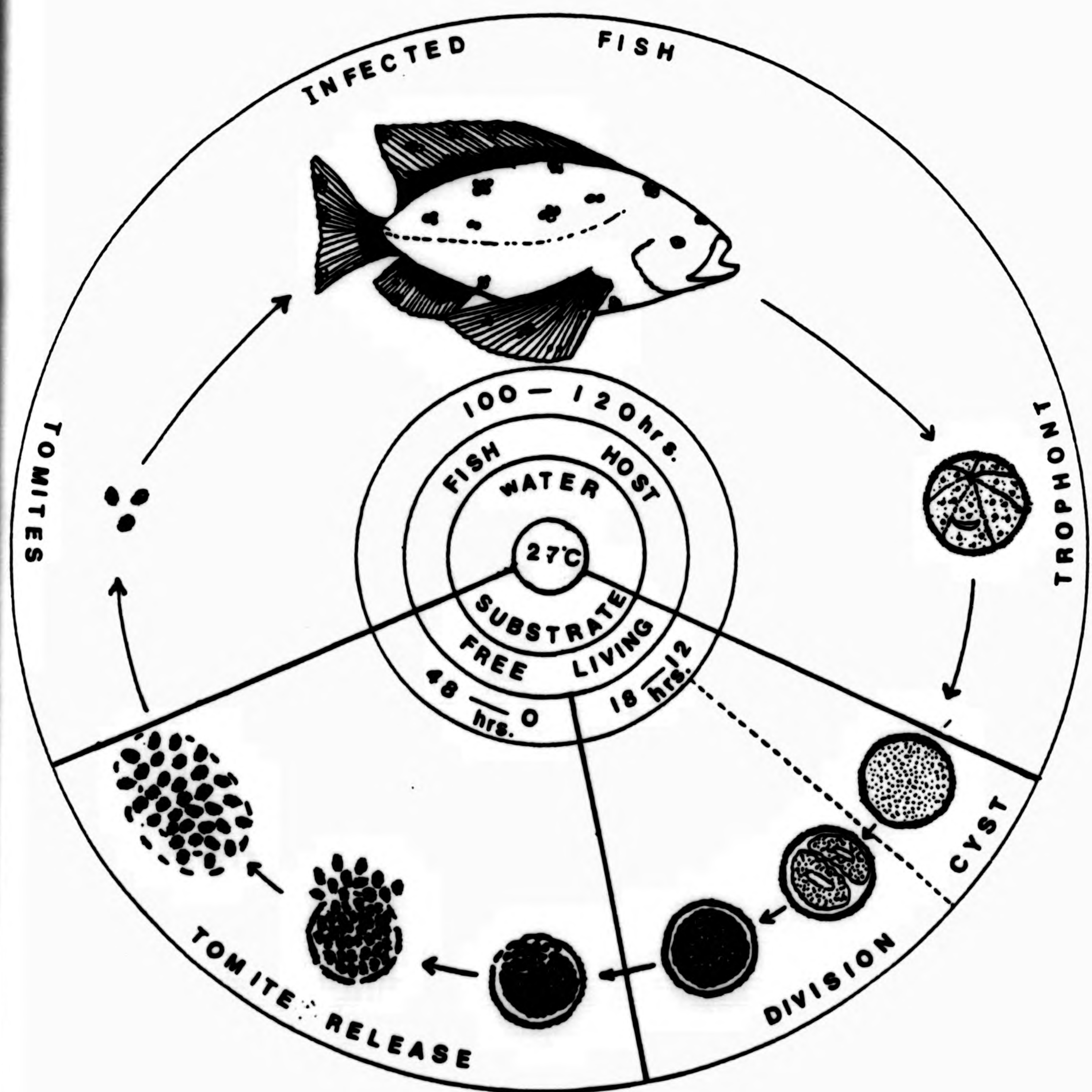
I.multifiliis is reported to be one of the most serious parasites of fresh water fish (Bauer 1958; Meyer 1969; Pearson 1970; Hines and Spira 1973a), and is considered a serious threat in intensive aquaculture. Mature parasites leave the fish host, encyst and produce large numbers of infective tomites (Figure 1). As intensive aquaculture practices create an environment which favours the rapid spread of the disease, I.multifiliis causes very high morbidity and mortality of cultured fish.

This parasite is cosmopolitan in distribution (Bauer 1958; Paperna 1972; Nigrelli, Pokorny and Ruggieri 1976; Hoffman 1978) and causes epizootics in wild fish populations too (Allison and Kelly 1963; Hoffman 1967; Chappell and Owen 1969).

The biology of the parasite has been well documented by many in the past including MacLennan (1935, 1937, 1942), Schaperclaus (1954) and Bauer (1959). The

Figure 1.

The life cycle of _I. multifiliis_ at 27'C.



(Based on the author's personal observations)

physiology, pathology and acquired immunity to the parasite has been quantitatively demonstrated by Hines and Spira (1973a, 1973b, 1974a, 1974b and 1974c).

Traditional methods of control by chemotherapy have been established (Meyer 1969; Cross 1972; Hoffman and Meyer 1974; Farlley and Heckman 1980), but they are not always effective, may cause pollution, can be costly and may be subjected to prohibitive legislation.

Though various species of fish have been shown to mount an immune response to the parasite (Hines and Spira 1974c; Govan, Dawe and Gratzek 1980; Wahli and Meier 1985), newer techniques of control using vaccines are still in an infant stage of development and many problems remain to be solved.

The severity of the reported outbreaks in culture systems (Butcher 1947; Johnson 1961; Bogdanova 1976; Valtonen and Keranen 1981), together with the considerable lack of information of Ichthyophthiriasis in cultured tilapias, generated a significant interest into this area of research.

Having understood the potential importance of the research into the environmental, pathogen and host interactions and relationships in recirculatory water tilapia hatcheries, the present investigation was designed to elucidate the following.

1. Dynamics of the microbiological, physical and chemical qualities of the water and their significance in the morbidity and mortality of eggs and early fry of O. mossambicus under recirculatory artificial hatchery conditions.

2. Host parasite interactions between O. mossambicus and I. multifiliis with special reference to the acquired and passive immunity.

CHAPTER 2.

GENERAL MATERIALS AND METHODS.

The methods commonly employed throughout the period of study are described in this chapter. The more specific methods exercised have been described in the appropriate chapters.

2.1. THE BROODSTOCK FISH.

Genetically pure O.mossambicus (as identified by McAndrew and Mujumdar 1983) supplied by the Tropical Fish Facility of the Institute of Aquaculture, University of Stirling were utilised throughout the investigation.

2.2. BROODSTOCK MANAGEMENT.

Individually tagged fish were maintained in a series of square glassfibre aquaria (120x120x50cm) supplied with recirculatory water. The quality of the water was maintained by means of a biological filtration unit together with a series of settling tanks filled with plastic biofilter rings. The detailed diagrams of the system are given in Figures 2 and 3.

A temperature of 27+1'C was maintained using a thermostatically controlled immersible heating element placed in the header tank. Each aquarium was individually aerated by diffusion of compressed air through an air

Figure 2.

Side elevation of the broodstock system.

P : Submersible water pump

S : Sump tank

H : Header tank

GA : Glassfibre aquaria

ST : Settling tanks

SP : Stand pipe

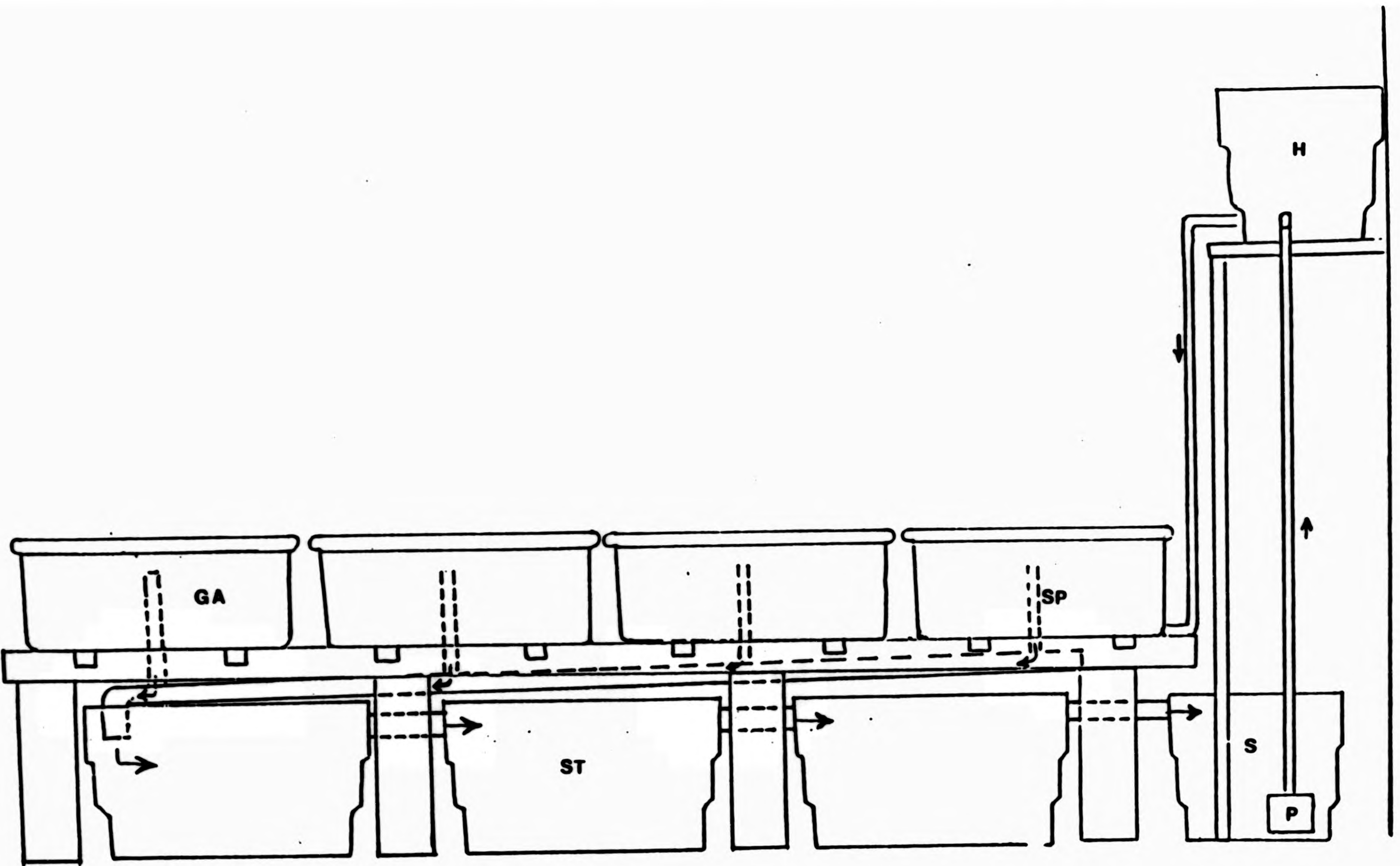

H
GA
SP
ST
S
P
1m

Figure 3.

Plan view of the broodstock system

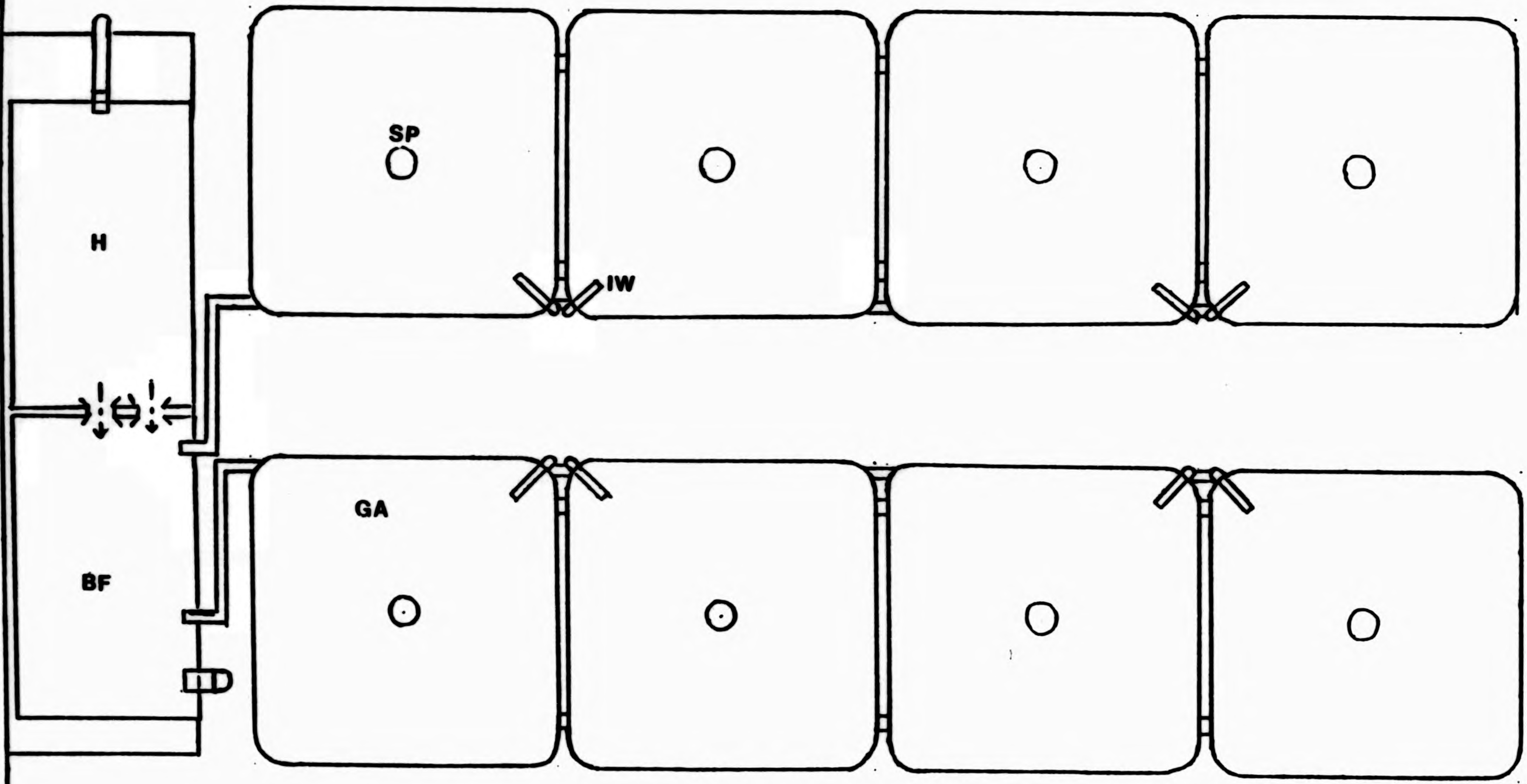
H : Header tank

GA : Glassfibre aquaria

IW : Inflow of water

SP : Stand pipe

BF : Biological filter



H
BF
SP
IW
GA
1m

stone. A photoperiod of 12 hours light and 12 hours dark was maintained by means of a time switch. Major water quality parameters such as dissolved oxygen (DO), pH, total ammonia-nitrogen (NH3-N), total nitrite-nitrogen (NO2-N) were monitored closely. On some occasions the recorded low pH levels were adjusted immediately by placing finely chopped oyster shells in the settling tanks. Care was taken to ensure good water quality and partial water changes were undertaken when necessary. The lower and upper levels of the water quality parameters measured during the period of investigation are described in Appendix 1.

Not more than 15 fish were stocked in an aquarium at any one time. In the case of the broodstock, maintained for the collection of eggs and fry, a limit of three females to one male was adhered to throughout. Fish were fed twice daily with No.4 commercial trout pellet (Ewos Baker Ltd., Bathgate, Scotland) containing 40% crude protein at a ration of 1-2% body weight per day.

2.3. COLLECTION AND HANDLING OF EGGS.

The broodstock females were checked twice daily to see if they were carrying eggs. As a general practice the eggs were removed from the buccal cavity between 12 and 24 hours post spawning unless otherwise for a special reason. The carrying females were individually netted out into a bucket of water using a hand net. Extreme

care was taken to ensure that the brooding female was disturbed as little as possible, as a slight disturbance during this process would cause the female to eject the eggs from the buccal cavity. The clutch of eggs was carefully removed by opening the mouth whilst the fish was under water. The eggs were then siphoned out into a plastic trough. The length and the weight of each individual female together with the clutch size were recorded at each spawning.

A sample of 50 or 100 eggs, depending on the size of the clutch, was then fixed in Bouin's fixative for estimating fertility of the clutch. As Bouin's fixative produces differential colouration of the developing embryo and the remaining yolk, it was possible to estimate the percentage fertility of the clutch by this technique. The remainder of the clutch was transferred to the incubation system.

2.4. THE INCUBATION SYSTEM A.

The schematic diagrams of the incubation system used are given in Figures 4 and 5. This was a recirculatory water system consisting of a sump tank, header tank, settling tank with plastic biofilter rings, trickle gravel biological filtration unit and a series of 12 litre transparent perspex aquaria (40x16x20cm). The water was pumped from the sump tank to the header tank by

Figure 4.

Side elevation of the Incubation System A.

P : Submersible water pump

S : Sump tank

H : Header tank

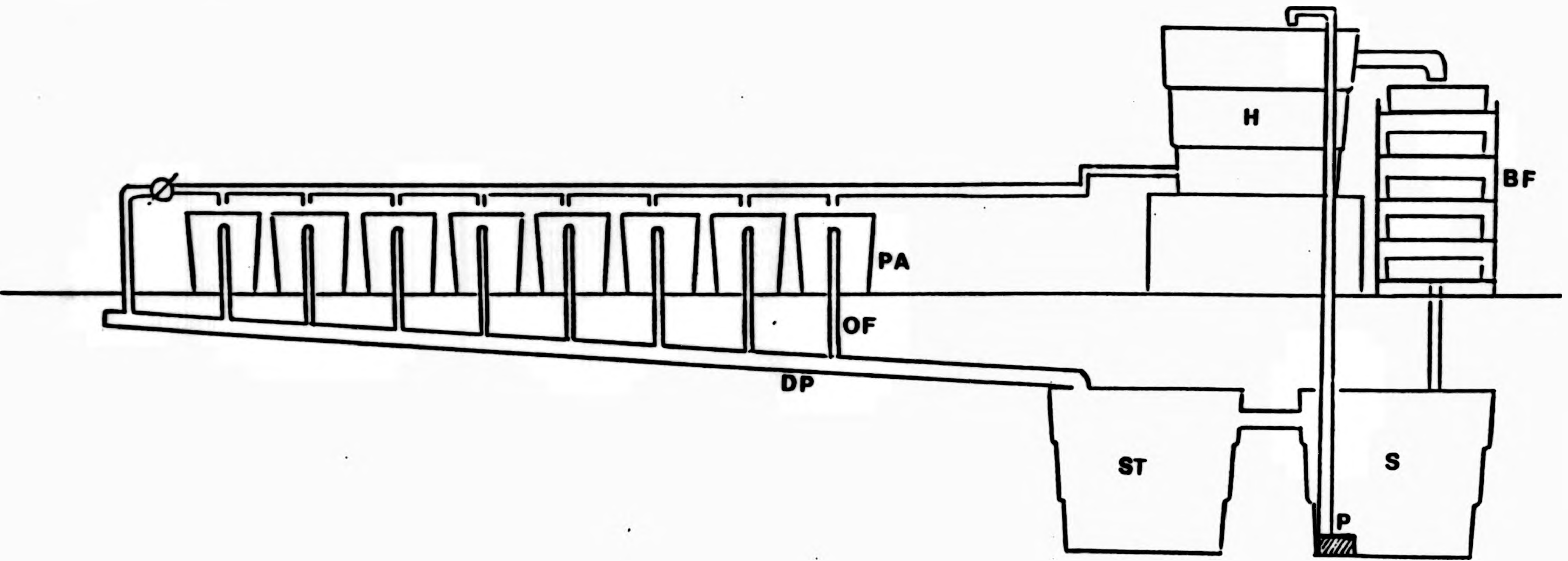
BF : Biological filtration unit

PA : Perspex aquaria

OF : Out flow of water

DP : Drain pipe

ST : Settling tank


H
BF
PA
OF
DP
ST
S
P

means of a submersible water pump (Otter by Beresford). The water was then gravity fed into the perspex aquaria and was then collected back in the sump tank via the settling tank through a common drain. A temperature of 27±1'C was maintained using several thermostatically controlled immersible aquarium heaters (Microtronic by Armitages) placed in the header tank. The water in the system was well aerated by means of compressed air diffused through air stones.

Four plastic cylindrical jars (0.75l) were placed in each perspex aquarium and used as incubators for the eggs. Each incubator was individually supplied with a jet of water through a small plastic tube mounted on to a controllable two-way valve (Fig.5). Gentle agitation of the eggs was ensured by controlling the water flow into the incubators. As the height of the incubators was greater than that of the water column in the perspex aquaria, the inflow water to the incubators continuously overflowed into the perspex aquaria and was then collected in the settling tank via a common drain pipe. Small volumes of water were added periodically to compensate for the water lost through evaporation.

This incubation system will hereafter be referred to as Incubation System A.

Figure 5.

Detailed diagram of the incubators in the Incubation System A.

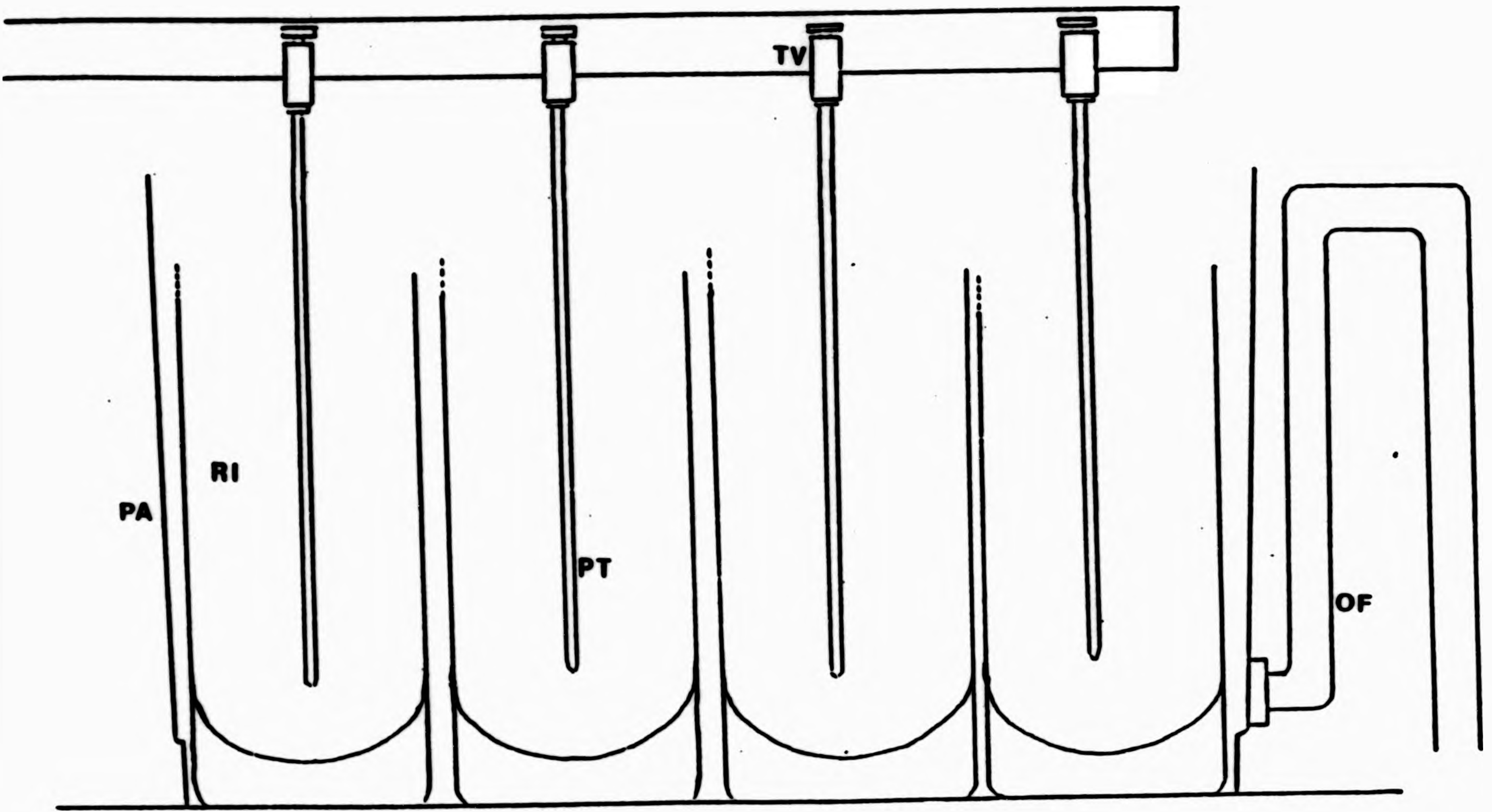
PA : Perspex aquarium

RI : Round-bottomed incubators

PT : Plastic tube (supplies water into the incubator)

TV : Two way valve (regulates the water flow into the incubator)

OF : Outflow of water into the drain pipe.


TV
RI
PA
PT
OF
30 cm

2.5. WATER QUALITY ANALYSIS.

Major water quality parameters such as DO, pH, NH3-N and NO2-N were measured during the course of the study. The standardised methods employed are given in Table 1.

2.6. MICROBIOLOGICAL PROCEDURES.

During the course of study microbiological procedures including estimation of viable bacterial and fungal spore numbers and identification of bacteria and fungi were carried out. The procedures commonly used are described below.

As the majority of bacterial fish pathogens are fastidious in their nutrient requirements, Tryptone Soya peptone Agar (TSA) is the medium of choice in most fish pathology laboratories. TSA supports the growth of all aeromonads, freshwater vibrios, pseudomonads, flavobacteria, enterobacteria, pasturellae, streptococci, micrococci and nocardiae likely to be encountered in fish diseases (Frerichs 1984). This medium was therefore used for the estimation of bacterial numbers as well as for isolation and identification of bacteria during the present investigation.

2.6.1. Estimation of total viable heterotrophic bacteria (TVHB).

TABLE 1.

The methods used for the measurement of the physicochemical qualities of the water.

Parameter	Method	Reference
Temperature and DO	DO/Temperature meter	Yellow Springs Instrument Co., Yellow Springs, Ohio. Model 57. Temperature accuracy 0.5 °C DO accuracy 0.1 mg/l
pH	pH/Salinity meter	Clandon Scientific Ltd. Lysons Avenue, Hampshire. Accuracy 0.05 pH unit
NH3-N	Phenol-Hypochlorite method for fresh water	Golterman, Clymo and Ohnstad (1978).
NO2-N	Sulphanilamide method	Mackereth, Heron and Talling (1978).

As a routine practice water samples were collected in duplicate in sterile stoppered bottles. Ten-fold serial dilutions of the samples were made using sterile distilled water and 0.5 ml aliquots were innoculated onto TSA (Difco) plates in duplicate. The spread-plate technique was adopted and plates were incubated at 20'C for a period of 72 hours. After incubation all plates were examined and the plates containing between 50 and 200 colonies were selected and counted using a digital colony counter (Gallenkamp). The mean of the duplicates was calculated and the TVHB was expressed in terms of numbers per millilitre of aquarium water.

2.6.2. Isolation and identification of bacteria.

The water samples were innoculated onto the TSA plates as described in section 2.6.1. The resultant growth was divided into groups using colonial appearance. Three to five representatives from each group were sub cultured on TSA to ensure that pure cultures were obtained. These pure cultures of bacteria were then identified using the standard procedures described by Cowan and Steel (1974) and Frerichs (1984). Certain identifications were confirmed by comparison with reference bacteria obtained from the Institute of Aquaculture, University of Stirling.

2.6.3. Estimation of total viable fungal spores (TVFS).

For the estimation of TVFS counts in the hatchery water, various methods were tried during the preliminary investigations. Because of the consistency of the estimates obtained, the following method was devised throughout the study.

Water samples were taken in duplicate in sterile stoppered bottles. These were diluted using sterile spring water to give a series of ten-fold dilutions. Ten ml of each dilution was filtered through an 8 um sterile membrane filter (Milipore) using a Buchner funnel with vacuum extraction. The filter membrane was carefully removed and placed face down on a Malt Extract Agar (MEA by Difco) plate into which was incorporated 200 IU per ml Penicillin with 200 ug per ml Streptomycin (Flow Laboratories). The trapped fungal spores were allowed to germinate into the agar medium during incubation at 20'C for a period of 48 hours. After incubation the filter membrane was carefully removed and a few drops of 1% cotton blue in lactic acid were added to the plate. After a few minutes the number of stained fungal colonies were counted under a binocular microscope. The plates containing between 50 and 200 colonies were selected for counting. During the study it was assumed that each colony was originated from a single viable spore. Each water sample was replicated

three times and the mean calculated. The number of TVFS was expressed in terms of numbers per millilitre of aquarium water.

2.6.4. Estimation of total viable Saprolegniaceae spores (TVSS).

The method employed in estimation of TVSS counts in the hatchery water was directly adopted from the method described by Willoughby and Pickering (1977). Water samples were taken in duplicate into sterile stoppered bottles. Ten-fold serial dilutions were made using sterile pond water. Three replicates of 0.5 ml aliquots from each dilution were plated onto Glucose Yeast Penicillin Streptomycin (GYPS) agar. The spread plate technique was used and after 48 hours of incubation at 16'C the Saprolegniaceae colonies were counted with the naked eye. The plates containing between 50 and 200 colonies were selected and counts were made. It was assumed that each colony originated from a single viable spore. The mean was calculated and the number of TVSS was expressed in terms of numbers per litre of aquarium water.

2.6.5. Isolation and identification of fungi.

The aquarium water innoculated MEA and GY-PS agar plates described in the respective sections 2.6.3. and 2.6.4. were used for the isolation of fungi. Random samples

of fungal colonies were cut out from the MEA plates under a binocular microscope without adding 1% cotton blue. The cut out colonies were then placed on MEA plates incorporated with 200IU/ml Penicillin and 200ug/ml Streptomycin. The plates were incubated at 16'C and subcultured to obtain pure colonies. A similar procedure was used for obtaining pure cultures of Saprolegniaceae. All the fungi isolated were identified using the monographs described by Coker (1923), Coker and Mathews (1937), Middleton (1943), Johnston (1956), Rifai (1969) and Toussoun and Nelson (1968).

CHAPTER 3.

INVESTIGATIONS INTO THE DYNAMICS OF THE PHYSICOCHEMICAL AND MICROBIOLOGICAL PARAMETERS OF THE HATCHERY WATER AND THEIR EFFECTS ON THE HATCHABILITY AND SURVIVAL OF EGGS AND FRY.

3.1. INTRODUCTION.

Artificial incubation of Oreochromis eggs has been carried out in the Institute of Aquaculture for several years. During early 1983 heavy egg mortalities were observed in one of the experimental incubation systems. Preliminary investigations by the author on the eggs, fry and the physicochemical and microbiological qualities of the water, indicated that poor water quality may have been associated with the reduction of eggs and fry survival. A considerable interest was generated in investigating the factors affecting the survival and mortality of eggs and fry as a result of this preliminary investigation.

Artificial incubation of tilapia eggs is generally carried out in recirculatory water systems. The quality of the water in a recirculatory system is usually maintained by means of a biological filtration unit. The recirculatory water systems in aquaculture, including waste removal and biological filtration, has recently been reviewed by Muir (1982).

Though the overall pattern of the fluctuation

of physical, chemical and biological qualities of the water in recirculatory water systems in general is understood (Muir 1982), the relationship between these fluctuations and the morbidity and mortality of eggs and fry of tilapias under artificial incubation is not clear. Further, the understanding of the presence or survival of pathogens within recirculatory systems and their transmission is also very poor (Muir, 1982). Therefore, a knowledge of these issues was considered to be important. This investigation was thus designed with the view to increasing the understanding of the dynamic relationship between physical, chemical and microbiological qualities including microbial pathogens of the water and their effects on the eggs and fry of O.mossambicus under artificial hatchery conditions. It was anticipated to examine this process in both untreated (without biological filtration) and treated (with biological filtration) recirculatory systems.

3.2. MATERIALS AND METHODS

3.2.1. Source of eggs

The eggs used in this study were obtained from the O.mossambicus broodstock maintained in the recirculatory water system described in chapter 2 section 2.2. The females weighed between 133.4 and 179.8g ($\bar{x}$=147.3g) and measured between 16.4 and 18.0cm

($\bar{X}$=17.6cm) in standard length.

3.2.2 Incubation system

For the first experiment (Experiment I) the incubation system described in Chapter 2 section 2.4 (System A) was utilized. For the second experiment (Experiment II) the incubation system A was modified by removing the biological filtration unit and the plastic rings placed in the settling tank. Hereafter this system will be referred to as System B. A diagram of the incubation system B is given in Figure 6. Prior to the commencement of experiment II system B was thoroughly cleaned and dried.

3.2.3 Experimental procedure

3.2.3.1 Experiment I

Three to four groups of 100 eggs were randomly separated from a single clutch of eggs and were placed in individual incubators as described in chapter 2 section 2.3. The remainder of the clutch was also placed in a separate incubator. Gentle agitation of the eggs was ensured and the eggs were incubated until hatching. The incubation generally took four days. Dead or decaying eggs were not removed from the incubator since this would interfere with the dynamics of the physicochemical and

Figure 6.

Side elevation of the incubation system B

P : Submersible water pump

S : Sump tank

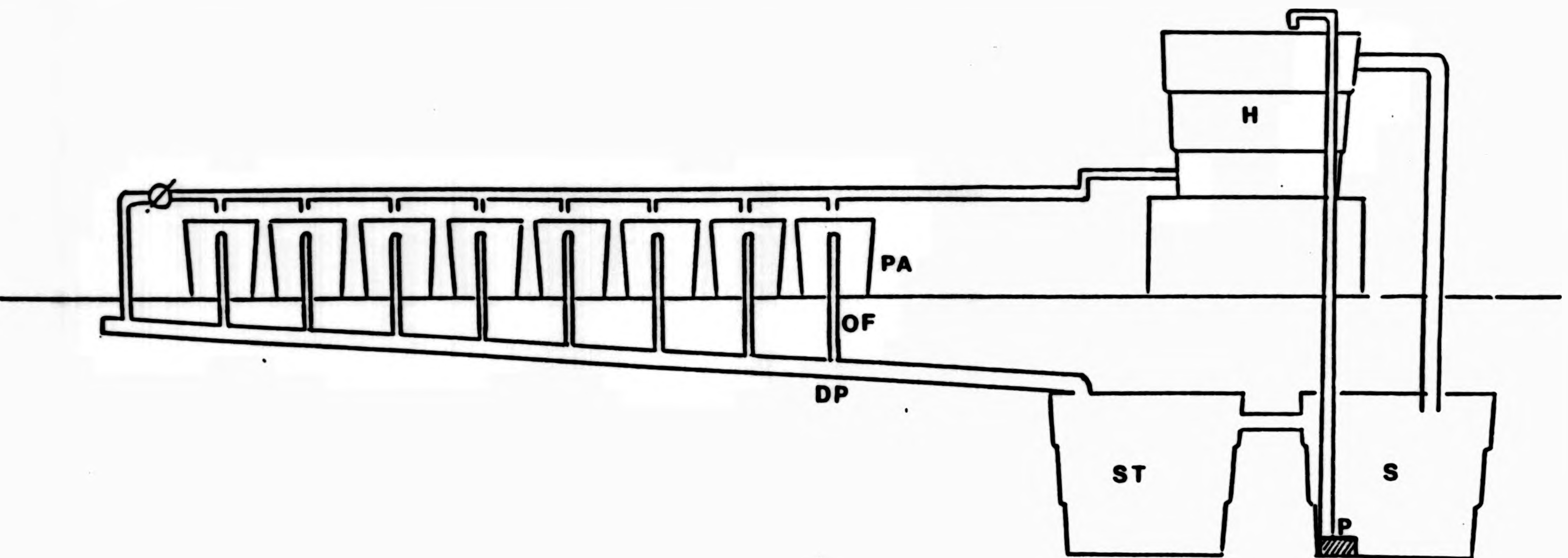
H : Header tank

PA : Perspex aquaria

OF : Outflow of water

DP : Drain pipe

ST : Settling tank



H
PA
OF
DP
ST
S
P

microbiological qualities of the hatchery water which was being assessed over a period of continuous incubation and rearing of fry.

Once the eggs had completed hatching the number of hatched fry were counted within twelve hours. The fry were left in the incubators for a further period of eight days. No food was given to the fry during this period. The numbers of surviving fry were counted at the end of day 8 post hatching and were removed and discarded. This process was continued for a period of 51 days and 17 batches of eggs were incubated.

3.2.3.2 Experiment II

The incubation system which did not include a biological filtration unit, System B, as described in section 3.2.2 was employed in this experiment. Identical experimental procedures were exercised and the same parameters were measured. The experiment was continued for a period of 51 days and 17 batches of eggs were incubated.

3.2.4 Water quality analysis

During the course of experiments I and II, the temperature, dissolved oxygen and pH of the hatchery was monitored daily. The total NH3-N and total NO2-N were measured every three days. The methods described in Chapter 2 section 2.5 were employed.

3.2.5 Microbiological parameters

The total viable heterotrophic bacterial counts (TVHB), total viable fungal spore counts (TVFS) and total viable Saprolegniaceae spore counts (TVSS) were measured every three days using the methods described in Chapter 2 sections 2.6.1, 2.6.3 and 2.6.4 respectively. This was carried out for both experiment I and II.

The bacteria and fungi were twice isolated and identified during each experiment using the techniques described in Chapter 2 sections 2.6.2 and 2.6.5 respectively.

3.3 RESULTS

3.3.1 Water quality parameters

3.3.1.1 Physicochemical parameters

The mean water temperature of both systems A and B during the period of investigation was 27.5±0.5'C.

The dissolved oxygen concentration of system A was always lower than in system B but neither of them showed any extreme fluctuations. They were maintained between 6.0 and 7.5 mg/l levels (Fig.7).

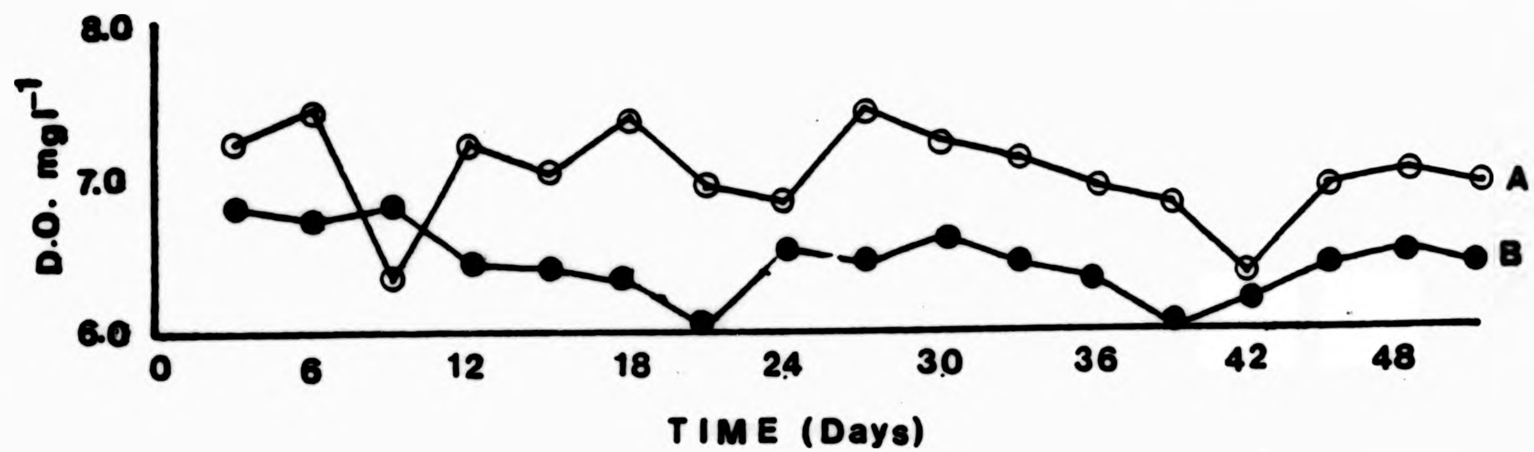
Figure 8 shows the fluctuation of the pH in both systems. The pH in system B decreased gradually from

Figure 7.

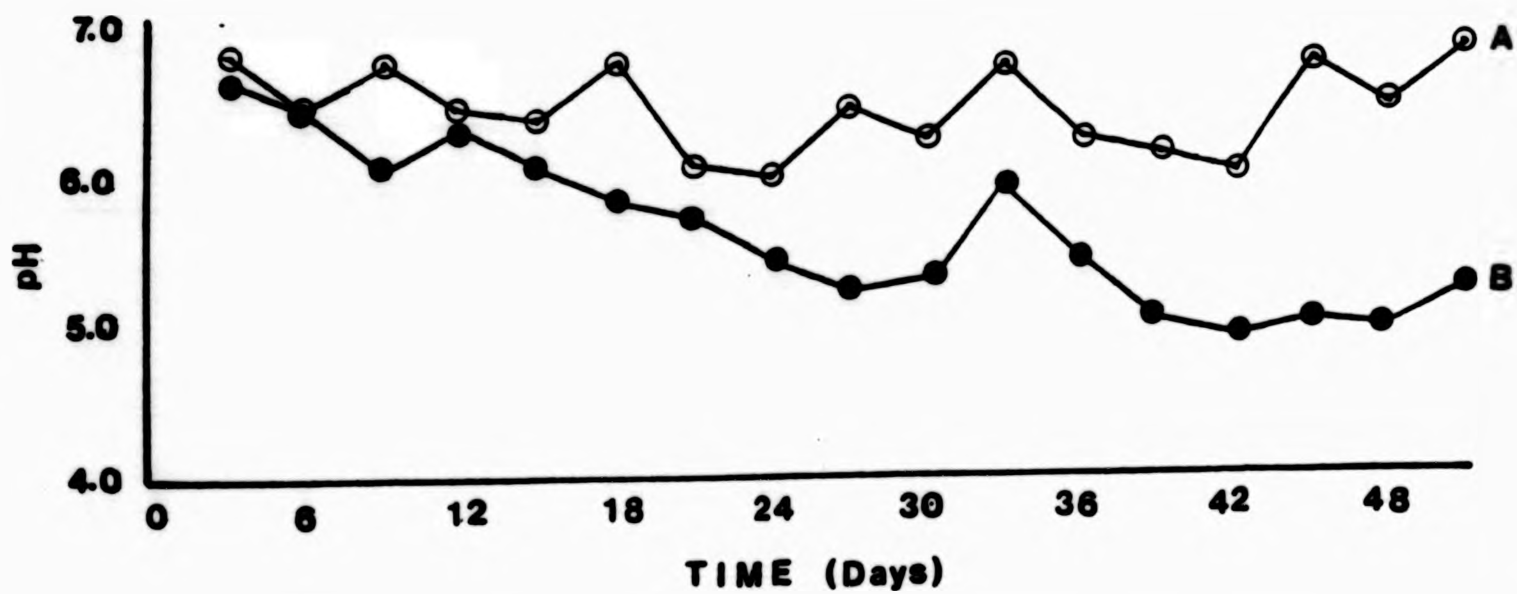
Graph showing the pattern of change of the dissolved oxygen (DO) levels in the incubation systems A and B over the period of investigation.

Figure 8.

Graph showing the pattern of change of the pH in the incubation systems A and B over the period of investigation.



8.0
7.0
6.0
D.O. mg l⁻¹
0
6
12
18
24
30
36
42
48
A
B
TIME (Days)



7.0
6.0
5.0
4.0
pH
0
6
12
18
24
30
36
42
48
A
B
TIME (Days)

6.62 to 4.93 during the first 42 days of experiment II and at the termination of the trial it was 5.22. In contrast the pH in system A fluctuated between 6.82 and 6.03 over the 51 day duration of experiment I and therefore showed a greater degree of stability.

At the beginning of the two experiments, I and II, the total NH3-N concentrations in systems A and B were respectively 0.08±0.00 mg/l and 0.03±0.00 mg/l (Fig.9). The NH3-N level in system B slowly increased to 0.56±0.03 mg/l over the first 21 days and began to increase rapidly thereafter reaching a concentration of 6.67±0.07 mg/l by day 48 (Fig.9). In contrast the total NH3-N concentration in system A only increased up to 0.49±0.02 mg/l over the entire experimental period.

The total NO2-N concentration in system A remained below 0.03±0.001 mg/l over the 51 days of experiment I (Fig.10). In contrast in incubation system B the total NO2-N concentration increased slowly from 0.01±0.00 mg/l to 0.34±0.12 mg/l over the first 27 days of experiment II and rose rapidly up to 2.83±0.08 mg/l over the remaining 24 days (Fig 10).

3.3.1.2 Microbiological parameters

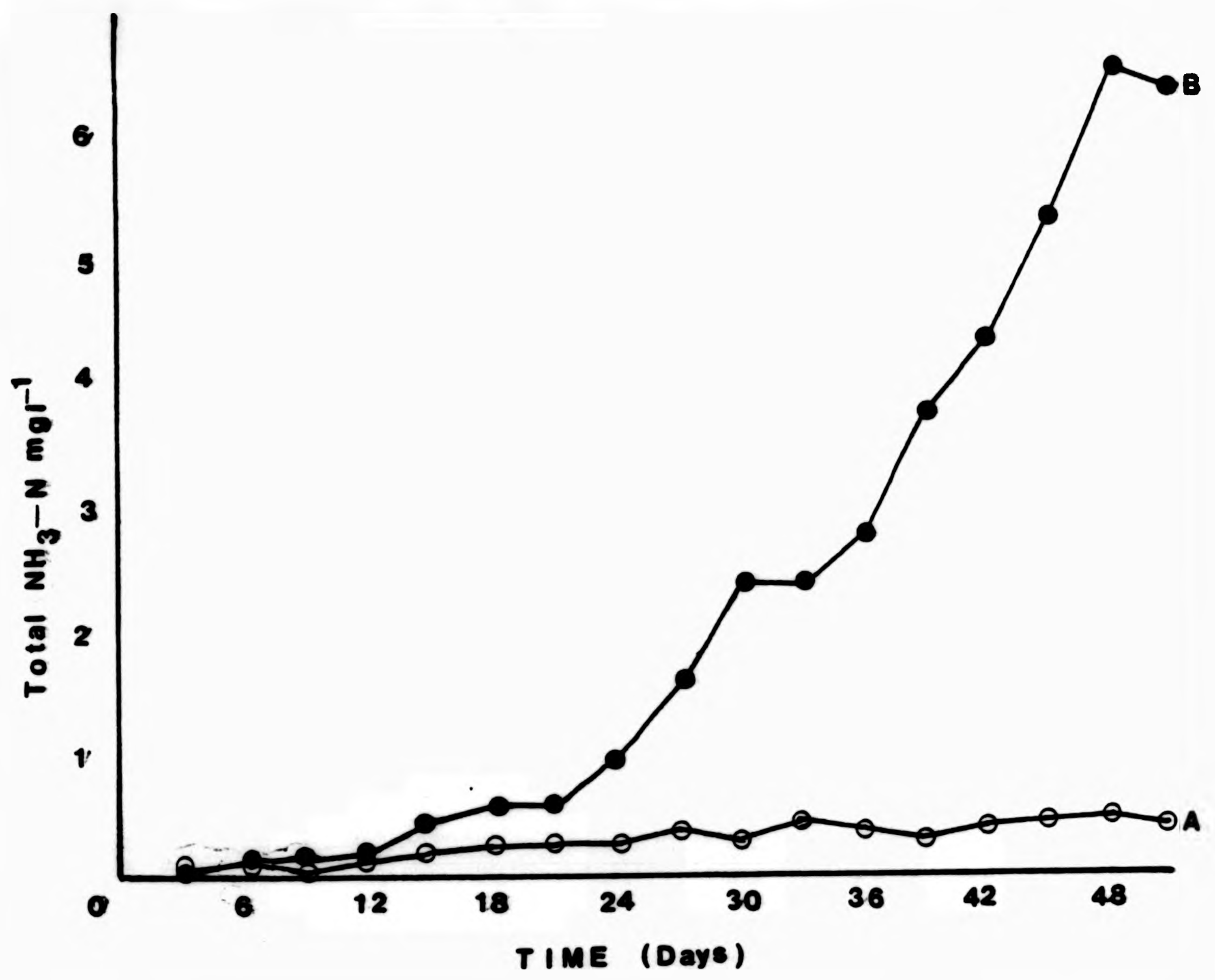
The pattern of change of TVHB counts in incubation systems A and B is illustrated graphically in Fig.11. The initial TVHB counts in systems A and B were

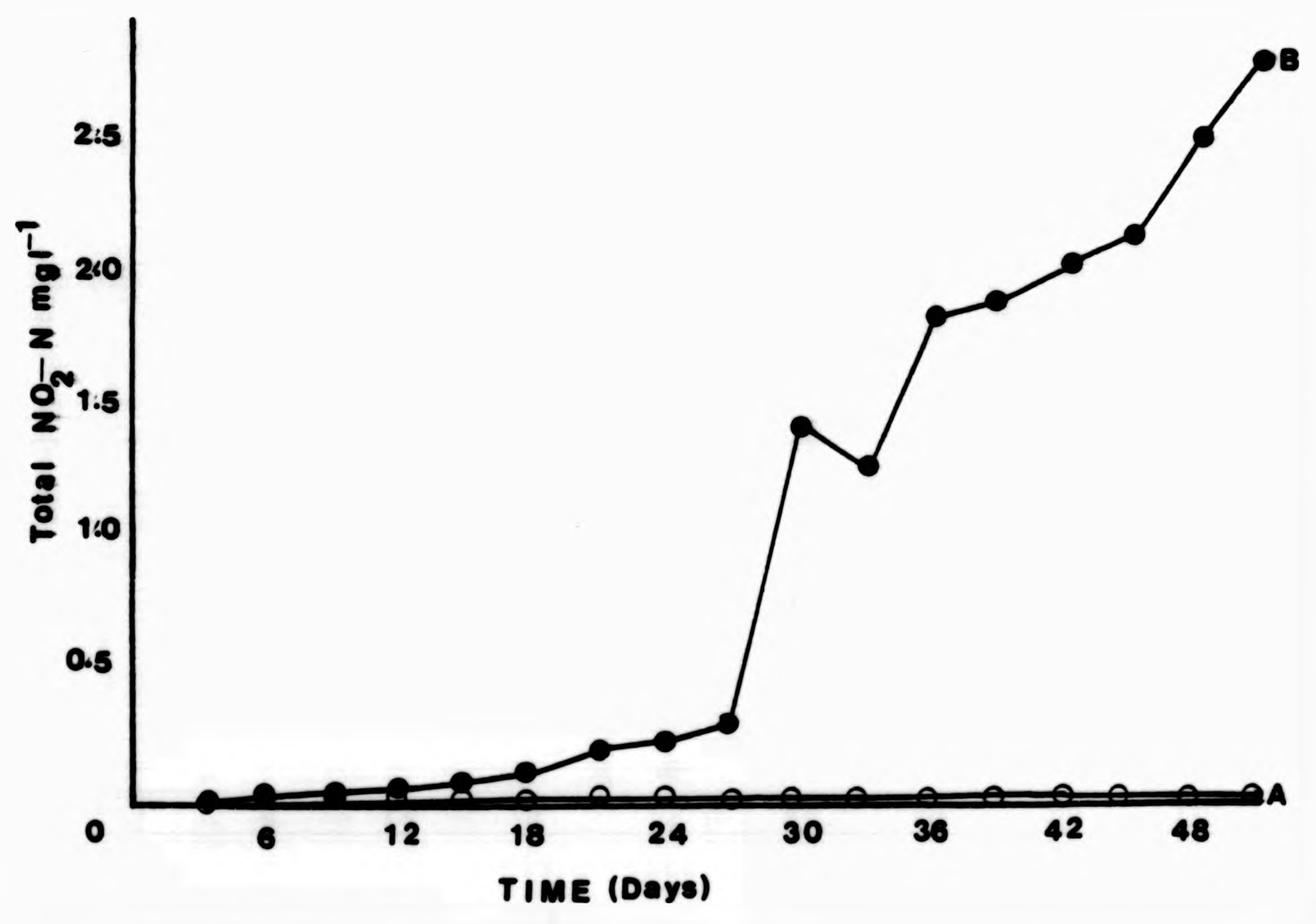
Figure 9.

Graph showing the change in concentration of the total NH_3-N in the incubation systems A and B over the period of investigation

Figure 10.

Graph showing the change in concentration of the total NO_2-N in the incubation systems A and B over the period of investigation.


Total NH3—N mgl-1
6
5
4
3
2
1
0
6
12
18
24
30
36
42
48
TIME (Days)
B
A


Total NO2—N mgl-1
2.5
2.0
1.5
1.0
0.5
0
6
12
18
24
30
36
42
48
TIME (Days)
B
A

respectively $1.41x10^3 \pm 0.31x10^3$ cells/ml and $8.50x10^2 \pm 5.3x10^2$ cells/ml. The counts in the sy stem B increased rapidly up to $1.96x10^7 \pm 0.96x10^7$ cells/ml over the first 15 days of experiment II. This was followed by a rapid decrease to $1.34x10^5 \pm 0.12x10^5$ cells/ml over the next three days (Fig.11). Thereafter the counts increased gradually up to $3.12x10^7 \pm 0.19x10^7$ cells/ml at day 24 and remained above 10^8 cells/ml level until the end of experiment II.

The pattern of the change in the TVHB counts was different in system A during experiment I. The counts increased only up to $4.49x10^4 \pm 0.05x10^4$ cells/ml over the entire period of 51 days of experiment I (Fig.11).

The TVFS counts in the two systems were significantly different (P>0.05) at the beginning of the two experiments, being $11.8x10^4 \pm 0.84x10^3$ and $8.85x10^3 \pm 0.77x10^3$ spores/l in systems A and B respectively. The counts gradually increased in both systems and, despite the initial higher count in system A, by day 48 of the trial the TVFS count in system B was $4.7x10^4 + 0.21x10^3$ spores/l compared to $3.52x10^4 \pm 7.7x10^3$ spores/l in system A (Fig.12).

The TVSS counts in system B was less than 250 spores/l during the first six days of the trial (the minimum sensitivity of the method was 250 spores/l). In contrast the initial counts in system A was $7.5x10^2 \pm 3.53x10^2$ spores/l (Fig.13). The total counts rose

Figure 11.

Graph showing the pattern of change in the total viable heterotrophic bacteria (TVHB) counts in the incubation systems A and B over the period of investigation.

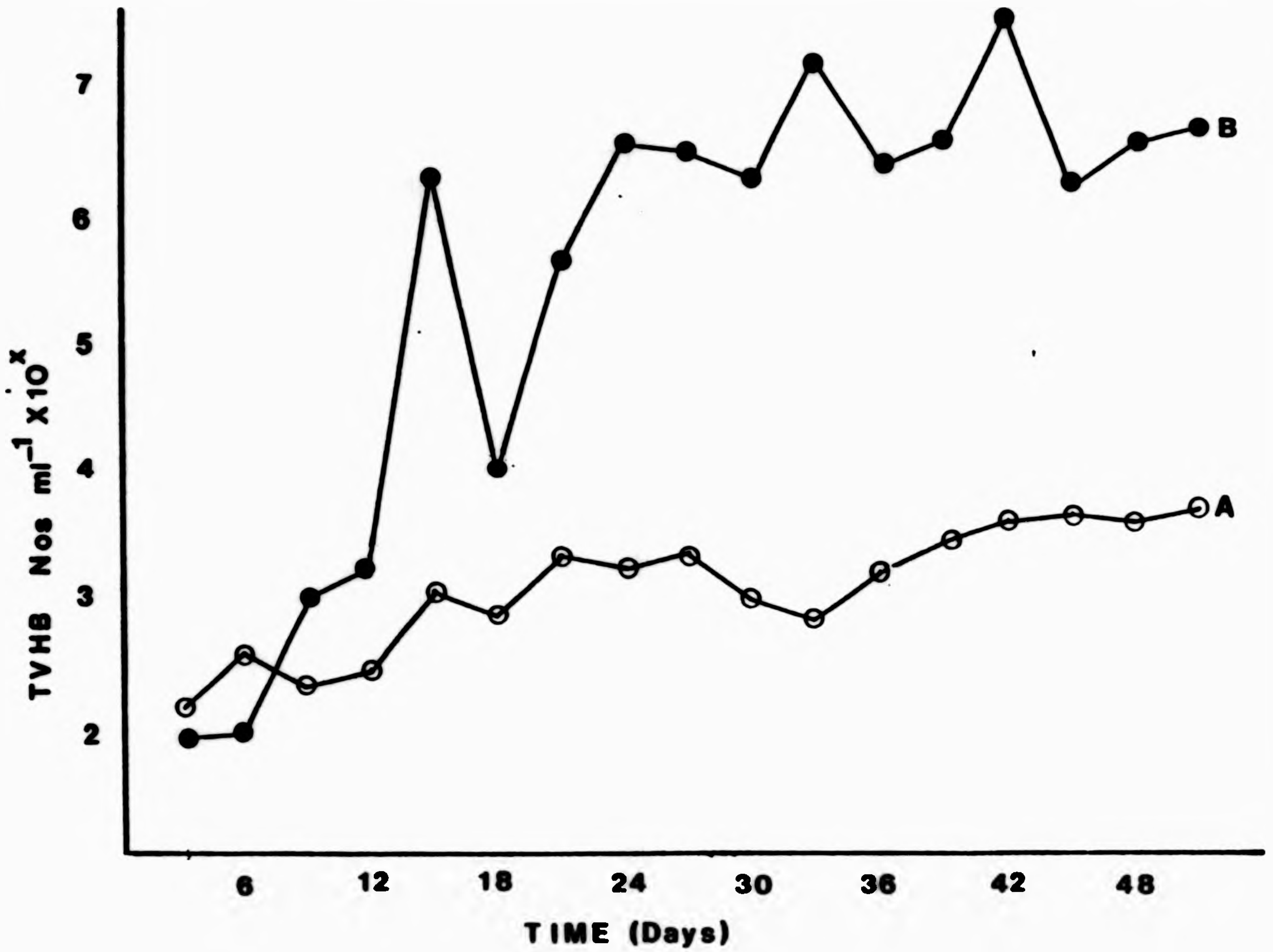
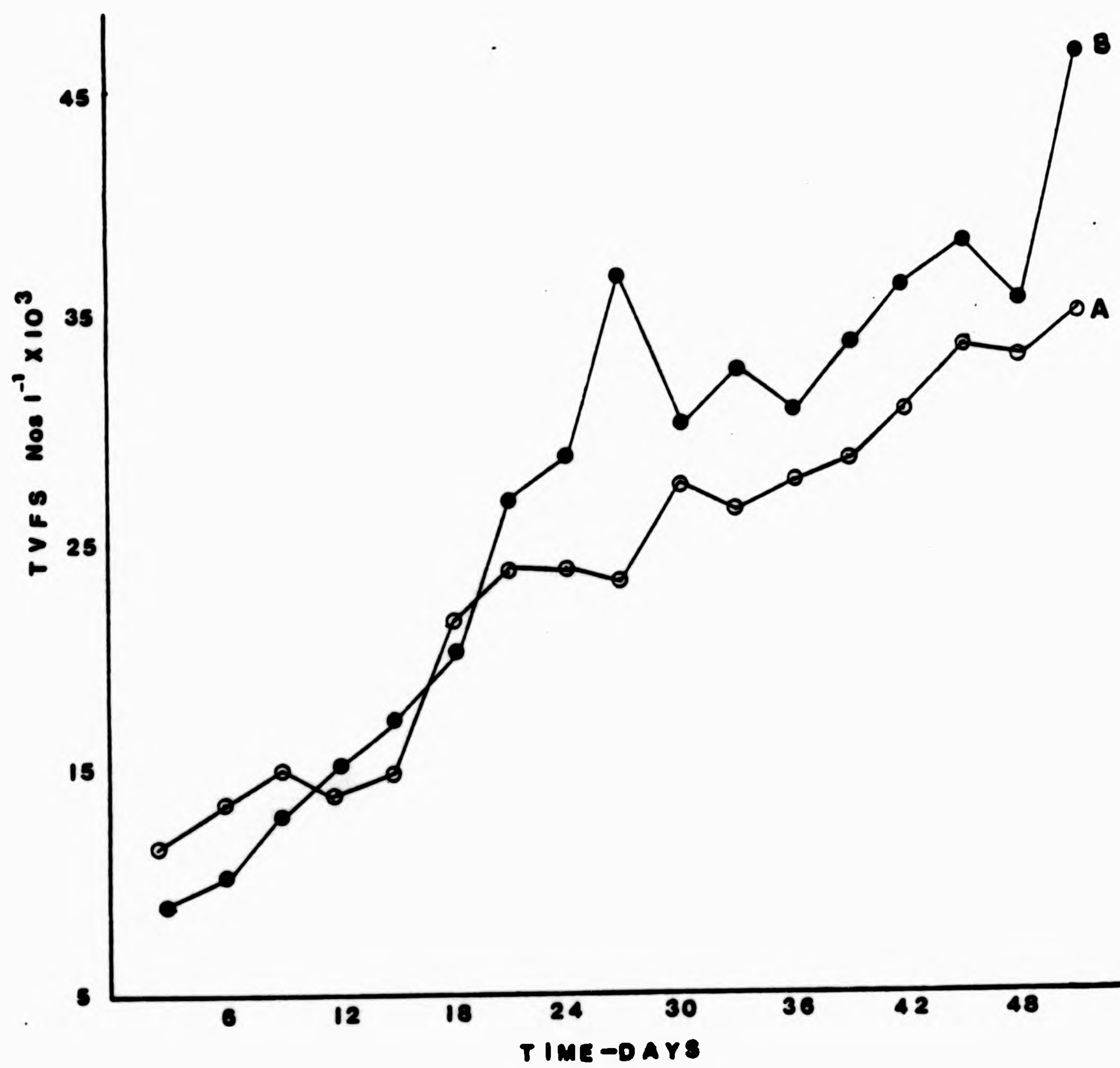

TVHB Nos ml^{-1} X10^{x}
7
6
5
4
3
2
B
A
6
12
18
24
30
36
42
48
TIME (Days)

Figure 12.

Graph showing the pattern of change in the total viable fungal spore (TVFS) counts in the incubation systems A and B over the period of investigation.


TVFS Nos l⁻¹ X 10³
45
35
25
15
5
6
12
18
24
30
36
42
48
TIME-DAYS
A
B

up to $9.0 \times 10^3 \pm 0.70 \times 10^3$ and $2.1 \times 10^4 \pm 0.7 \times 10^3$ spores/l in systems A and B respectively, so that the counts in system B had considerably overtaken those in A by the end of the experiment; Figure 13 shows clearly the 100 fold increase in TVSS counts in system B compared to the 10 fold increase in system A.

3.3.2 Hatchability and fry survival

Figure 14 describes the percentage hatchability of the viable eggs in systems A and B over the experimental period. The percentage hatchability in system A fluctuated between 47.2±0.0 and 67.2±0.8 over 47 days during experiment I. With the exception of day 17 (percentage hatchability 47.2±0.0) the hatchability was above 50% throughout (Fig.14). In contrast, the hatchability of viable eggs in the incubation system B was 60.3±3.6% at day 14 of experiment II and decreased rapidly thereafter. It remained within 34.8±1.6 and 19.8±1.1% until the termination of the experiment.

The survival of the yolk sac fry up to 8 days post hatch in systems A and B over the experimental period is given in Figure 15. In system B the fry survival was above 62% until day 13 of experiment II and then dropped to 38.4±2.3% by day 16. From day 26 until day 46 the percentage fry survival fluctuated between 0 and 24.26 after which it remained at zero. On the other hand, in

Figure 13

Graph showing the pattern of change in the total viable saprolegniaceae spore (TVSS) counts in the incubation systems A and B over the period of investigation.

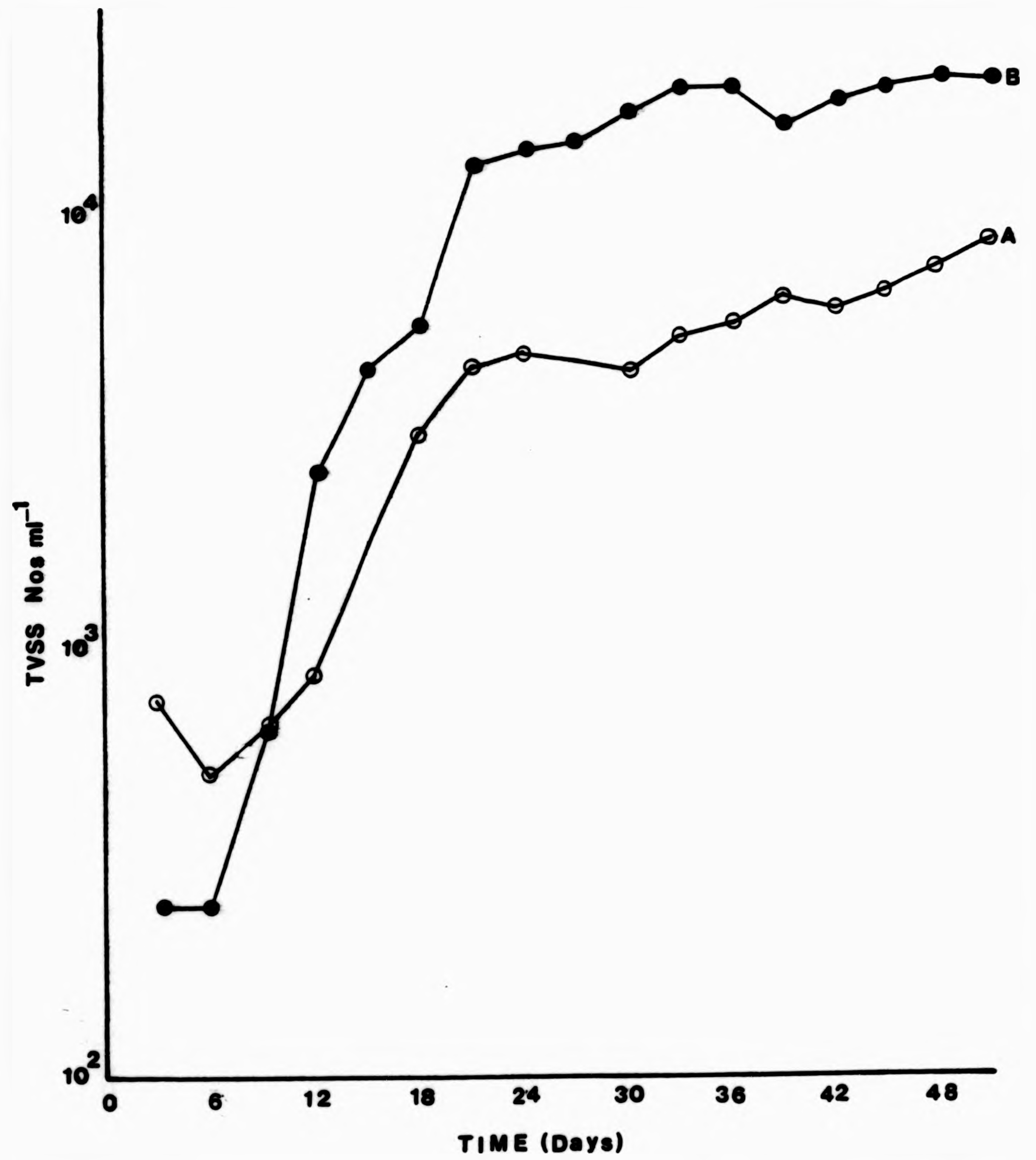
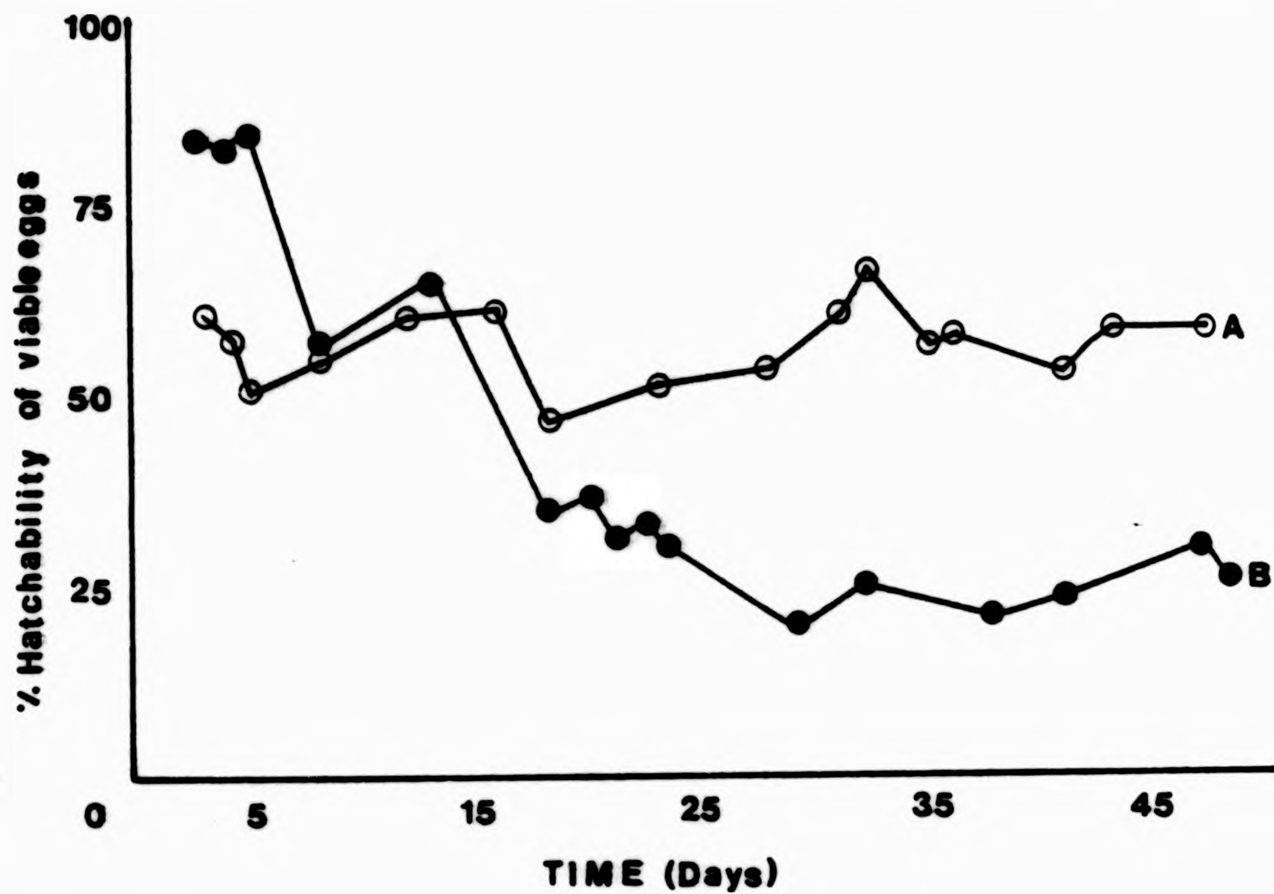

B
A
TVSS Nos ml^{-1}
10^4
10^3
10^2
0
6
12
18
24
30
36
42
48
TIME (Days)

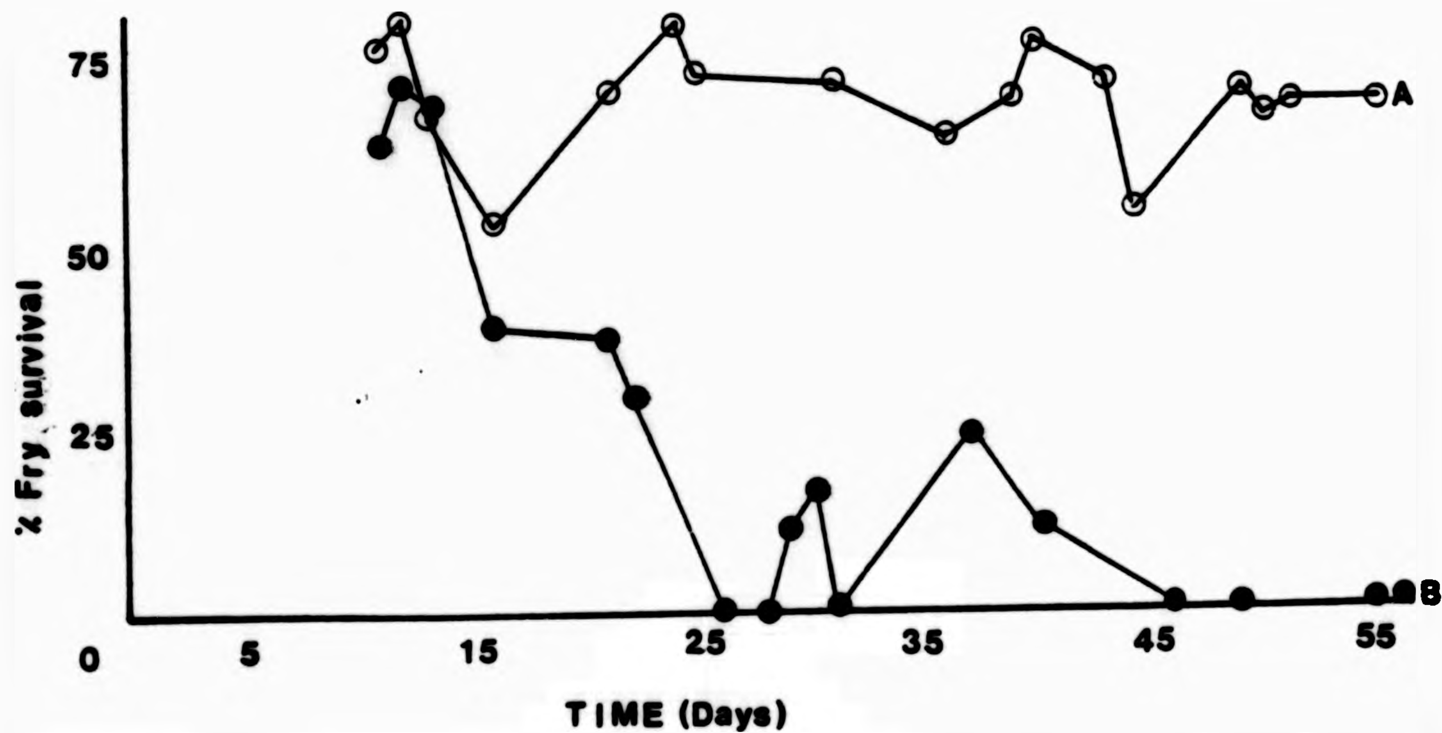
Figure 14.

Graph showing the percentage hatchability of the viable eggs in the incubation systems A and B over the period of investigation.

Figure 15.

Graph showing the percentage survival of the yolksac fry in the incubation systems A and B over the period of investigation.





Each point represents the hatchability of the viable eggs or the survival of yolksac fry from a single clutch. Seventeen consecutive egg clutches were incubated during the study.

incubation system A, no significant difference ($P<0.05$) in the survival of yolk sac fry was observed throughout the period of experiment II. The survival rates fluctuated between 52.5±12.8 and 78.5±4.9% (Fig.15).

3.3.3 Identified pathogenic bacteria and fungi

Table 2 lists the bacteria and fungi isolated and identified from the two incubation systems during the course of investigation.

3.4 DISCUSSION

It is evident from the results that the changes in the physicochemical and microbiological qualities of the water in incubation system B were more pronounced than those in system A. The number of egg batches introduced into both systems during the entire period of investigation was constant. It is known that O.mossambicus has a similar fecundity within a given size range. As the females used for the collection of eggs during the present investigation were grouped into a narrow size range, the biomass of the eggs incubated in the two systems could be considered to be very similar. The only major difference between the two incubation systems was the well conditioned trickle gravel biological filtration unit incorporated into system A. Hence, the difference observed in water quality between the two systems was

TABLE 2.

List of bacteria and fungi isolated and identified from the incubation systems A and B.

Type of the organism	Occurrence	
	System A	System B
Bacteria		
Aeromonas hydrophila	+	+
Aeromonas sp.	+	+
Pseudomonas fluorescens	+	+
Pseudomonas sp.	+	+
Pseudomonas sp.	+	-
Chromobacterium violaceum	+	+
Flavobacterium sp.	+	+
Micrococcus sp.	+	+
Bacillus sp.	+	+
Fungi		
Achlya prolifera	+	+
Achlya flagellata	+	+
Achlya sp.	-	+
Saprolegnia sp.	+	+
Fusarium sp.	+	+
Fusarium sp.	+	-
Pythium sp.	+	+
Allomyces sp.	+	-
Trichoderma sp.	+	+

attributed to the presence and absence of the biofiltration capabilities in the two systems.

Ammonia is the major end product of nitrogen metabolism in teleosts (Forster and Goldstein 1969). Biochemical oxidation of ammonia to nitrate, ie nitrification, is carried out by two groups of autotrophic bacteria. The process of oxidation of ammonia to nitrite is carried out principally by the genus Nitrosomonas and the oxidation of nitrite to nitrate is mainly accomplished by species of Nitrobactor. The significance of nitrification is the conversion of toxic ammonia and nitrite to nitrate which is less toxic to teleosts (Sharma and Ahlert 1977).

It appears that when a nitrifying flora is being built up on a biological filter the Nitrosomonas bacteria become established first, so that ammonia is oxidized to nitrite initially. The Nitrobactor bacteria which oxidizes nitrite to nitrate becomes established more slowly and the conversion of nitrite to nitrate takes place subsequently (Lloyd 1981). This could be the reason for the rapid increase in the nitrite level in system B observed a few days after the ammonia level increased above 1.5 mg/l.

The efficiency of the nitrification process is affected by the factors that limit the proliferation of nitrifying bacteria or inhibit their biochemical activities. According to Spotte (1979) the most significant factors are toxic substances in the water, temperature, pH,

the concentration of dissolved oxygen, salinity and surface area available for microbial attachment.

Toxic substances such as certain antibiotics, organic dyes and inorganic salts have been found to inhibit nitrification at different levels (Collins, Gratzek, Dawe and Nemetz 1976; Lavine and Meade 1976). However, in the present study there were no such substances introduced to either of the systems knowingly. Hence, it appears that the elevated ammonia and the nitrite levels experienced in the incubation systems were not affected by an inhibition of the nitrification process by toxicants.

The nitrification is most efficient at warm temperatures (Spotte 1979). Carlucci and Strikland (1968) demonstrated that certain cultures of nitrifying bacteria did not oxidize ammonia to nitrate at 5'C even after 3-4 months of incubation. As the temperature of both systems was maintained at 27'C it is very unlikely that temperature played any significant role in arresting the nitrification process in system B.

Srna and Baggaley (1975) analysed the data available on ideal pH ranges for efficient nitrification and suggested that nitrifying bacteria can be conditioned to function throughout a fairly wide pH range, provided that there was sufficient time to adjust. On the other hand according to Haug and McCarty (1972) the nitrification process slows at pH 6.0 and seized completely below pH 5.5

but they also stated that nitrifying bacteria can adjust to pH values as low as 5.5 in about ten days. The observed pH values were low in incubation system B and were below pH 6 for most of the experimental period. This may have contributed to the slowing down of the initiation of the nitrification process. However, the drop of pH was gradual and it is likely that adjustment by the nitrifying bacteria took place. If this is the case the pH may have had only a little effect on nitrification process.

The dissolved oxygen concentration and salinity found would not have caused any effect on the nitrification process in incubation system B during the experiment as the salinity of the water in the incubation system was negligible and the inhibition of nitrification takes place only at the dissolved oxygen levels below 0.6 mg/l (Forster 1974). The DO levels were well above 6.0 mg/l throughout the experiment in both systems.

The final factor which could have affected the nitrification process is the surface area available for attachment of the nitrifying bacteria. According to Spotte (1979) the physical presence of added attachment sites is significant in nitrification. As nitrifying bacteria live on surfaces (Lloyd 1981), a large surface area is required in an aquarium system. The addition of biofilter rings and gravel filter beds facilitates ample surface area for the nitrifying bacteria assuring a complete and efficient

nitrification process. In the present study the low ammonia levels and nitrite levels in system A appeared to be due to the more efficient and complete nitrification process occurring in the system. In contrast, the elevation of total NH3-N levels above 6.4 mg/l and total NO2-N levels above 2.8 mg/l in the incubation system B seem to be a result of inadequate surface area or attachment sites for the nitrifying bacteria which arrest an efficient and complete nitrification process.

Though there is ample literature available on nitrification in aquarium systems (Hirayama 1965,1966,1970; Speece 1973), all these studies have dealt with conditioned systems which Spotte (1979) defined as "one in which the nitrifying bacteria are in dynamic equilibrium with routine formation of their energy sources". Collins, Gratzek, Shotts, Dawe, Campbell and Senn (1975) demonstrated the changes in water chemistry and bacterial counts during and after establishment of the nitrification process in closed recirculatory system. In contrast to the present study, they used 70 l glass aquaria equipped with a commercial filter containing one litre of quartz gravel. Those aquaria were stocked with Ictalurus punctatus Rafinesque fingerlings giving an approximate total biomass of 150 g per aquarium. They were also fed daily with a commercial fish diet at a rate of 3% of total body weight per day. During the present investigation there was no nitrogenous

material introduced into the incubation systems in the form of food. The only possible source of nitrogenous material was either ammonia from the eggs and fry as their metabolic end product or from the dead and decaying eggs and fry accumulated during the experimental period. The average biomass of eggs and fry being incubated compared to the volume of water in the systems was very little (approximately 15g). Hence, the input of nitrogenous material into the hatchery water was relatively small. Though the input of ammonia was low, the failure of nitrification process could cause an unacceptable increase in ammonia and nitrite levels. Thus looking at the changes of physicochemical qualities of the water observed in system B, it is clear that only inefficient or partial nitrification was facilitated, resulting in a gradual increase in both ammonia and nitrite levels.

Collins, Gratzek, Shotts, Dawe, Campbell and Senn (1975) reported an increase of TVHB counts up to 1.2×10^6 cells/ml over a period of 28 days during an establishment of the nitrification process. In the present study TVHB counts increased up to 1.96×10^7 in incubation system B over a 15 day period. On the other hand Jana and Barat (1983) estimated the relative abundance of heterotrophic bacteria in aquaria as affected by two size groups of fed and unfed <u>Clarius batrachus</u> (Linnaeus). It was noted that the fish have a defined effect on the

population size of heterotrophic bacteria mediated through the excretion of metabolic waste in the aquatic environment. Therefore, it appears that the accumulation of organic nitrogen rich material results in an enriched medium which is conducive to the rapid multiplication of those heterotrophic bacteria. It seems possible therefore that the observed increase in TVHB counts in system B was a direct result of gradual enrichment of hatchery water by an accumulation of nitrogenous material.

The TVFS counts exhibited 2.9 and 5.3 fold increment in systems A and B respectively. The counts at the end of the experiment were 3.52×10^4 and 4.70×10^4 spores/l in systems A and B respectively. Akpata and Ekundayo (1983) estimated some fungal populations in Lagos lagoon in Nigeria and found that the counts were as high as 5.6×10^4 colonies/l. They concluded that the high numbers were due to the decomposition of sawdust in the water and sediment originating from a nearby saw mill and the isolated fungi were mainly terrestrial. However, the lack of reports on the TVFS counts in aquatic environments where fish were being reared, restricts comparison of the present data. In contrast, Willoughby (1962) estimated the total saprolegniales counts in a fish hatchery which consisted of several large ponds. He found that the counts never decreased below 400 spores/l with a peak of 4600 spores/l. In the present study the TVSS counts increased

from <250 spores/l to 2.1×10^4 spores/l over a 51 day period. As unconsumed fish food and dead fish constitute the most important substrate for the growth of Saprolegniaceae in fish hatchery situations (Willoughby 1962) it seems probable that this almost 100 fold increment of TVSS counts in system B was supported by the dead and decaying eggs and early fry.

The increment of TVSS counts in system A was only approximately twelve fold. The egg and fry survival in system A was significantly higher (P>0.05) than that in system B. Therefore the fewer dead eggs and fry may have resulted in the lower increment of the TVSS numbers observed in system A. The TVSS counts in a fish hatchery might also be determined by the quality of the water supply into the system and the quantity of the total fish population (Willoughby 1962). Little information is available on the Saprolegniaceae spore counts in natural waters; the only work reports exceptionally high numbers of fungal spores. This was carried out by Suzuki (1960) and his estimation of water mould spores was 50000-300000 per litre. Unfortunately the method used by Suzuki (1960) was different from that of the present study, hence, no comparison is possible. More information is needed on fungal spore counts, especially Saprolegniaceae counts in hatchery water supplies to try to correlate with the level of organic loading.

Low egg hatchability and early fry survival under artificial hatchery conditions have been reported frequently. However, the different reports have attributed the mortalities to a variety of causes. Wright and Snow (1975) reported high mortalities of largemouth bass, *Micropterus salmoides* (Lacepede) embryos associated with *A. liquefaciens* infections whereas McFadden (1969) said that the organism responsible for protracted lethal epizootics of salmonid fry was *A. liquefaciens*. Schachte (1979) and Colesante, Engstrom-Heg, Ehlinger and Youmans (1981) stated that New York State's muskellunge, *Esox masquinongy* (Mitchill) hatchery sustained total or near total losses of early fry at or near "swim up" stage due to *A. hydrophila* infections.

Apart from these bacterial infections which cause mortalities in hatcheries, fungal infections are also reported to cause severe egg and early fry mortalities in hatcheries. Taylor and Bailey (1979) reported nearly 100% egg mortality due to *Saprolegnia diclina* infections. Srivastava and Srivastava (1975, 1976) reported almost 100% egg mortalities of *Cyprinus carpio* L., and *Channa striatus* (Bloch) eggs due to parasitism by *Achlya orion* and *Achlya prolifera* respectively. Certain other species of fungi including other members of the family Saprolegniaceae have been reported to cause heavy losses of eggs and early fry under artificial hatchery

conditions (Scott and O'Bier 1962).

Microbial fish pathogens are not the only factors associated with egg and fry mortalities. Jana, S arkar and Kundu (1985) reported that the hatching success of the Indian carp, Cirrhirnus mrigala (Hamilton), eggs in a traditional type hatchery was 30-40% and could improve up to 75-85% by maintaining a more favourable temperature, high oxygen levels and optimum water quality throughout. However, they did not specify the particular cause of mortality.

In the light of this information it is rather difficult to suggest which of these factors might have accounted for the different levels of egg and fry mortality observed in the two incubation systems during the present study. The percentage hatchabilities of the viable eggs and survival of yolk sac fry observed in the two incubation systems were significantly different (P>0.05). It is possible that incubation system A provided somewhat more favourable physicochemical and microbiological conditions for the developing eggs and fry than system B. The low ammonia and nitrite levels could have been the major contributing factor towards the observed higher percentage hatchability in system A compared to that of system B.

The increased ammonia levels and the nitrite levels in system B showed a high correlation with the hatchability of eggs and survival of fry. However, the

tolerance levels of O.mossambicus eggs and fry to ammonia and nitrite have not been investigated and their influence on morbidity and mortality could not be assessed at this stage.

One of the interesting observations made was the rapid decline in hatchability of eggs and survival of fry observed 15 days after the commencement of experiment II in system B. Beyond 15 days the TVHB counts in system B was significantly higher ($P>0.05$) than that in system A. The increased numbers of TVHB were highly correlated ($r=0.727$, $r=0.783$) with the decreasing hatchability and the fry survival in system B. Therefore, it seems probable that when TVHB numbers rise above a certain level the egg hatchability and fry survival decrease accordingly as in system B, ie there seems to be a critical level of TVHB numbers which governs the survival of eggs and fry of O.mossambicus under artificial hatchery conditions. Although Blaxter (1981) stated that herring (Clupea harengus Linnaeus) larvae did not show enhanced mortalities with total viable bacterial counts in the water as high as 10^3/ml; those bacteria were non pathogenic.

The question of the possible cause of approximately 50% mortality of the eggs observed in system A still remains unanswered. Could it be possible that the heterotrophic bacterial and fungal flora were responsible for the mortalities? Of the species of fungi identified

from the incubation systems. genus *Saprolegnia* is said to be truly parasitic (Willoughby pers com). Other species such as *Achlya*, *Pythium*, *Allomyces* and *Fusarium* have also been reported to be fish pathogenic (Srivastava and Srivastava 1975, 1976; Scott and O'Bier 1962). During the course of investigation the genus *Saprolegnia* was only isolated in small proportions whereas *Achlya* was found to be the most predominant member of the family Saprolegniaceae. *Fusarium* was isolated only once during this study. The higher rate of increase of TVSS counts in system B compared to system A was attributed to the accumulation of dead eggs and fry occurring during the latter half of experiment II. However, this does not exclude the possibility that the mortalities may have also been associated with pathogenic species of bacteria found amongst the heterotrophic bacterial population. The bacteria isolated are known to be pathogenic to fish (Richards and Roberts 1978; Frerichs 1984; Cowan and Steel 1974).

According to Blaxter (1981) the earliest mortality of eggs occurs as a result of some eggs being infertile and in addition the very early developmental stages of embryos are comparatively delicate and sensitive. The eggs used during the present investigation were aged between 12 and 24 hours, and may not have passed this delicate stage of development, thus contributing to

the observed mortalities. However, the eggs introduced into the two systems were of the same age, the differences in hatchability observed could not possibly be associated with this early delicate stage of development.

Considering all the above discussed reports on the egg and fry mortalities in fish hatcheries together with the results of the present investigation, it is not possible to draw any conclusion as to the cause of mortality of O. mossambicus eggs and fry. As previously mentioned, the cause of mortality appeared to be a single or combined result of physical, chemical and microbiological qualities of the hatchery water. In order to elucidate the relative influence of these factors on egg mortalities and fry survival further information is required. Therefore, a series of experimental investigations were carried out and are described in the forthcoming chapters.

CHAPTER 4.

EFFECTS OF SOME ENVIRONMENTAL VARIABLES ON THE HATCHABILITY AND SURVIVAL OF EGGS AND YOLK SAC FRY.

4.1. INTRODUCTION.

Water quality management in the intensive culture of aquatic animals has been of major interest to the aquaculturist over the last decade. This has been due, in part, to the interest in the use of partially or totally recirculated water systems in aquaculture. Though these systems provide very high yields, they certainly require close control of environmental variables such as temperature, dissolved oxygen, pH and nitrogenous compounds.

According to Colt and Armstrong (1981), in aquaculture recirculatory systems, the toxicity of excreted nitrogen compounds is singularly the most limiting factor once adequate dissolved oxygen levels are maintained. In hatchery operations where first feeding fry are being fed at 20-30% body weight per day, the amount of ammonia excreted by the fish as the major end product of protein catabolism is very significant.

The ammonia is oxidised to nitrate in a two-way process by two groups of aerobic bacteria, Nitrosomonas and Nitrobacter. This nitrification is a slow

process and as a result the major nitrogen species in a flowthrough system is ammonia and urea. However, in a well aged recirculatory system with biological filtration, the rate of ammonia oxidation is equal to the rate of nitrite oxidation. Under such steadystate conditions the nitrite levels are typically low. However under certain conditions, as a result of the growth of nitrifying bacteria being slow or the fish being stocked and fed in a system where the nitrifying bacteria have not been established properly, then lethal levels of nitrite could be produced (Collins et al, 1975). Therefore, as the ammonia and nitrite concentrations could reach sublethal and/or lethal levels in culture systems incorporating either static or recirculatory water, it is important to identify the tolerance levels for these products by the organisms under culture.

The toxicity of an aqueous ammonia solution is directly related to the amount of Unionised Ammonia (UIA) present; the proportion of UIA to Ionised Ammonia (IA) increases with increasing pH and temperature (Trussell 1972). In contrast nitrite in low concentrations can be toxic to fish but increased dissolved chloride and calcium concentrations and pH decreases or inhibits its toxicity (Perrone and Meade 1977; Russo, Thurston and Emerson 1981).

Apart from the toxicity effects produced

by the chemical constituents of the aquatic environment the physical characteristics also play an important role in influencing the performance of fish. Temperature, pH and dissolved oxygen concentration are by far the most important physical environmental variables which affect the fish under artificial culture conditions.

Though many studies concerning the effects of environmental variables such as ammonia, nitrite, temperature and pH on cultured fish have been reported, very few have dealt with eggs and larvae of warm water fish and none address the effects on eggs and yolk sac fry of tilapias. The artificial hatchery rearing of tilapias has become a particularly important aspect in fast moving practice of aquaculture and the establishment of tolerance levels and effects of environmental variables on eggs and fry needs urgent attention.

In the light of this information the present study was designed to estimate the tolerance levels of eggs and yolk sac fry of O.mossambicus to certain environmental variables under artificial hatchery conditions. The tolerance levels of ammonia, nitrite and temperature and their effects on eggs and yolk sac fry of O.mossambicus was investigated. It was hoped that the knowledge of tolerance levels to these parameters might help interpretation of the findings in Chapter 3.

4.2. MATERIALS AND METHODS.

4.2.1. Source and handling of eggs.

The eggs used in this experiment were obtained from a broodstock of twenty four females. The mean weight of the females was 128.8g (range 102.7-179.3g), and the mean standard length was 16.9cm (range 15.2-18.1cm). The maintenance of the broodstock and the collection and handling of eggs is described in Chapter 2 sections 2.2 and 2.3 respectively. All the eggs used in this experiment were aged between 48 and 72 hours post spawning at the commencement of the trial.

4.2.2. Incubation system.

As *Oreochromis* eggs need continuous churning during incubation for their survival, it was necessary to design an incubation system which fulfilled this requirement which would normally be performed by the female parent during oral incubation. A series of identical incubation systems were made to be used for the range of concentrations of the toxicants and the temperatures to be tested. A cross sectional diagram of an incubation system is shown in Figure 16.

The system was comprised of a 15l perspex aquarium (40x12x20cm) with six cylindrical round bottomed plastic vessels (0.75l) used as incubators and a power

Figure 16.

Side elevation of an incubation system used for the tolerance trials of ammonia, nitrite and temperature.

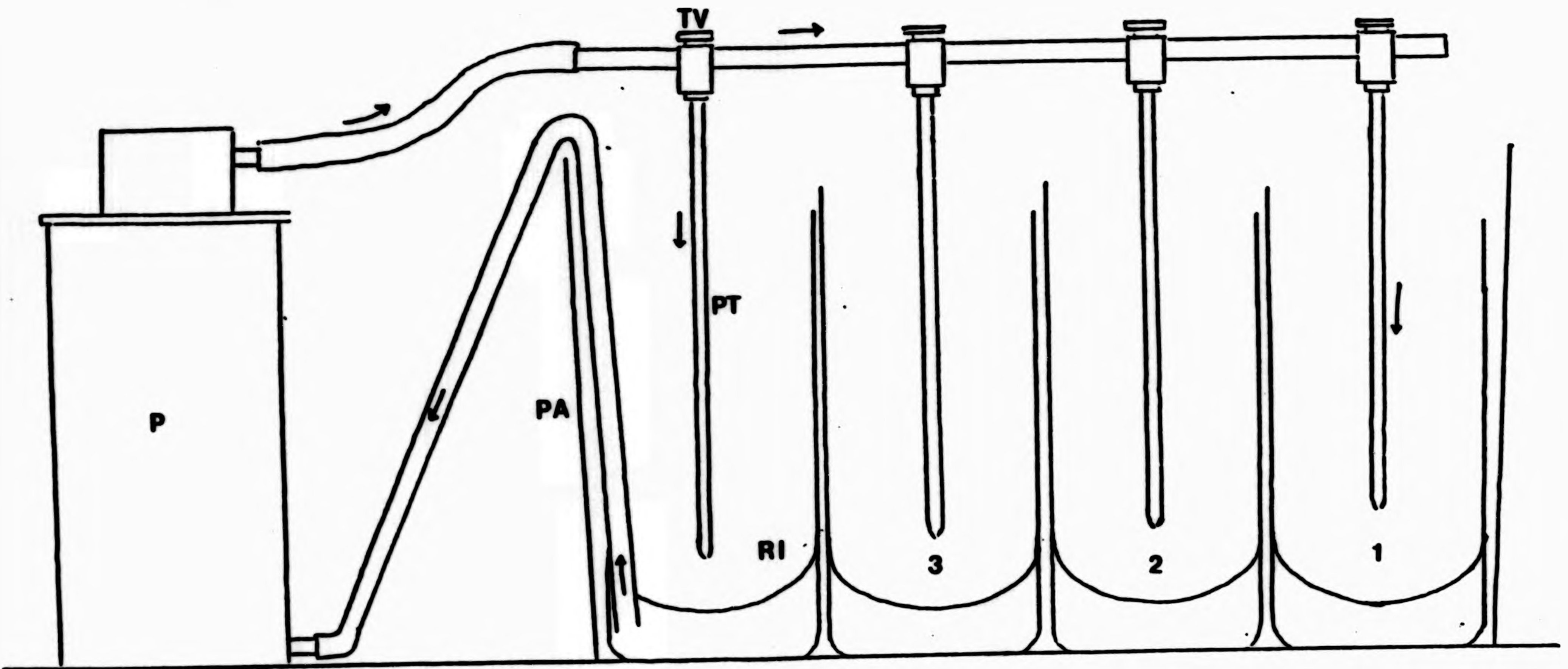
P : Power pump

TV : Two-way valve (regulates the water flow into the incubator)

PT : Plastic tube

RI : Round-bottomed incubator

PA : Perspex aquarium



TV
PT
P
PA
RI
3
2
1

filter unit (Eheim 1021, 23 watt, 6.3 l/min). The power filter was used as a pump (no filter material was incorporated) to recirculate the water in order to provide a current of water for the continuous churning of eggs. The recirculation of the water also ensured identical test conditions for the replicates. The adjustable two way valves were found beneficial in achieving a controlled flow of water into the incubators as a gentle agitation of the eggs was an essential requirement for their survival.

4.2.3. Dilution water.

Throughout the investigation "dilution water" was used as test water as recommended by the Ministry of Agriculture Fisheries and Food for the fish toxicity testing experiments (Anon 1969). The dilution water was prepared to provide a theoretical hardness of 50mg per litre. The preparation procedure with the formula for the dilution water is given in Appendix 2. The chemical characteristics of the dilution water is given in Appendix 3.

4.2.4. Test parameters.

The experiment was carried out in three separate trials to investigate the effects of ammonia, nitrite and temperature on the hatchability and survival of eggs and yolk sac fry.

4.2.5. Test chemicals

All the chemicals used in the preparation of the dilution water were reagent grade, produced by the British Drug House. The test chemicals (toxicants) used were analytical grade from the same manufacturer. Ammonium chloride (NH_4Cl) and sodium nitrite ($NaNO_2$) were used as a source of ammonia and nitrite respectively.

4.2.6. Experimental procedure.

The procedures used in carrying out the ammonia, nitrite and temperature trials are described separately.

4.2.6.1. Trial 1 Ammonia.

Six identical incubation systems were used for a range of five different concentrations of ammonia and a control. Each system was filled with 15L of well-aerated dilution water. A thermostatically controlled immersion aquarium heater was placed in each aquarium for the maintenance of a constant temperature. Each aquarium was well-aerated by a single airstone. A photoperiod of 12 hours artificial light and 12 hours dark was exercised.

Twelve hours prior to the commencement of the trial, a calculated weight of NH_4Cl based on the molecular weight was added to each aquarium to render a

range of five theoretical concentrations of total ammonia nitrogen. The sixth aquarium was used as a control and no NH4Cl was added. The weights of the test chemicals together with the theoretical concentrations are given in Table 3.

A clutch of eggs was removed from the mouth of a brooding female and was gently transferred to a plastic trough. The eggs were examined under a binocular microscope and only the healthy, developing eggs were selected. These were divided into 12 batches of 30 eggs and placed in incubators 1 and 2 of each incubation system. The remaining developing eggs of the clutch were equally divided into six groups and were placed in incubator 3 in each incubation system (Figure 16). The number of eggs placed in incubator 3 was not less than sixty. The water flow into the incubators was adjusted to ensure gentle agitation of the eggs. The egg batches were carefully observed for hatching and the numbers of hatched fry in incubators 1 and 2 in each aquarium were counted within twelve hours of hatching.

The dead or unhatched eggs were removed from all three incubators in each aquarium as soon as observed. The trial was continued and the numbers of surviving fry in incubators 1 and 2 in each aquarium were counted daily. The dead fry in all the incubators were removed twice daily. When there was 100% fry mortality observed in incubators 1 and 2 of any test concentration

TABLE 3. Physico-chemical conditions of the NH_3-N trial

Treatment	Weight of NH_4Cl used g/l.	Expected NH_3-N concentration mg/l.	Measured NH_3-N concentration mg/l.
1	0.0	0.0	<0.01 0.0-0.01
2	0.229	5.0	6.2 5.93-6.38
3	0.458	10.0	13.6 12.74- 14.05
4	0.916	20.0	23.9 21.73-24.89
5	1.832	40.0	56.3 54.21-57.92
6	3.664	80.0	101.4 98.45-103.37
pH	7.56 (7.51-7.63)		
DO mg/l.	7.75 (7.23-7.87)		
Temp.°C	29.1 (29.0-29.2)		

the trial was terminated for that particular concentration. The entire trial was terminated after nine days post hatch.

The fry in the 3rd incubator of each aquarium were used for growth studies.

4.2.6.2. Trial 2 Nitrite.

An identical experimental procedure was employed as described in section 4.2.6.1. for the nitrite trial. The weights of NaNO2 used with their theoretical concentrations are given in Table 4.

4.2.6.3. Trial 3 Temperature.

For the temperature tolerance trial, seven incubation systems were used. These systems were held in a cold room where the temperature was maintained at 12+1'C. A range of temperatures were facilitated by placing a calibrated thermostatically controlled immersion aquarium heater in each incubation system. The range of temperatures are given in Table 5. A similar experimental procedure was practised as described in section 4.2.6.1.

4.2.7. Water quality analysis.

4.2.7.1. Physical parameters.

During each trial, the temperature, pH and the dissolved oxygen concentration of the test water in

TABLE 4. Physico-chemical conditions of the NO2-N trial

Treatment	Weight of NaNO2 used g/l.	Expected NO2-N concentration mg/l.	Measured NO2-N concentration mg/l.
1	0.0	0.0	<0.001
2	0.0394	8.0	7.18 (7.23 -7.14)
3	0.0788	16.0	17.29 (16.27 - 18.31)
4	0.1577	32.0	35.86 (35.44 - 36.29)
5	0.3154	64.0	62.63 (61.78 - 63.42)
6	0.6308	128.0	139.87 (137.91-141.83)

pH	7.73 (7.61 - 7.86)
DO mg/l	7.30 (7.0 - 7.6)
Temp. °C	27.75 (27.0 - 28.5)

TABLE 5. Physico - chemical conditions of the temperature trial.

Treatment	Temperature°C	Range
1	11.05	10.1 - 12.0
2	17.10	17.0 - 17.2
3	20.05	20.0 - 20.1
4	24.30	24.2 - 24.4
5	29.75	29.4 - 30.1
6	34.55	34.3 - 34.8
7	40.00	40.00

pH	7.55	(7.2 - 7.9)
DO mg/l.	7.65	(6.2 - 9.1)
Total NH3-N mg/l.	0.20	(0.02 - 0.38)
Total NO2-N mg/l.	0.012	(0.001 - 0.024)

each aquarium was measured daily using the methods desribed in Chapter 2 section 2.5.

4.2.7.2. Chemical parameters.

At the beginning and at the end of the ammonia and nitrite trials, the test water in each aquarium was analysed to estimate the actual concentrations of the toxicants used. The analytical methods used are as described as in Chapter 2 Section 2.5.

In the ammonia trial the UIA concentration in each test solution was calculated using the formula:

$$(NH3\text{-}N) = (Ammonia\text{-}N)/1+10.0^{(pKa-pH)}$$

where,

HH3-N = unionized ammonia as nitrogen,

Ammonia-N = the measured concentration of ammonia,

pKa = the acidity constant for the reaction,

pH = the measured pH of the solution.

(Colt and Armstrong 1981).

4.2.7.3. Microbiological parameters.

The total viable heterotrophic bacterial counts, total viable fungal spore counts and total viable Saprolegniaceae spore counts were made at the beginning

and the end of each trial for each test concentration and temperature separately. The methods used are described in Chapter 2 Section 2.6.1, 2.6.3 and 2.6.4 respectively.

4.2.8. Growth Studies.

In each trial within 12 hours of hatching a sample of 20 fry was removed from the incubator 3 of each aquarium. They were divided into two equal batches. The yolk was removed from one batch of fry by disecting under a binocular microscope, after being killed by a high dose of anaesthetic (Benzocaine). The disection was carried out very gently and extreme care was taken not to remove any internal organs. The two groups were then dried separately in an oven (105'C) for 12 hours. The dry weight of the fry with and without yolk was then measured. This procedure was repeated every third day for each concentration of toxicant or temperature tested until the trials were terminated at day nine post hatch.

4.2.9. Histology.

On the day the trial was terminated the remaining fry in incubators 1 and 2 of each test aquarium were fixed in 10% buffered formalin. The fixation was carried out after the fry were killed by an over dose of anaesthetic Benzocaine. The fixed material was processed in an automatic tissue processor (Tissuetec) and histological

sections were made. They were stained routinely using Hematoxylin and Eosin. The detailed procedure of processing and staining is given in Appendix 4.

4.3 RESULTS

4.3.1 Ammonia trial

The percentage survival of the eggs and fry over the experimental period in the different ammonia concentrations is shown in Figure 17. The hatchability of the eggs was not affected by the total NH3-N concentration up to 101.4 mg/l. One hundred percent fry mortalities were observed only at the test concentrations above 13.6 mg/l. The percentage survival of the fry exposed to the total NH3-N concentration of 6.2 mg/l was 81.5 at day 10 post exposure. The concentration of 101.4 mg/l total NH3-N caused 100% fry mortality within 4 days of exposure.

The physicochemical parameters measured during the experiment are given in Table 3. From the table it can be seen that the pH and the temperature fluctuated only slightly (pH 7.44-7.56 and temperature 29.0 -29.1'C) during the period of investigation. As the observed fluctuations were slight it is safe to assume that there would be no effect on the unionized ammonia (UIA) levels in the test solutions during the trial.

The Spearman Karber estimation of the LC50

Figure 17.

Graph showing the percentage hatchability and survival of eggs and fry at different ammonia concentrations (Bars indicate ± standard deviation).

A : Control

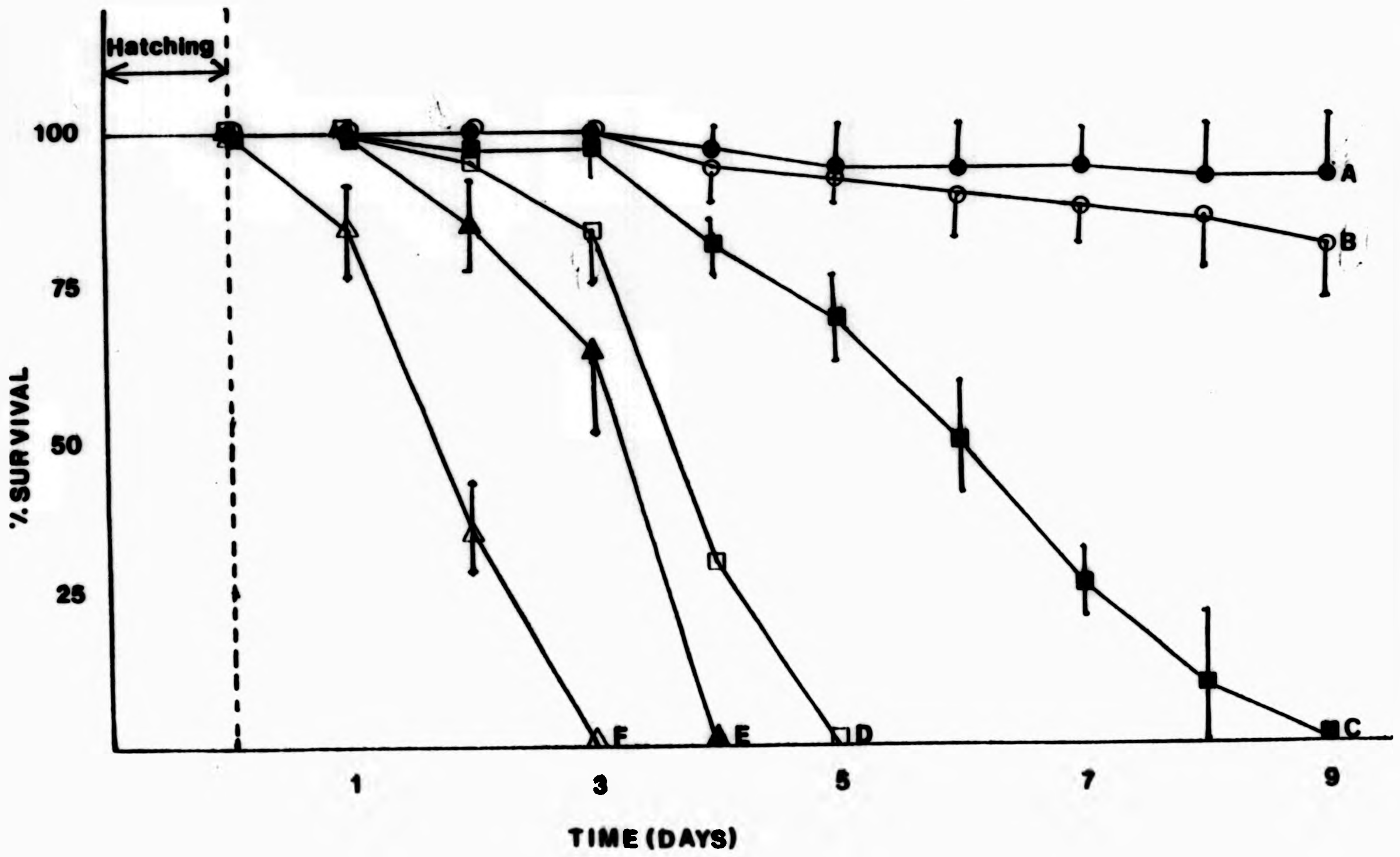
B : 6.2 mg/l total NH3-N

C : 13.6 mg/l "

D : 23.9 mg/l "

E : 56.3 mg/l "

F : 101.4 mg/l "



Hatching
100
75
50
25
% SURVIVAL
1
3
5
7
9
TIME (DAYS)
A
B
C
D
E
F

(the concentration at which 50% mortality occurs) values are given in Table 6. As the observed mortalities of the yolk sac fry among all the test concentrations were less than 50% after one day post hatch (Figure 17), the calculation of the LC50 values for the one day (24 hours) old fry was not possible. The pattern of change of LC50 values with time for the hatched fry over the rest of the experimental period is given in Figure 18. It is evident from the graph that the LC50 values changed from 1.905 mg/l UIA to 0.195 mg/l UIA respectively between day 2 and day 9 post hatch.

As there was only one test concentration of ammonia which resulted in less than 80% fry mortality over the experimental period, the effects of ammonia on the growth of the yolk sac fry were not studied. However, incidental observation made over the trial period indicated that exposure to ammonia had a marked effect on growth and yolk absorption of *O. mossambicus* fry (Figure 21).

4.3.2 Nitrite trial

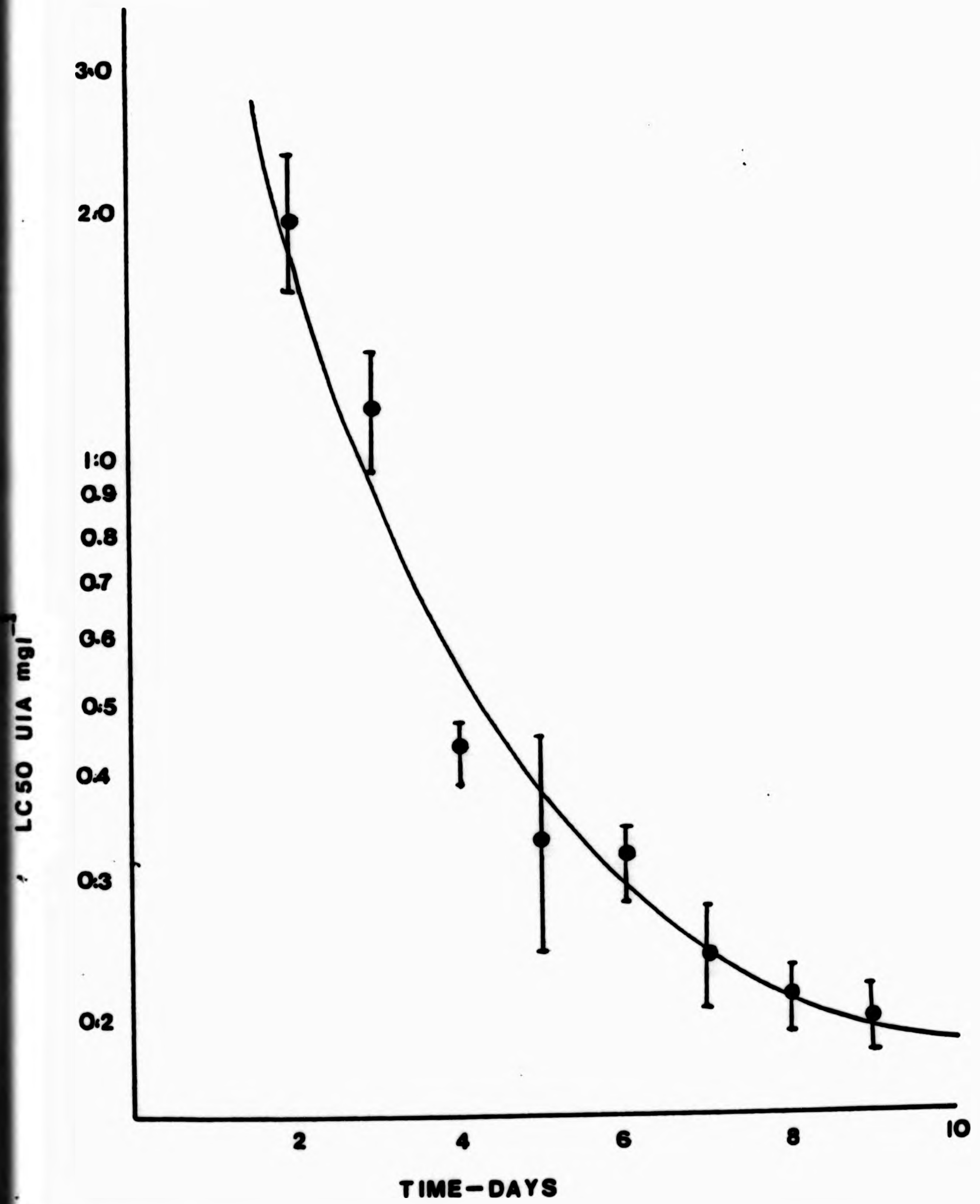
The physicochemical parameters measured during this trial are given in Table 4. The observed hatchability of the eggs was less than 100% for all the concentrations tested including the control (Figure 19). However, the percentage hatchability was not significantly different ($P<0.05$) between the treatments including the

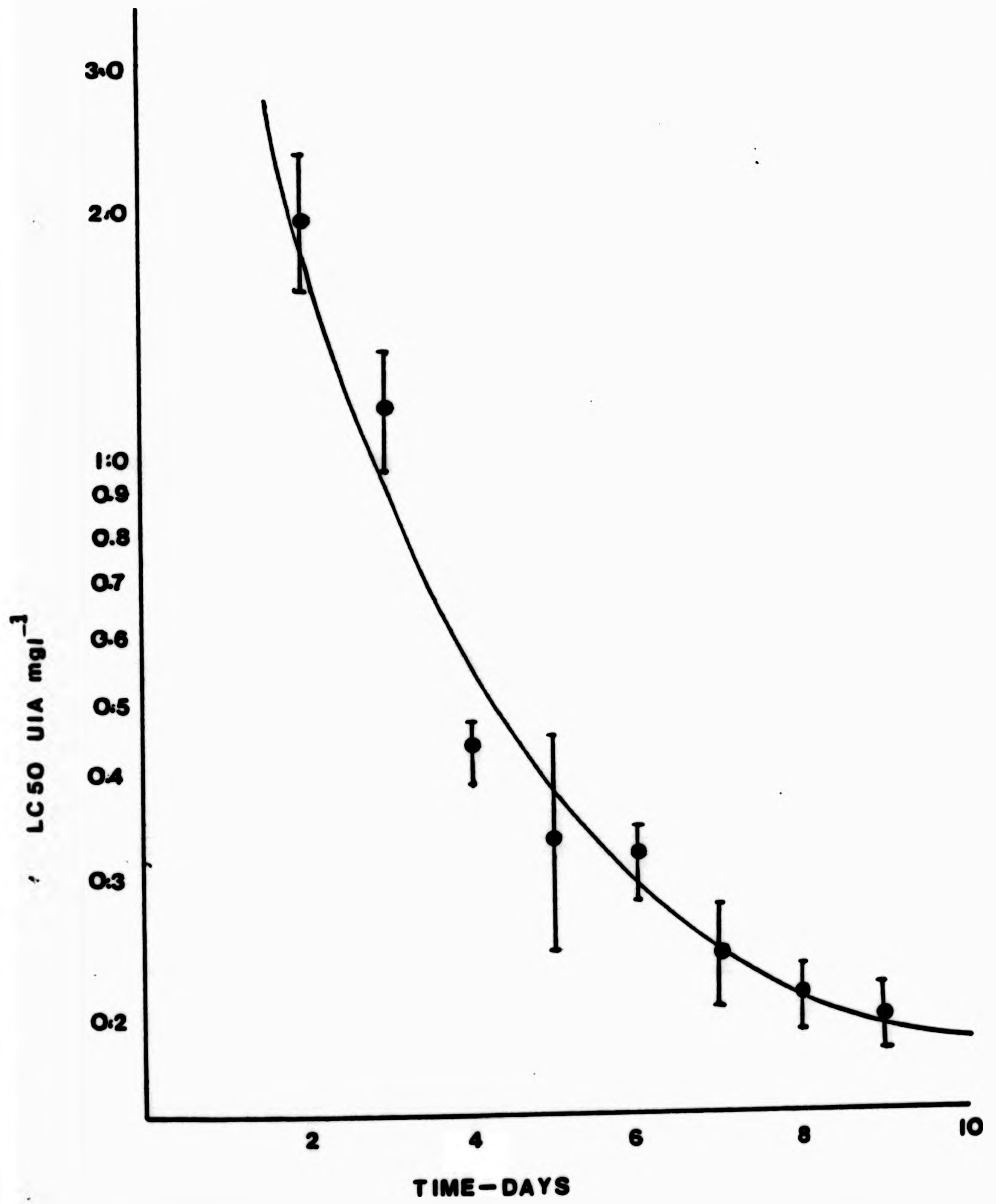
TABLE 6. Spearman-Karber estimation of the LC50 values of Unionised Ammonia (UIA) for newly hatched fry at different exposure times.

Time of exposure (Days)	LC50 (95% confidence intervals) of total NH3-N mg/l.
1	Not calculable
2	1.905 (1.578 - 2.300)
3	1.111 (0.947 - 1.304)
4	0.432 (0.386 - 0.459)
5	0.327 (0.239 - 0.447)
6	0.312 (0.278 - 0.310)
7	0.235 (0.203 - 0.271)
8	0.209 (0.191 - 0.229)
9	0.195 (0.178 - 0.213)

Figure 18.

The toxicity curve of Unionised Ammonia (UIA) for newly hatched fry . Bars indicate 95% confidence limits.


3·0
2·0
1·0
0.9
0.8
0.7
0.6
0·5
0.4
0·3
0·2
LC50 UIA mgl⁻¹
2
4
6
8
10
TIME—DAYS


3·0
2·0
1·0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
LC50 UIA mgl⁻¹
2
4
6
8
10
TIME—DAYS

control.

One hundred percent fry mortality was observed at concentration above 35.85 mg/l total NO2-N whereas 14% fry mortality occurred in the control over the nine day trial. However, a significant difference (P>0.05) between the control and the test concentration was found to occur as low as 7.18 mg/l NO2-N.

The Spearman Karber estimation of the LC50 values for the yolk sac fry are given in Table 7. The observed fry mortalities were less than 50% for all the concentrations tested including the control until 2 days post hatch. Therefore, the Spearman Karber estimation of LC50 values for the first two days of post hatching were not possible.

The estimated 50% lethal concentrations of NO2-N for fry exposed for 3 and 9 days were 38.57 and 11.0 mg/l respectively. The pattern of change of the LC50 values for the yolk sac fry aged between 3 and 9 days post hatch is given in Figure 20. As the LC50 curve reached a phase which runs parallel to the time axis the median threshold concentration (TCM) of NO2-N for O.mossambicus yolk sac fry appeared to be around 11.0 mg/l.

Only one concentration tested (7.18 mg/l) resulted in less than 80% fry survival over the experimental period and therefore, though the fry were sampled for growth studies the effect of NO2-N on growth

Figure 19.

Graph showing the percentage hatchability and survival of eggs and fry at different nitrite concentrations (Bars indicate ± standard deviation

A : Control

B : 7.18 mg/l total NO2-N

C : 17.29 mg/l "

D : 35.86 mg/l "

E : 62.63 mg/l "

F : 139.87 mg/l "

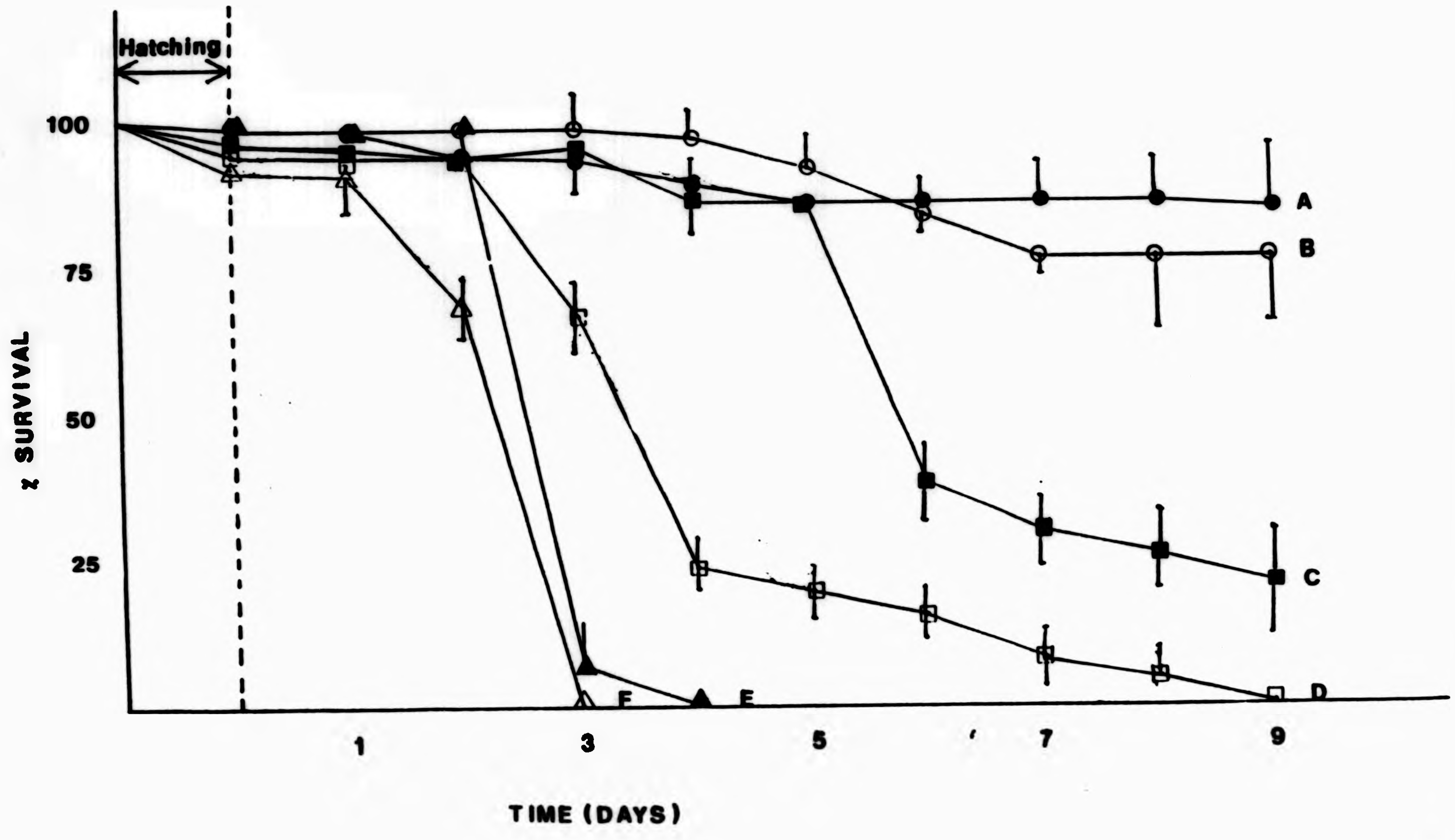
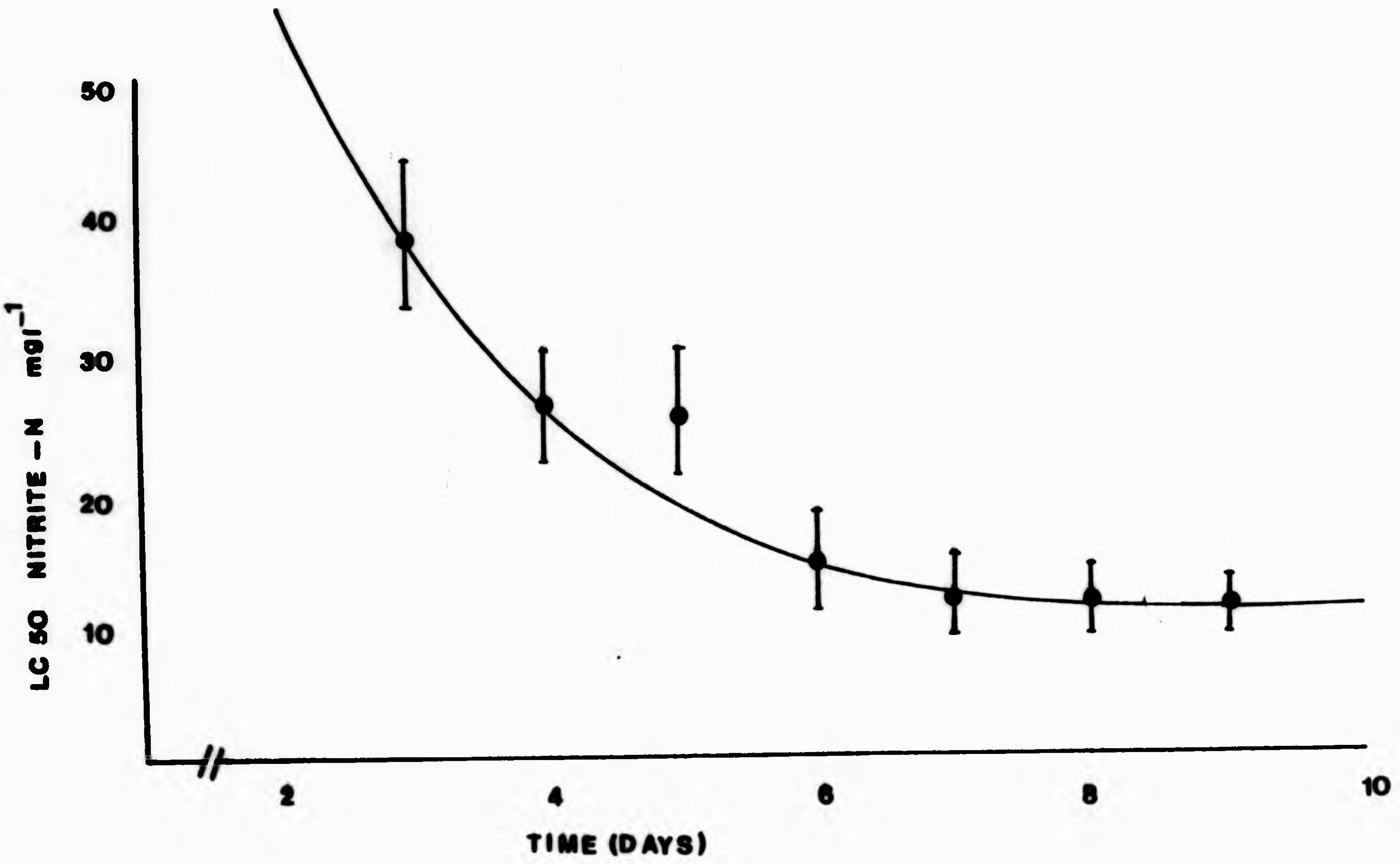

Hatching
100
75
50
25
% SURVIVAL
A
B
C
D
E
F
1
3
5
7
9
TIME (DAYS)

TABLE 7. Spearman - Karber estimation of the LC50 values of NO2-N for newly hatched fry at different exposure times.

Time of Exposure (days)	LC50 (95% confidence intervals) of total NO2-N mg/l.
1	Not calculable
2	Not calculable
3	38.572 (33.228 - 44.696)
4	26.111 (22.377 - 30.467)
5	25.673 (21.783 - 30.258)
6	14.353 (11.280 - 18.264)
7	11.929 (9.433 - 15.084)
8	11.493 (9.348 - 14.129)
9	11.000 (9.258 - 13.071)

Figure 20.

The toxicity curve of NO_2-N for newly hatched fry. Bars indicate 95% confidence limits.


LC 50 NITRITE -N mgl-1
50
40
30
20
10
2
4
6
8
10
TIME (DAYS)

was not investigated. However, the observations indicated a reduction in yolk absorption and growth in yolk sac fry when exposed to NO2-N concentrations above 7.18 mg/l. Figure 22 illustrates the size difference between a 6 day old control fry and a fry exposed to 17.29 mg/l NO2-N.

4.3.3 Temperature trial

Table 5 describes the mean temperature and the physicochemical parameters of the test water measured during the investigation. The time taken for hatching of the eggs differed between some of the temperatures tested (Figure 23). At 11.1'C the eggs failed to hatch whilst at 40'C all the eggs hatched and died within 24 hours of exposure. The eggs incubated at 17.1 and 20.1'C took 4 and 3 days respectively to hatch. The rest were hatched within 48 hours of incubation.

At the two extreme temperatures 11.1 and 40.0'C and also at 17.1'C the mortality was 100% (Figure 23). The observed fry survival at 24.3, 29.8 and 34.6'C were almost 100% whilst at 20.1'C the fry survival was less than 60%.

4.3.4. Growth studies

The initial dry weight of the fry hatched at the temperatures between 20.1'C and 34.6'C were not significantly different at 5% level. The relationship

Figure 21.

A photomicrograph showing differences in growth and yolk absorption between six day old fry exposed to 6.2mg/l NH3-N and control.

A : Control

B : Exposed

Note the lack of pigmentation in the exposed fry.

Figure 22.

A photomicrograph showing the differences in growth and yolk absorption between six day old fry exposed to 17.29mg/l NO2-N and control.

A : Control

B : Exposed

Figure 23. Graph showing the percentage hatchability and survival of eggs and fry at different temperatures ('C). Bars indicate ± standard deviation.

A : 11.05

B : 17.10

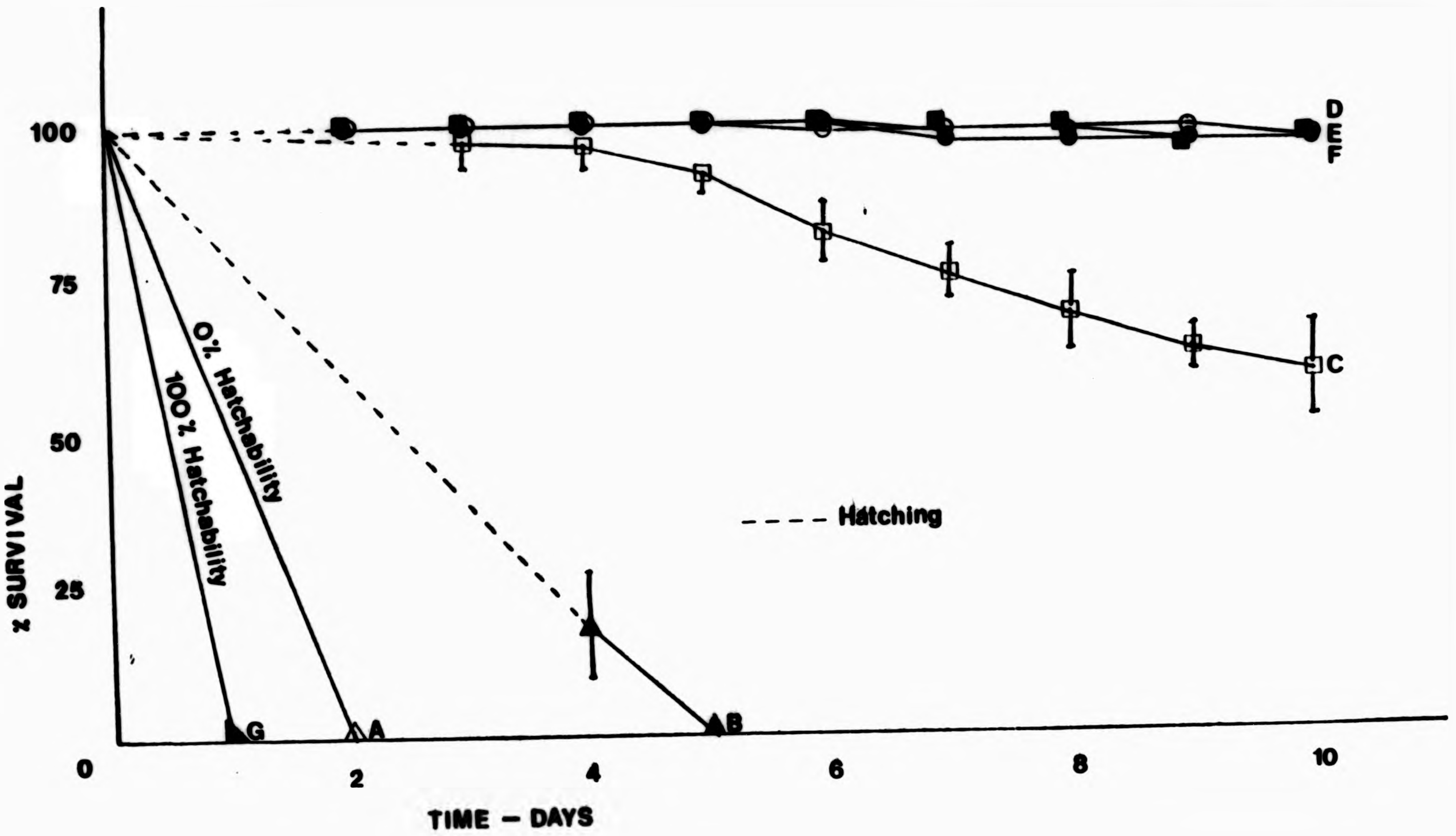
C : 20.05

D : 24.30

E : 29.75

F : 34.55

G : 40.00


% SURVIVAL
100
75
50
25
0
2
4
6
8
10
TIME — DAYS
D
E
F
C
A
B
G
0% Hatchability
100% Hatchability
Hatching

between the dry weight of the fry without yolk (somatic weight) and time measured at four different temperatures over the investigation is shown in Figure 24. The graph shows that the dry somatic weight of fry reared at 34.6'C began to decrease 6 days after hatching, whilst the other group continued to increase in weight up to 9 days post hatching when the trial was terminated. The dry somatic weight of the fry after 9 days post hatch reared at 34.6'C was approximately 0.65 mg and was lying between the final weights at 24.3 and 29.8'C.

The relationship between the log mean dry somatic weight of the fry and time at different temperatures is given in Figure 25. As the dry somatic weight of the fry at 34.6'C after 9 days post hatch was less than that after 6 days, the dry weight at day 9 post hatch was not used to obtain the regression equation. However, regression lines for the rest of the temperatures were calculated up to 9 days post hatch. The somatic weight increment of the fry at temperatures 20.1, 24.3, 29.8 and 34.5'C was significantly different (P>0.001).

The specific growth rate of the fry at different temperatures are given in Table 8. At the highest temperature, 34.6 C, a negative specific growth rate was observed for 6-9 days post hatching. However, the overall specific growth rate of the fry over the first 6 days was 31.29% per day, which was more than four times higher than

TABLE 8. Specific growth rate (SGR) of fry estimated as the somatic growth at different temperatures.

Temperature 'C	Age (days after hatching)			
	0 - 3	3 - 6	6 - 9	0 - 9
20.05	6.82	8.25	8.32	7.89
24.30	12.68	13.17	14.75	13.53
29.75	23.22	28.13	12.57	20.43
34.55	39.45	23.14	-8.43	31.29a

a = SGR estimated for 0-6 days post hatching

$$SGR\ (\%/day) = \frac{(\log_e W\ tn - \log_e W\ ti)}{tn - ti} \times 100$$

W tn = final mean dry embryo weight at time tn

W ti = initial mean dry embryo weight at time ti

tn = final time

ti = initial time

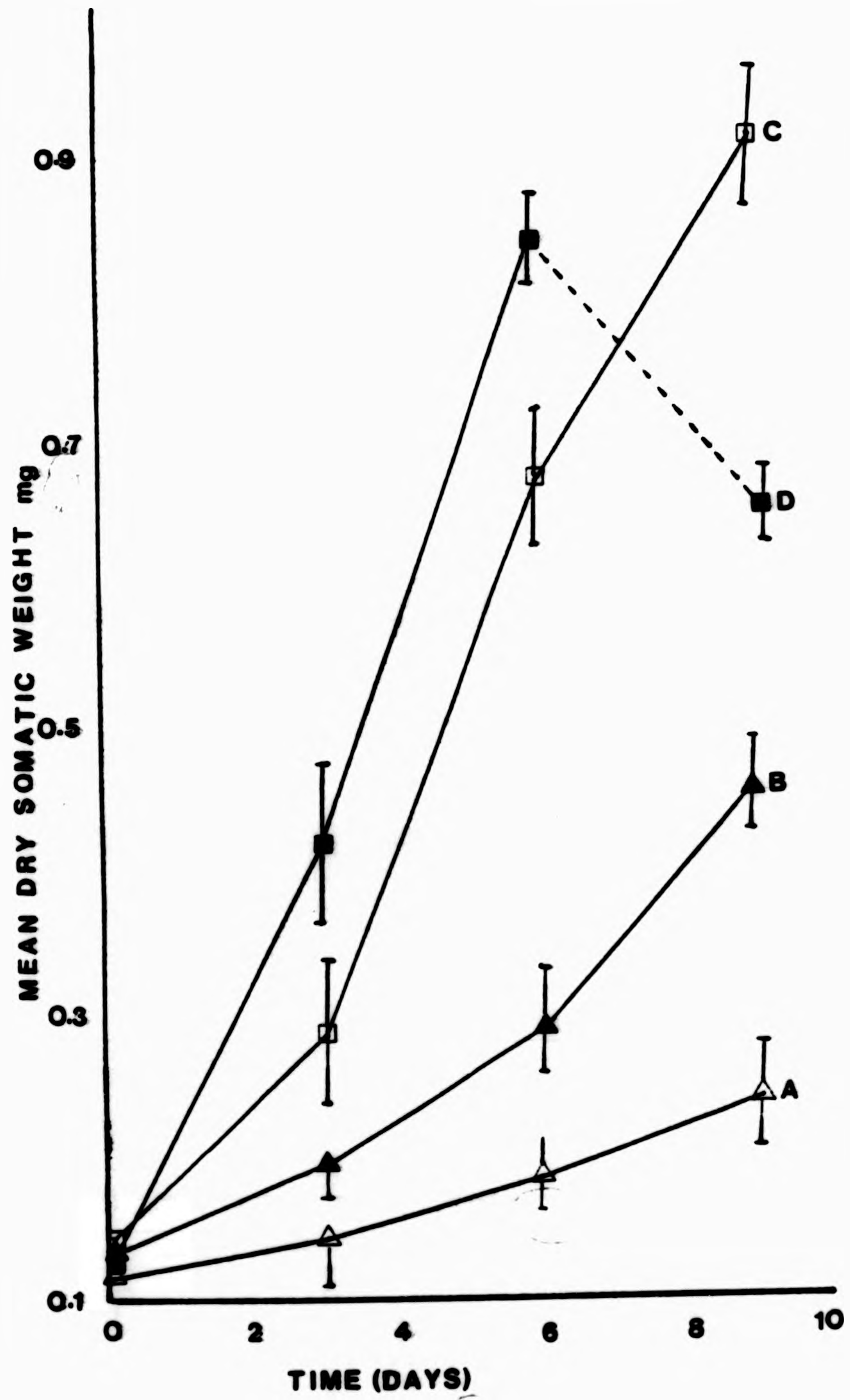
Figure 24.
Graph showing the somatic weight increment of yolksac fry at different temperatures ('C). Bars indicate ± standard deviation.

A : 20.00

B : 24.30

C : 29.75

D : 34.55



MEAN DRY SOMATIC WEIGHT mg
0.9
0.7
0.5
0.3
0.1
TIME (DAYS)
0
2
4
6
8
10
A
B
C
D

Figure 25.

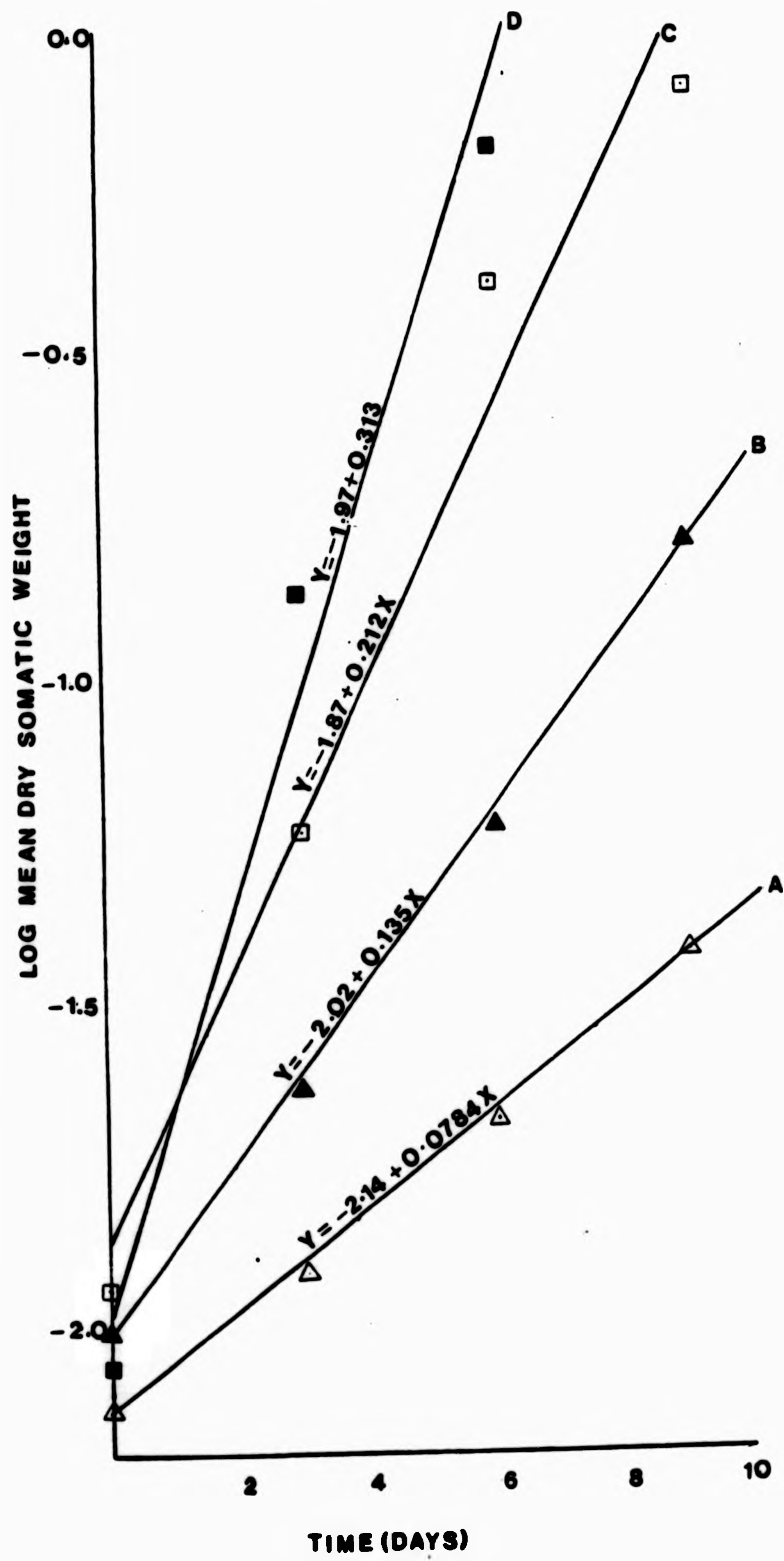
Graph showing the relationship between log Dry Somatic Weight of yolksac fry and Time at different temperatures ('C). (Semi-log transformation of Figure 24).

A : 20.05

B : 24.30

C : 29.75

D : 34.55



D
C
B
A
0.0
-0.5
-1.0
-1.5
-2.0
LOG MEAN DRY SOMATIC WEIGHT
Y=-1.97+0.313
Y=-1.87+0.212X
Y=-2.02+0.135X
Y=-2.14+0.0784X
2
4
6
8
10
TIME (DAYS)

that at 20.1'C over first 9 days. The specific growth rates at the different temperatures tested were significantly different (P>0.001).

4.3.5. Histopathology

The histopathology of the fry reared only at sublethal concentration of NH3-N and NO2-N was studied. The sublethal concentration was justified in terms of more than 50% survival at the end of the trial. Therefore, changes in the histological picture were described only for the lowest concentrations of ammonia and nitrite used together with the controls.

The skin and gills of the controls showed no apparent cellular changes, with the exception of a few hypertrophic areas at the junctions of the primary and secondary lamellae. The development of the secondary lamellae was not complete in any of the samples as the fry were only nine days old. This incomplete development caused a great difficulty in interpreting the histopathological changes. However, the fry reared over a period of 9 days at 6.2mg/l NH3-N concentration showed marked histopathological changes when compared to the controls. The observed changes were not very consistent amongst the samples. They mainly consisted of epithelial hypertrophy and oedema of primary and secondary lamellae. Areas of mild hyperplasia together with epithelial cell necrosis, of the lamellae as

evidenced by the presence of pyknotic nuclei, were also evident. Lifting of the hypertrophic epithelium of the secondary lamellae was convincingly evident. A slight degree of interlamella fusion was also noted. These epithelial changes were mainly confined to the gills and the dermal epithelium was not distinctly changed. There were no histopathological changes found in any internal organs when compared to their controls. The figures 26 and 27 illustrate some of the observed histological changes in the 9 day old fry exposed to 6.2 mg/l NH3-N concentration.

In the nitrite trial 9 days post exposure to 7.18 mg/l NO2-N, the gills exhibited histological changes such as an oedematous epithelium of the secondary lamellae, hypertrophy of the epithelial cells of both primary and secondary lamellae and mild hyperplasia of the lamella epithelium. Increased mucus cells were also evident in certain areas of the secondary lamellae. The changes were relatively less distinct than those in the ammonia trial and were not very consistent amongst the individual samples. Figures 28 and 29 describe some of the histopathological changes observed in the 9 day old fry exposed to 7.18 mg/l NO2-N concentration.

The spleen and the liver of the fry exposed to nitrite did not exhibit any pathological changes when compared to their controls. In contrast, the kidneys showed a low degree of tubular necrosis. Certain amorphous

Figure 26.

Photomicrograph showing branchial histopathology of nine day old fry exposed to 6.2mg/l NH3-N. H&E X 250.

Note the separation of the secondary lamellae epithelium and hypertrophy of primary and secondery lamellae epithelial cells.

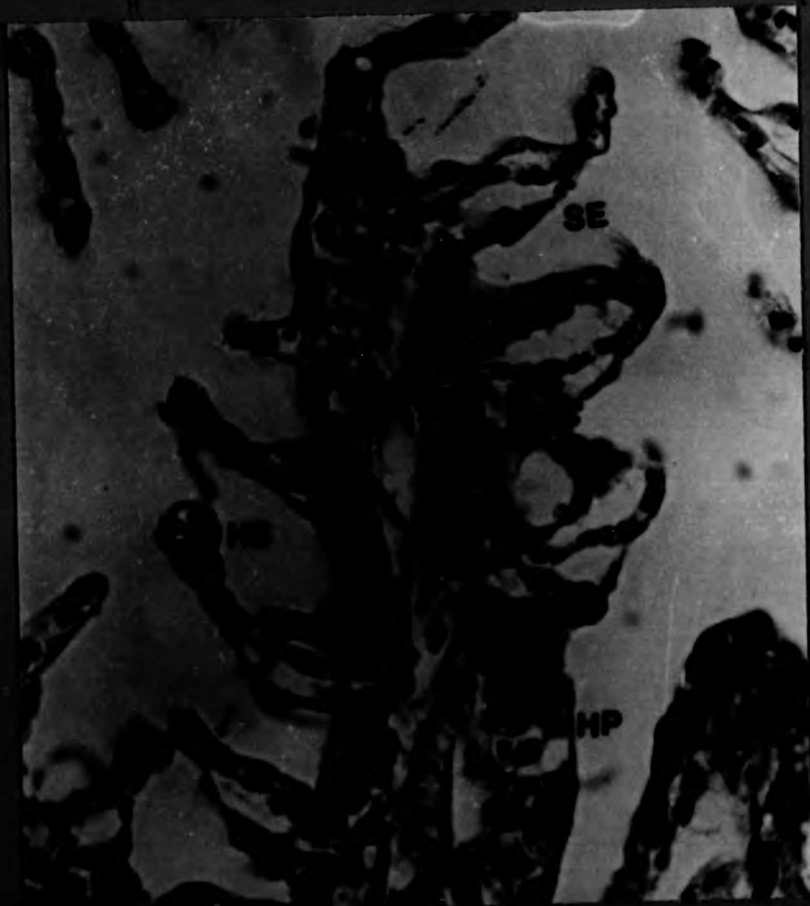
Figure 27.

Photomicrograph showing branchial histopathology of nine day old fry exposed to 6.2mg/l NH3-N. H&E X 400.

SE : Separation of secondary lamellae epithelium

HP : Heterotrophic primary lamellae epithelial cells

HS : Heterotrophic secondary lamellae epithelial cells


SE
HP

SE
HS
HP

Figure 28.

Photomicrograph showing branchial histopathology of nine day old fry exposed to 7.18mg/l NO2-N. H&E X 250.

Note the separation of secondary lamellae epithelium and hypertrophy of the primary and secondary lamellae epithelial cells.

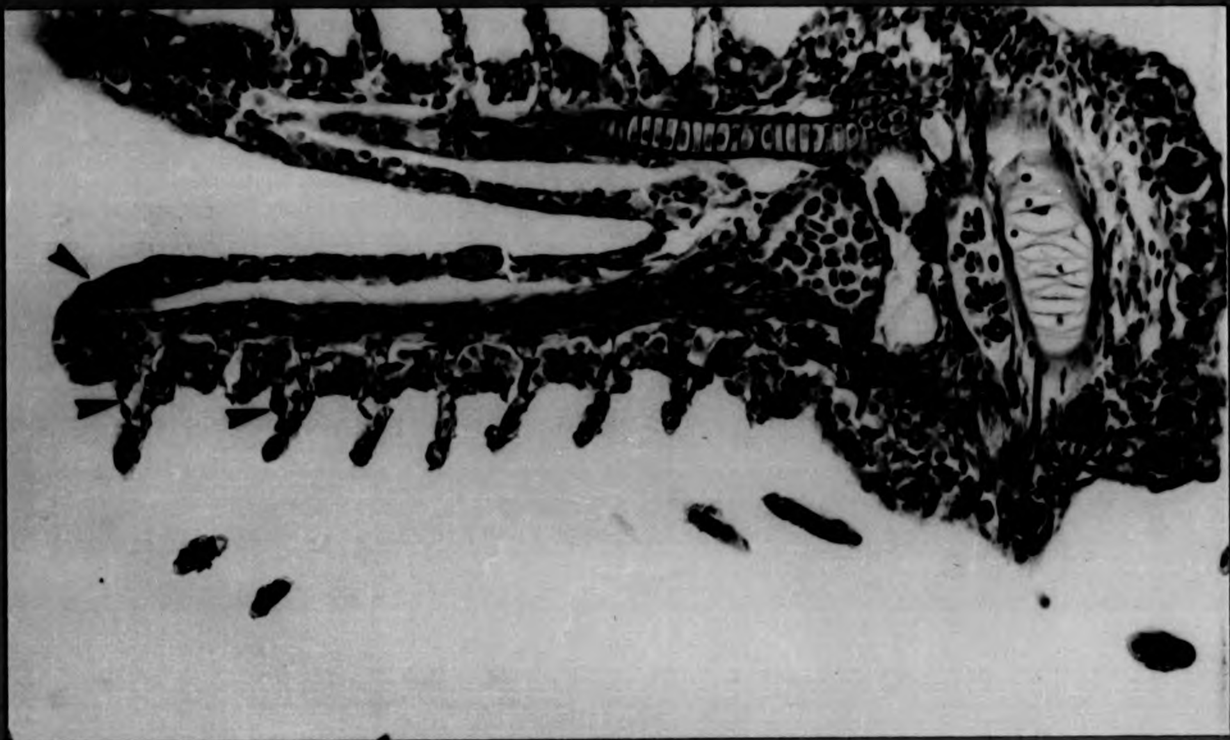
Figure 29.

Photomicrograph showing branchial histopathology of nine day old fry exposed to 7.18mg/l NO2-N. H&E X 400.

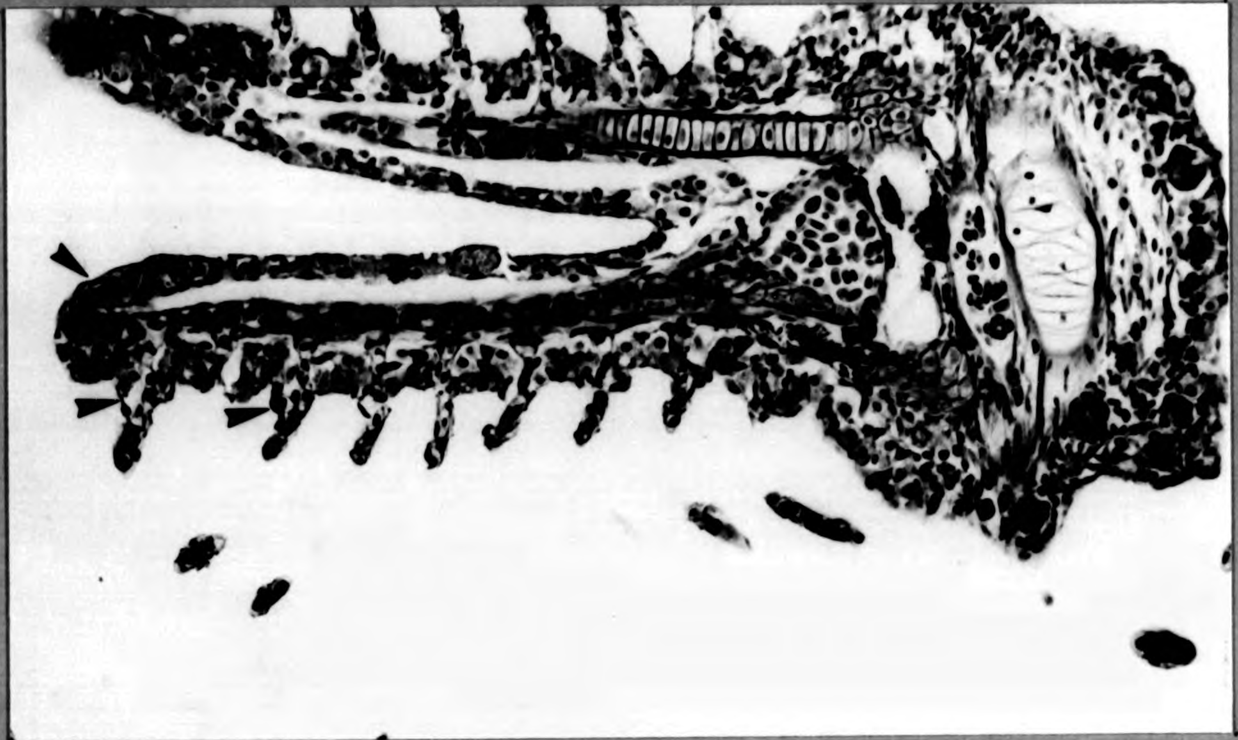
SE : Separation of secondary lamellae epithelium

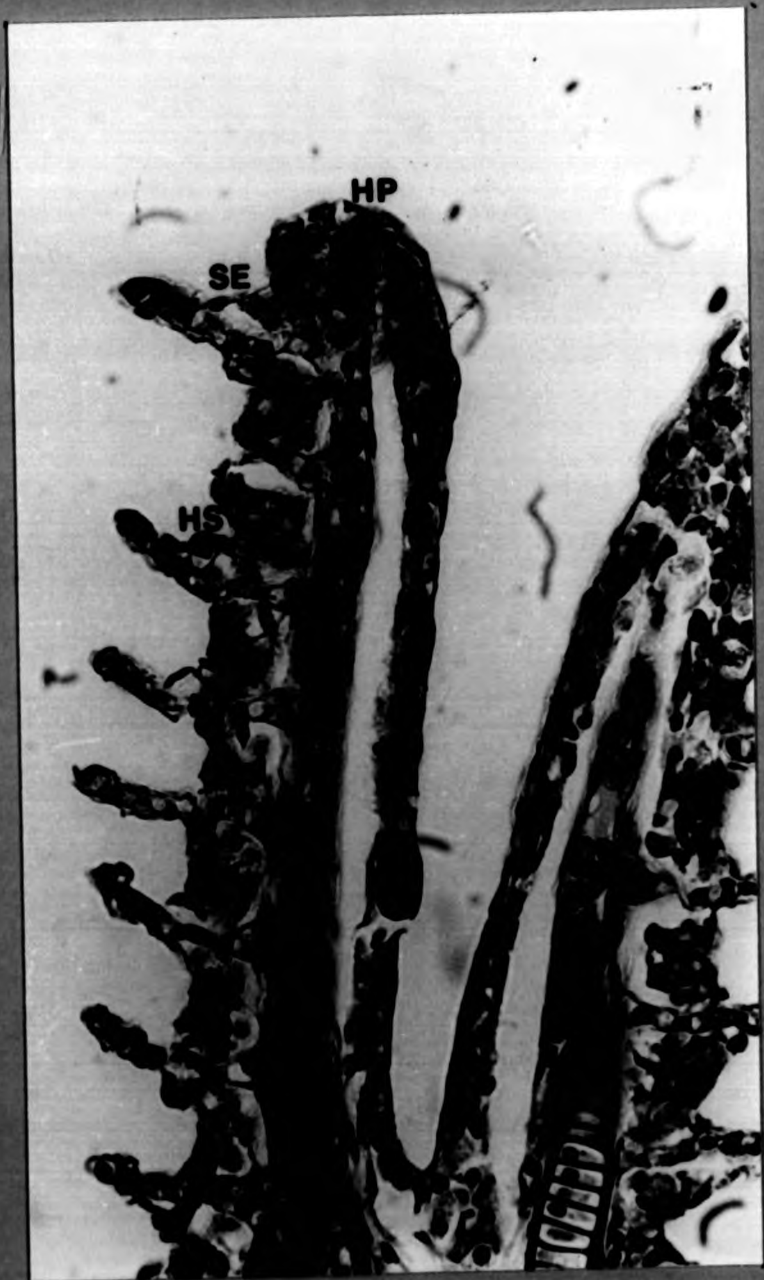
HP : Hypertrophy of the primary lamellae epithelial cells

HS : Hypertrophy of the secondary lamellae epithelial cells



HP
SE




HP
SE
HS

granular deposits were also evident in nephrotic interstitial tissues. Those granules stained blue with Perls Prussian Blue stain, and were considered to be haemosiderin deposits (Figure 30). These deposits were not found in any other organs in the exposed fish or in any organ of the control fish examined. The gills, skin, liver, kidney and spleen of the control fish were considered to be normal without apparent histopathological changes.

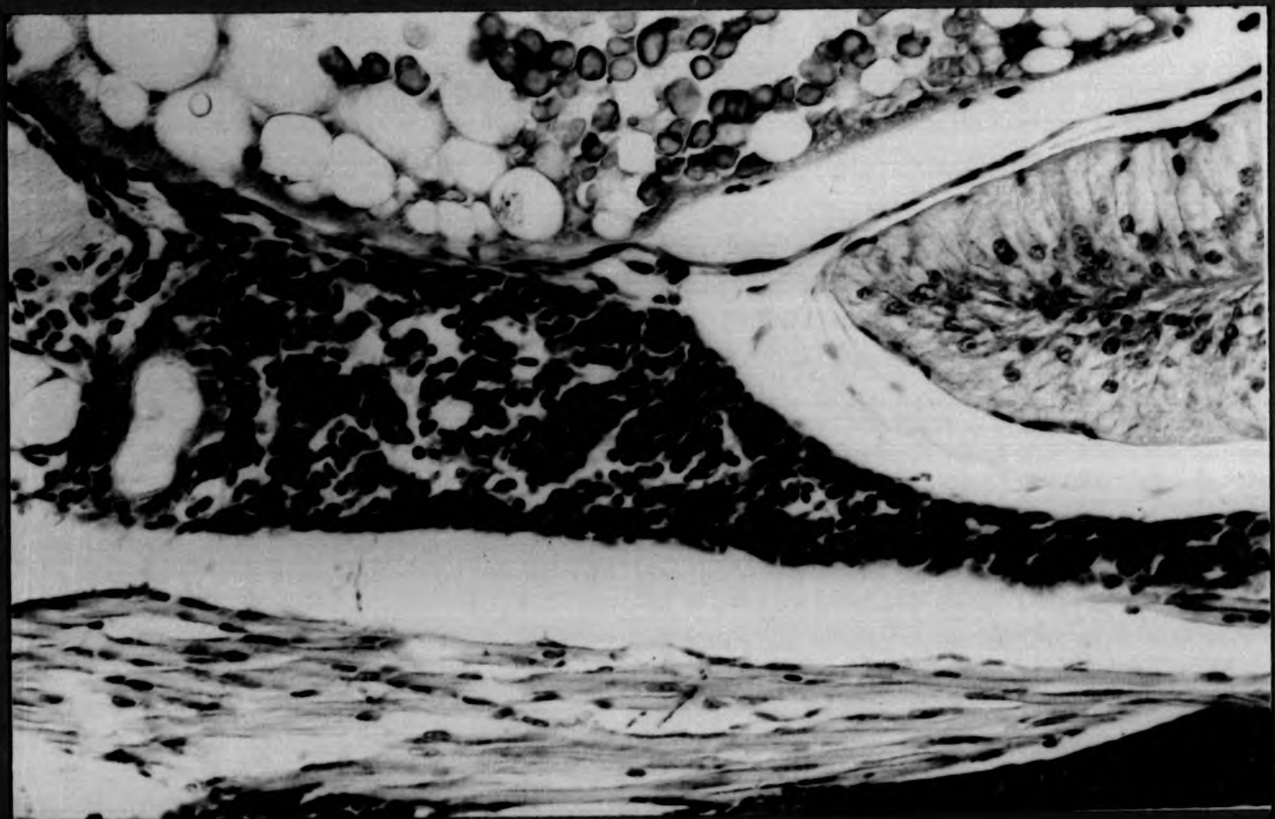
4.4 DISCUSSION

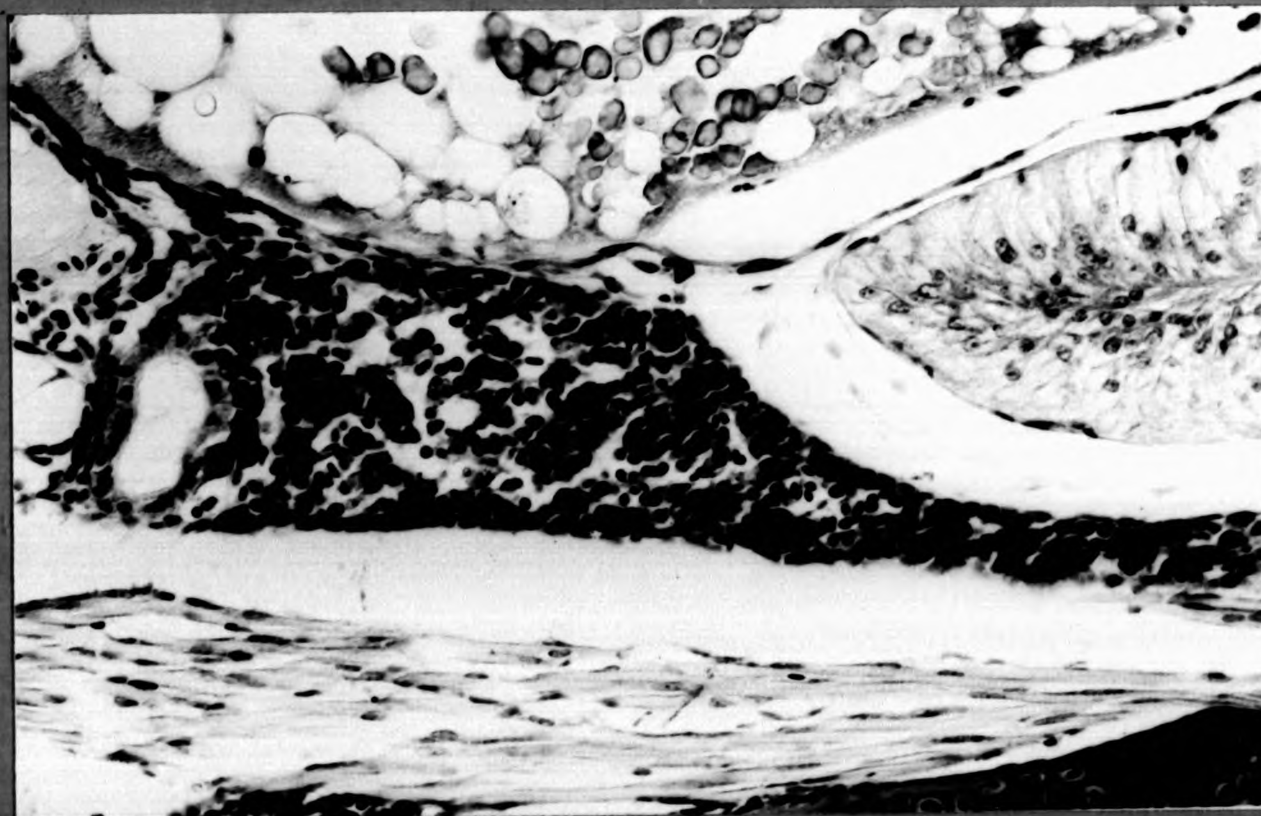
The most widely used methods for the estimation of median lethal concentration (LC50) of a toxicant are based on either the integrated normal (Probit) or logistic (Logit) models. However, according to Hamilton, Russo and Thurston (1977) the x% trimmed Spearman Karber procedure for the estimation of LC50 is overall a better method than the methods based on the Probit and Logit models. This conclusion was based on their experience with real and hypothetical data and taking into account the accuracy, precision, comparability and robustness of the method. Hence, in the present study all the estimations of LC 50 values were carried out using the x% trimmed Spearman Karber method described by Hamilton _et al_ (1977). A computer programme written in FORTRAN was obtained from the Montana State University, U.S.A and used throughout the analysis. The details of the programme were also described

Figure 30.

Photomicrograph showing haemosiderin deposits in the kidney interstitial tissue of nine day old fry exposed to 7.18mg/l NO2-N. Perls Prussian Blue X 250.

H : Haemosiderin deposits.





by Hamilton *et al* (1977).

None of the concentrations of ammonia or the nitrite used in the present study produced any significant difference ($P<0.05$) in hatchability of eggs between controls and treatments. This indicates a high degree of resistance of *O. mossambicus* eggs to ammonia and nitrite. Similarly Penaz (1965) cited by Rice and Stokes (1975) found that early developing eggs of *Salmo trutta* (Linnaeus) were resistant to ammonia as high as 50 mg/l UIA. Rice and Stokes (1975) found that neither fertilized eggs, embryos nor alevins of *Salmo gairdneri* (Richardson) were susceptible to 24 hour exposure of UIA at a level of 3.58 mg/l until about the fiftieth day of development. Holt and Arnold (1983) also found that the mean hatching success of red drum, *Sciaenops ocellatus* (Linnaeus) eggs was between 88 to 97% at UIA levels as high as 7.2 mg/l. They also reported that the nitrite concentrations up to 100 mg/l did not significantly increase the mortality of red drum eggs. Therefore, it is evident that the developing fertilized eggs are generally relatively less susceptible to higher levels of ammonia and nitrite than adult fish.

Fish eggs and yolk sac fry are reported to possess a certain degree of resistance to various other chemicals too. Trout eggs and sac fry were only slightly susceptible to endrin, an organophosphate pesticide at concentrations that seriously affected adults (Wenger 1973)

whilst the eggs of two species of salmonids were found to be about one tenth as sensitive to a commercial formulation of rotenone as fry at the same temperature (Garrison 1968). Burdick, Harris, Dean, Walker, Skea and Colby (1964) observed that a high proportion of lake trout, Salvelinus namaycush (Walbaum) fry, from eggs which appeared normal but which contained about 3 ppm DDT only died at the completion of their yolk sac absorption. Moreover, the eggs of zebra fish, Brachydanio rerio (Hamilton) were found to be relatively less susceptible than newly hatched fry to zinc (Skidmore 1965). Therefore, it appears that not only the developing fertilized eggs but also the early yolk sac fry are relatively less susceptible to toxicants than other life stages of many fish.

One of the obvious explanations for this relative resistance is the possible protection of the embryos provided by the surrounding egg membranes. However, Skidmore (1966) showed that the zebra fish eggs with ruptured outer membrane ie exposed embryos, survived as long as the intact control embryos when exposed to similar concentrations of zinc. If the protective barrier was the outer egg membrane the embryos would not have survived as long as the controls with intact membranes. The study of Holt and Arnold (1983) convincingly showed that the sensitivity to ammonia by red rum eggs and larvae varies with the stage of development and that the most critical

time is the larval transition from the yolk sac stage to exogenous feeding. Rice and Stokes (1975) found that S.gairdneri fry exhibited a dramatic increase in the excretion of ammonia and sensitivity to ammonia at the completion of the yolk sac absorption. However, the reasons for this higher early resistance followed by the decline in resistance towards the completion of yolk sac absorption in early fry, as observed in many studies including the present study, have not yet been satisfactorily explained.

Owing to the nature of the study an estimation of a LC50 value over a constant exposure time for various developmental stages of the embryos and fry was not possible. However, the observed high resistance during the very early life of the fry could also be attributed to the stage of development of the branchial apparatus during their very early stages of development. In oviparous teleosts just after hatching the principal site of gaseous exchange is the vascularized surface of the yolk sac. All the newly hatched fry undergo a transition from cutaneous respiration to gill respiration as they develop (Holten 1971). Further, at very early stages of development the demand for energy is less as the fry are relatively inactive. The surface area to volume ratio and the area available for the gaseous exchange is relatively low in such early developing fry compared to the fry with properly developed gills. Therefore, as the fish complete absorption

of yolk and move on to branchial respiration the surface area available for the gaseous exchange increases rapidly; they become less resistant to toxicants including ammonia and nitrite.

Most threshold lethal concentrations of UIA reported for salmonids have been in the range of 0.2 to 0.4 mg/l (Lloyd 1961; Ball 1967; Lloyd and Orr 1969). Among non salmonid fish such as chub (Leuciscus cephalus Linnaeus) and perch (Perca fluviatilis Linnaeus), the 24 hour LC100 was as high as 14.5 mg/l UIA whilst in feathered minnow (Phoxinus phoxinus Linnaeus) and bluegill sun fish, Enneacanthus gloriosus (Holbrook), the 48 hour LC 50 was 8.5 and 9.0 mg/l UIA respectively (McKee and Wolf 1963). According to Has an and Mcintosh (1986a) the 48, 96 and 168 hour LC 50 for 0.3 g carp (C.carpio) was 1.76, 1.74 and 1.64 mg/l UIA. The estimated 48 hour LC50 for the newly hatched O.mossambicus fry was 1.905 thus, the 48 hour LC50 value observed for O.mossambicus was higher than that for C.carpio. This could be due to the specific differences between fish as well as to the greater resistance of the relatively early stage of development of the O.mossambicus. However, the 168 hour value LC50 value was lower for O.mossambicus fry than C.carpio. This may be associated with the effects of the preceding period of exposure as well as the increase in the susceptibility towards the end of yolk sac absorption.

The existing literature suggests that the toxicity to nitrite varies a great deal amongst fish species. Salmonids are more susceptible than the other freshwater fish species. The 96 hour LC50 range from 0.2 mg/l NO2-N for rainbow trout (Russo and Thurston 1977) to 86 mg/l for blue gill sun fish (Huey, Wooten, Freeman and Beitinger 1982 cited by Palachek and Tomasso 1984). The 96 hour LC50 for carp (C.carpio) was 40 mg/l (Solbe' 1981) whilst for roach (Rutilus rutilus Linnaeus) it was 12 mg/l (Cooper and Solbe' 1980). Palachek and Tomasso (1984) suggested that these differences may also have associated with the water quality characteristics that exist at the individual research facility as well as to the genetically based differences in nitrite susceptibility among species.

The 72 and 96 hour LC50 obtained for newly hatched O.mossambicus fry during the present investigation was 38.5 and 26.1 mg/l NO2-N respectively. Palatchek and Tomasso (1984) observed a 96 hour LC50 of 16.2 mg/l NO2-N for approximately 3.5 g size T.aurea. This difference in low LC50 value obtained by Palatchek and Tomasso (1984) could be associated with the species, stage of development and low chloride ion concentration in the dilution water used during the present study. Russo and Thurston (1977) and Hasan and Macintosh (1986b) clearly showed that the increased chloride ion concentration resulted in decreased nitrite toxicity.

Exposure of fish to ammonia generally raises the blood ammonia levels (Fromm and Gillette 1968). There is evidence that the fish respond to increased blood ammonia levels either by increasing the conversion of ammonia to urea, a non toxic excretory product, or by increased production of glutamide which helps to detoxify ammonia in the blood (Haywood 1983).

Ammonia toxicity manifests itself chiefly as a neurological disorder which results in secondary distress symptoms (Ruffier, Boyle and Kleinschmidt 1981). It has also been shown that the increase in ammonia level causes a decrease in growth (Sadler 1981). In an experiment on effects of prolonged exposure to ammonia on yolk sac fry of *S.gairdneri*, Burkhalter and Kaya (1977) demonstrated an inhibition of growth and development of sac fry by long term exposure to concentrations of ammonia as low as 0.05 mg/l UIA. During the present study reduction of growth was also observed at all the test concentrations. Though there were no growth studies carried out on the fry exposed to ammonia, the degree of growth inhibition appeared to be concentration dependent (eye estimation).

Ammonia has also been found to produce histopathological changes in fish gill structure. Chronic exposure to ammonia causes proliferative lesions in epithelium which result in hyperplasia of lamellar epithelium, fusion of secondary lamellae, fusion of gill

filaments, epithelial cell hypertrophy and epithelial oedema (Burrows 1964, Larmoyeux and Piper 1973, Smith and Piper 1975, Smart 1976 and Thurston et al 1978). Though it was relatively difficult to compare the degree of histopathological changes observed during the study as the developmental stage of the fish used was different, most of the above mentioned histological changes were evident on the fish exposed to ammonia.

According to Daoust and Ferguson (1984) exposure of rainbow trout to very high levels of UIA did not produce any lesions in the gills or any other organs. The exposure level was 0.4 mg/l; twenty times the maximum level recommended in a fishery by the European Inland Fisheries Advisory Commission (1973). The results of the present study are not in complete agreement with Daoust and Ferguson (1984). However, the lack of histological changes in the tissues other than gills noted in the present study is interesting. In contrast Soderberg (1985) observed reduced "vacuolation" (sic) in the liver of rainbow trout exposed to very low levels of UIA in static ponds. Further Larmoyeux and Piper (1973) and Thurston et al (1978) also reported reduction in trunk kidney haemopoetic tissue and degenerative changes in fish exposed to high levels of UIA. However, the exposure times were prolonged. The lack of apparent histopathological changes in the tissues other than gills in the present study could be associated with

the comparatively short term exposure as well as the difference in the stage of development.

The mechanism of nitrite toxicity to fish is different from that of ammonia. Nitrite enters the fish through the respiratory surface and passes into the circulatory system. The best known effect of nitrite is the conversion of haemoglobin to methaemoglobin, a derivative incapable of binding and transporting oxygen (Brown and McLeay 1975 and Wedemeyer and Yasutake 1978). The appearance of methaemoglobin is associated with nitrite ions entering the blood plasma and the rate of methaemoglobin formation is closely related to the concentration of NO2 ions in the blood (Eddy, Kunzlik and Bath 1983).

According to Wedemeyer and Yasutake (1978) steelhead trout (S.gairdneri) showed varied degrees of slight epithelial hypertrophy, hyperplasia and lamella separation, 3 weeks post exposure to sublethal levels of nitrite. The observation of similar pathological changes in the gills of newly hatched yolk sac fry of O.mossambicus during the present investigation suggests that the fry are vulnerable to sublethal exposure to nitrite over a nine day period. Though the magnitude of the damage was not as high as observed by many other workers, sublethal nitrite could be detrimental depending on the extent of damage.

One of the interesting observations made

during the study was the marked reduction in growth and yolk absorption in the yolk sac fry exposed to nitrite concentrations above 17.29 mg/l. This reduction was possibly due to the impaired metabolic activity which resulted from increased methaemoglobin levels and anoxia. In contrast Wedemeyer and Yasutake (1978) reported that steelhead trout (S. *gairdneri*) showed no significant growth reduction 6 months post exposure to 0.03 mg/l NO2-N. Their concentration was sublethal.

Arillo, Gaino, Margiocco, Mensi and Schenone (1984) working on the acute toxicity mechanism of nitrite in rainbow trout, stated that "....... tissue hypoxia due to the nitrite induced high methaemoglobinemia is too low to be directly responsible for animal death. Nevertheless, liver hypoxia is thought to be at the root of the nitrite acute toxicity mechanism by producing suitable conditions for toxic potentialities. Thus irreversible and deadly damage arises in liver biochemistry and ultrasructure ....". However, there were no histopathological changes observed in the liver tissue during the present study under light microscopic levels. In contrast, the observation of haemosiderin deposits in the kidney interstitial tissue suggests that nitrite induced methaemoglobinemia may have resulted in breakdown of erythrocytes in the haemopoeitic tissues in the kidney. Usually in haemolytic conditions where there is heavy

breakdown of erythrocytes the deposition of haemosiderin occurs in the melanomacrophage centres of the haemopoeitic tissue (Roberts 1978). However, though the Perl's Prussian blue stain positively demonstrated the presence of haemosiderin deposits in the kidney haemopoeitic tissue of yolk sac fry during the present study, no signs of accumulation in the melanomacrophage centres were noted.

It is clear from the results that the upper lethal temperature for the developing O.mossambicus eggs is above 40'C and the lower lethal temperature is between 17 and 11'C. However the upper and lower lethal temperatures for the yolk sac fry were different from those of the developing eggs, and appear to lie between 34-40'C and 17-20'C respectively. This indicates that the developing egg stage is more heat resistant at both ends of the scale compared to yolk sac fry. Somewhat similar observations were made by Hassler (1982) during his study on the survival of northern pike (Esox lucius Linnaeus) embryos and yolk sac fry.

Guma'a (1978) observed a clear cut, inverse relationship between the rate of development of the perch (P.fluviatilis) embryos and the incubation temperature. He noted that the time required for 90% hatching at 8'C was more than 5 times longer than that at 20'C. During the present study the eggs incubated at 40'C were hatched within 24 hours of incubation whilst those

incubated at 17'C took 96 hours to hatch. As the eggs used were 48-72 hours incubated at 27'C before being transferred to the experimental system, the estimation of the overall time required for hatching at the temperatures tested is not possible. However, it is clear that there is an inverse relationship between the hatching time and the incubation temperature.

One of the observations made during the study was the quality of the fry hatched at 17'C. Not only was their survival low but also the proportion of deformed fry was high. The low survival time of the hatched fry may have been associated with these deformities and abnormalities. Under normal hatching conditions at 27'C the percentage abnormalities observed in a batch of fry hatched from a clutch of O.mossambicus eggs was less than 2% (Rana 1986b). The rough estimations made on the larval abnormalities at 17'C during the present study were between 17-23%. Similarly Hasler (1982) observed 8-10% abnormalities in northern pike larvae hatched at low temperatures. As in the present study those fry did not survive to "swim up" stage. Further Guma'a (1978) also observed deformed premature hatching at low temperatures.

Thermal tolerance of fish eggs changes depending on the acclimation temperatures (Blaxter 1969). During the present study certain technical difficulties restricted the temperature acclimation of eggs prior to

experimentation. The favourable temperature for *O.mossambicus* to spawn is between 25-30'C. The broodstock was therefore kept at 27'C. The hatching time for the eggs incubated at 27'C is around 96 hours. Hence the normal acclimation regime of 1'C a day was not practical. Therefore, the spawning and incubation temperature of 27'C was allowed to gradually adjust to the appropriate test temperatures. This may have had some effect on the actual observed hatching time as well as the pre and post hatching survival. Therefore, as a result of this as well as the magnitude of the temperature intervals used, the estimation of absolute upper and lower lethal temperatures for the developing eggs and early fry was not possible.

Certain developmental stages of the fish eggs are known to be very sensitive to thermal impacts. For carp eggs acclimated at 25'C, cleavage and blastopore closure stages were more susceptible to thermal damage (Frank 1974). According to Kiyono and Shinshima (1983), the 8-16 cell stage, the eye forming stage and the beginning of the heart beat stage were found to be susceptible to thermal impact. Hassler (1982) noted that the northern pike embryos were most susceptible to temperature change and handling within the first five hours of development. The stage of development at which the eggs were introduced to the test incubators during the present study (48-72 hours post hatch) was retinal pigmentation stage (Rana 1986b).

This would indicate that the actual key morphogenic stages which are more sensitive to thermal impact were already exceeded at the beginning of the trial. This reflects the observed high temperature tolerance of eggs (above 40'C) when compared with the upper lethal temperatures for *O.mossambicus* reported by Nyambi (1982) and Allanson (1966) as 36'C and 38'C respectively. However, these figures were for first feeding fry and fingerlings respectively.

It is well known that high temperatures accelerate and low temperatures retard the growth (Blaxter 1981). According to Blaxter (1969) the growth is influenced in the early stages by the ratio between the embryo and yolk weight. The influence of temperature on the metabolic process of the larvae is reflected in the changing yolk utilisation efficiency at different temperatures. The marked difference in the somatic weight of the fry observed at different temperatures reflects this phenomenon. Ishibashi (1974) noted that newly hatched *Tilapia sparmarnii* (Steindachner) reared at 30'C took 2 days to grow to the first feeding stage whilst at 24'C it was 4 days.

Rana (1985) during his investigations of the effect of the egg size on the growth of unfed *O.mossambicus* fry, recorded a specific growth rate of 48.2% per day for the first 3 days of development at 28'C for the "large eggs". During the present study the SGR recorded for

the first three days of development was 39.5%/day at 34.6'C. This difference could be associated with the possible differences in the size of the eggs utilised.

It has been suggested that the transition of exogenous feeding may be related to full swimbladder infla tion (Doroshev, Cornacchia and Hozan 1981). Rana (1985) reported that in the O. mossambicus fry reared at 25'C the swimbladders were fully inflated six days post hatching suggesting that they were capble of exogenous feeding by six days post hatching. The rapid decline of growth rate in the days 6-9 at temperatures 25°C and 29°C suggests that most of the yolk was used for maintenance rather than growth during this period. In contrast, the SGR was negative between the 6-9 days period at 34.6'C. This indicates the thermal impact on yolk utilisation; at the elevated temperatures the yolk utilisation is faster and the fry begin to starve and lose weight earlier than normal. Therefore, it is important to consider the rearing or incubating temperature under artificial hatchery conditions in judging the commencement of exogenous feeding.

CHAPTER 5

SCANNING ELECTRON MICROSCOPIC STUDIES OF THE ARTIFICIALLY INCUBATED EGGS

5.1 INTRODUCTION

The investigations made on the hatchability of O. mossambicus eggs under artificial incubation (described in Chapter 3) indicated that the egg mortalities are likely to be associated with physicochemical and/or microbiological qualities of the hatchery water. The effects of some of the physicochemical qualities of the water on the hatchability and survival of O. mossambicus eggs and fry were later investigated (Chapter 4). It was concluded that the most important physicochemical qualities such as ammonia, nitrite and temperature had not directly contributed to the mortality of eggs. This suggested that the reduced hatchability of eggs may be associated with the pathogenic microbes in the hatchery water. However, the possible mechanism of the microbial action on the eggs which might lead to mortality under artificial incubation is unknown.

In teleosts the developing embryo is surrounded by a thick, highly differentiated, egg membrane. Depending on the species, the morphology of this membrane varies and may reflect adaptation to diverse ecological

conditions. This membrane consists mainly of carbohydrates and proteins and the exact mechanism by which the egg membrane is formed in teleost oocytes is unknown. However, it is known that the egg membrane acts as a protective covering to the developing embryo (Monroy and Moscona 1979). Therefore it was thought that if microbial pathogens are involved in the reduced egg hatchability described in Chapter 3, examination of the surface properties of the egg membrane might elucidate the mechanism of this microbial action.

Therefore, the present study was designed to investigate the surface properties of the artificially incubated O. mossambicus eggs using Scanning Electron Microscopy (SEM) with the view to elucidating the possible mechanism of their mortality.

5.2 MATERIALS AND METHODS

5.2.1 Source and handling of eggs

The eggs used in the present study were obtained from the broodstock females maintained in the glassfibre aquarium system described in Chapter 2 section 2.2. The mean weight of the fish was 163.4g (range 147.4 - 173.2g) and the mean standard length of the fish was 16.4cm (range 16.2-16.7cm). The collection and handling of the eggs was carried out as described in the Chapter 2 section 2.3. All the eggs used in this study were aged between 12

to 24 hours post spawning.

5.2.2 Incubation system

The incubation system used in the current investigation (system A) is described in Chapter 2 section 2.4.

5.2.3 Experimental procedure

The eggs were removed from the brooding females into plastic sterile petridishes. The viable, developing eggs were separated by observing them under a binocular microscope. For this purpose a soft hair brush was used to minimise damage to the egg membrane. The separated viable eggs were divided into groups of 100 eggs. The number of groups depended on the clutch size but fell between 5 and 8 per clutch. Each group was placed in a separate incubator. Ten eggs were randomly sampled from each group prior to incubation, 12, 24, 48 and 72 hours post incubation.

This whole procedure was repeated four times ie. four clutches of eggs were used in the investigation.

5.2.4 Scanning electron microscopy

The egg samples were first fixed in 2.5% glutaraldehyde. They were post-fixed in cacodylate buffered 1% osmium tetroxide. The detailed procedure of fixation is

given in Appendix 5.

Each sample of eggs was dried using the critical point method in a critical point dryer. The dried egg samples were then carefully orientated on separate SEM stubs and were coated with gold/paladium mixture in a sputter coater. The eggs were then examined in an ISI 600 scanning electron microscope.

5.2.5 Water quality analysis.

During the course of the investigation, the main physicochemical and microbiological qualities of the water were monitored as described in Chapter 2 section 2.5 and 2.6. Samples of water were analysed at the beginning, at the midpoint and at the end of the investigation.

5.3 RESULTS

No obvious differences were observed between viable and nonviable eggs immediatly after removal from the buccal cavity. On observation, the egg surfaces appeared very smooth and showed no evidence of mechanical damage or bacterial or fungal activity (Figure 31).

Twelve hours post incubation, injuries to the egg membrane could be observed. These injuries sometimes appeared as "scratches" on the egg surface and sometimes as rupture of the chorionic membrane. The rupture of the chorionic membrane resulted in exposure of the yolksac to

Figure 31.

Scanning Electron Micrograph of a 24 hour old viable egg freshly removed from the buccal cavity.

A. Whole egg X $0.029 \cdot 10^3$

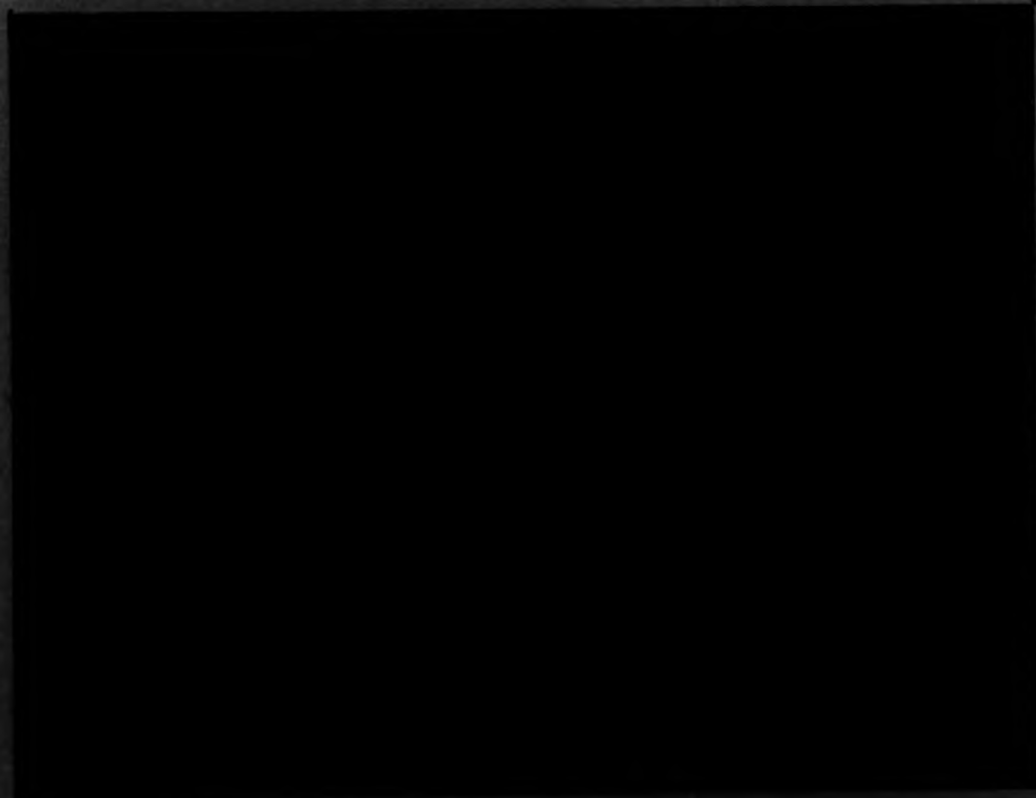
B. A magnified area of an egg X $1.14 \cdot 10^3$

Note the lack of bacterial and fungal activity.

P : Pores

M : Mucus

D : Debris?



the outer environment. In most such cases, the yolksac was observed to be herniating through the injury and forming obvious "yolk blebs" (Figure 32).

Deposition of foreign material on certain areas of the egg surfaces was also evident. The nature of these deposits was not clear but appeared to be organic debris, probably from the aquatic environment. Some eggs clearly showed localized bacteria on the surface and most of these plaques were found to be centered around an area of organic debris (Figures 33 and 34).

Twenty four hours post incubation, some of the observed eggs were found to be completely covered with a colony of bacteria (Figure 35). In others, the bacterial plaques were more localised. The chorionic damage and yolk bleb formation was still evident. Some of the eggs showed evidence of fungal growth on the surface. However, developing fungal hyphae were only seen on the eggs which were completely, or almost completely, covered with bacteria (Figure 36). No germinating fungal spores or developing hyphae were apparent on the eggs without considerable bacterial activity.

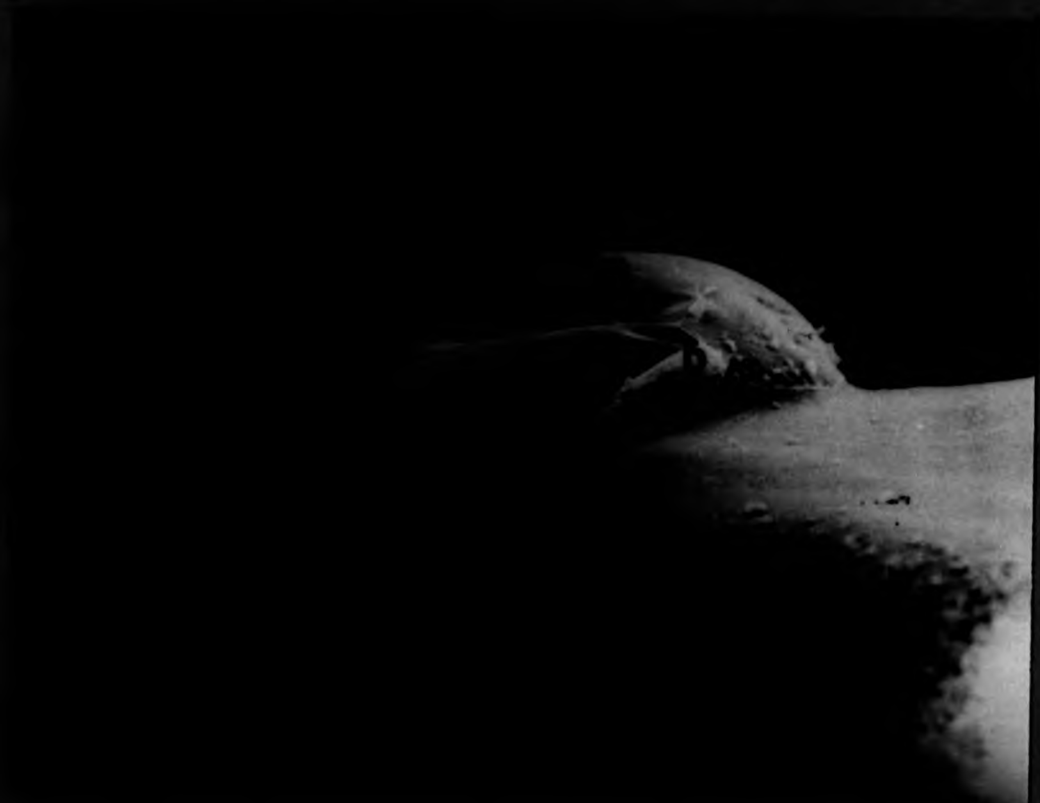
The eggs examined 48 hours post incubation consisted of eggs with little or no microbial flora on the surface together with eggs showing all the stages observed 24 hours post incubation. In addition, eggs completely covered with fungal hyphae were also present

Figure 32.

Scanning Electron Micrograph of an egg artificially incubated for 12 hours showing chorionic damage and yolk blebs. X $0.230 \cdot 10^3$

D : Damaged chorionic membrane

YB : Yolk bleb



YB
D

Figure 33.

Scanning Electron Micrograph of an egg artificially incubated for 12 hours showing localized bacteria.

X $0.029 \cdot 10^{3}$

L : Localized bacteria

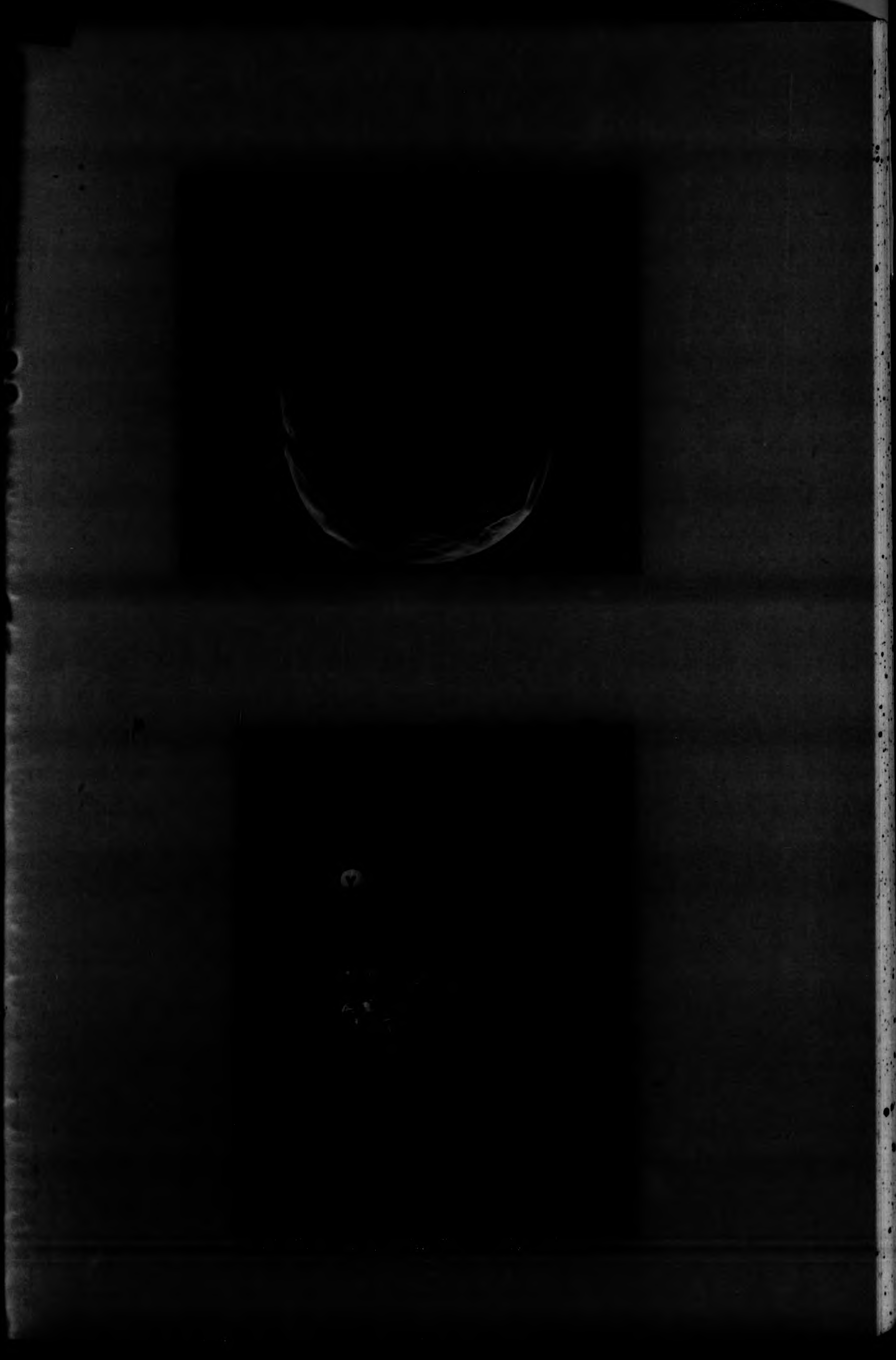
Figure 34.

Scanning Electron Micrograph of an area of an egg artificially incubated for 12 hours. X $0.960 \cdot 10^{3}$

L : Localized bacteria

Y : Yolk platelet escaped from a damaged egg

D : Debris - Organic material?



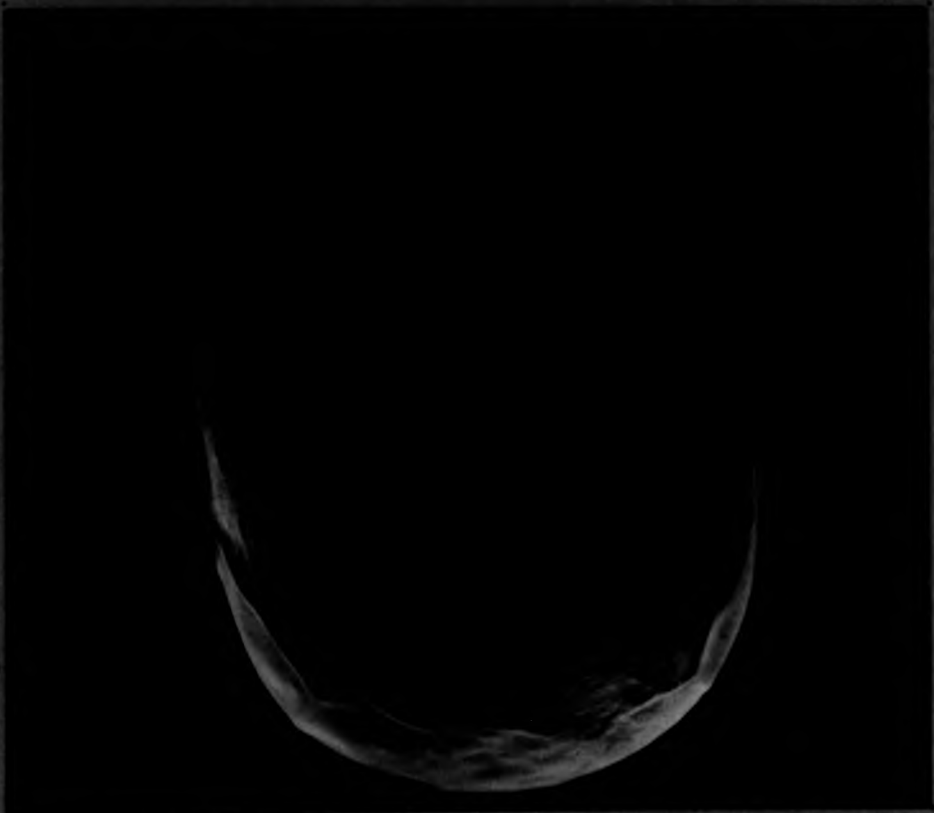


Figure 35.

Scanning Electron Micrograph of an area of an egg artificially incubated for 24 hours showing surface bacteria. X 1.50×10^3

B : Bacteria

D : Debris - Organic material?

Y : Yolk platelet

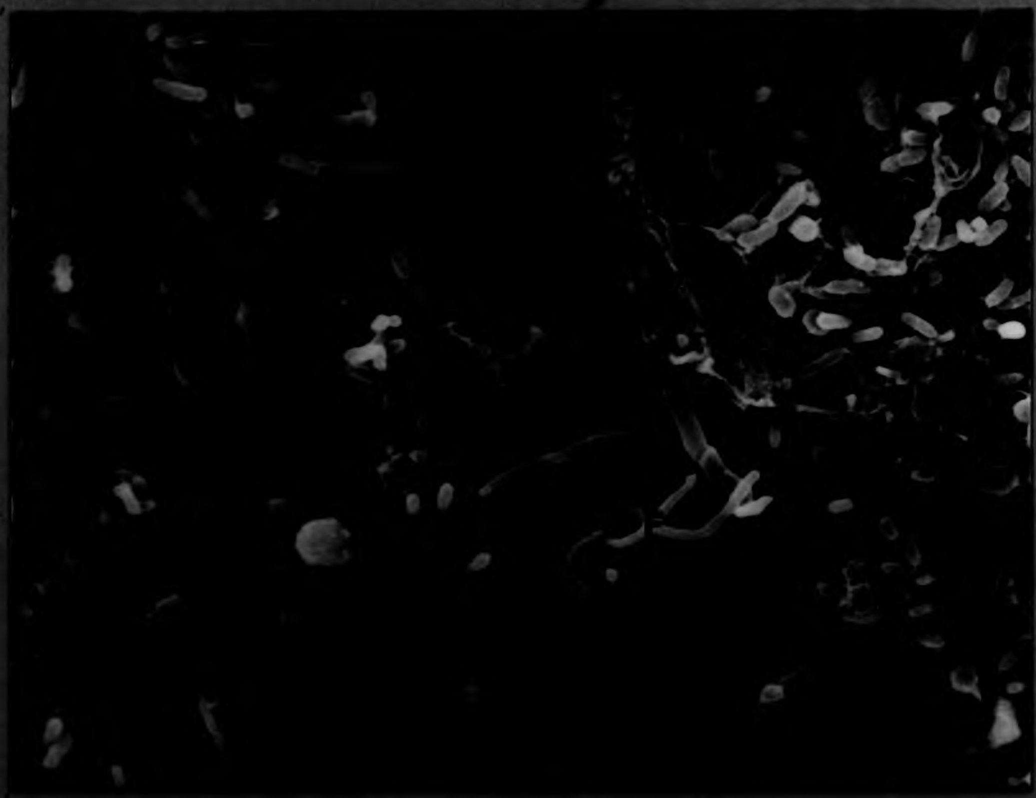
Figure 36.

Scanning Electron Micrograph showing developing fungal hyphae on an egg artificially incubated for 24 hours.

X 2.90×10^3

F : Fungal hyphae

B : bacteria



(Figure 37).

Examination at higher magnifications of the eggs covered with bacteria clearly showed damage to the egg membrane appearing as deep cavities (Figure 38).

Seventy two hours post incubation the eggs showed almost all the stages including localised bacterial plaques, spreading of bacteria and yolk blebs observed at 24 and 48 hours post incubation. At this stage, a further type of rupture to the chorionic membrane was first evident, associated with the hatching process (Figure 39). In such areas the chorionic membrane appeared stretched and thin and the surface of the embryo was clearly visible beneath the ruptured areas (Figure 40). This natural rupture of the chorionic membrane in conjunction with the hatching process was clearly distinguishable from the previously observed ruptures possibly due to mechanical stress.

The results of the physicochemical and microbiological analysis of the water measured over the period of investigation are presented in Table 9. All the parameters measured were found to lie within the same range as observed to occur in the same incubation system during the investigation described in Chapter 3.

5.4 DISCUSSION

It is clear from the results of the study

Figure 37.

Scanning Electron Micrograph of a 48 hour post incubated egg covered with fungi. X $0.031 \cdot 10^3$

F : Fungi

Figure 38.

Scanning Electron Micrograph of an area of an egg 48 hours post artificial incubation showing damage to the chorionic membrane. X $6.70 \cdot 10^3$

C : damage to the chorionic membrane appearing as a deep cavity.

B : Bacteria

F : Fungal hyphae

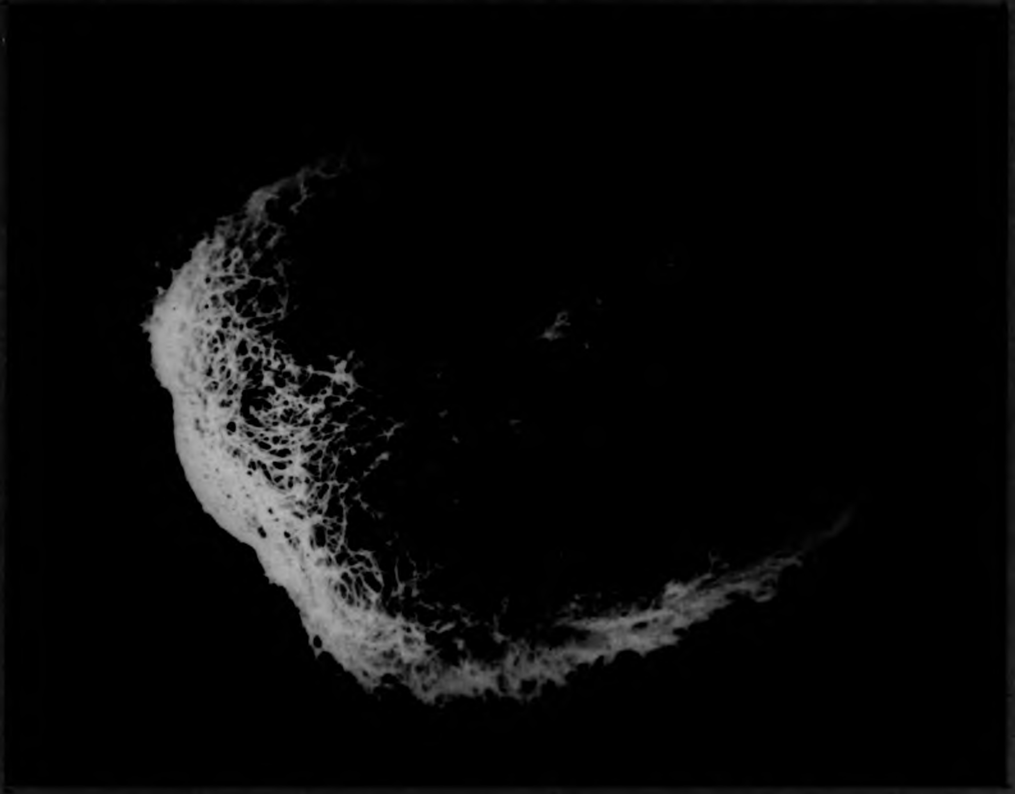


Figure 39.

Scanning Electron Micrograph showing ruptured chorionic membrane of an egg due to the hatching process. X $0.038 \cdot 10^3$

Figure 40.

Scanning Electron Micrograph showing ruptured chorionic membrane of an egg due to hatching and mechanical damage. X $0.038 \cdot 10^3$

RH : Rupture due to hatching

E : Embryo

RM : Rupture due to mechanical damage

Y : Yolk bled

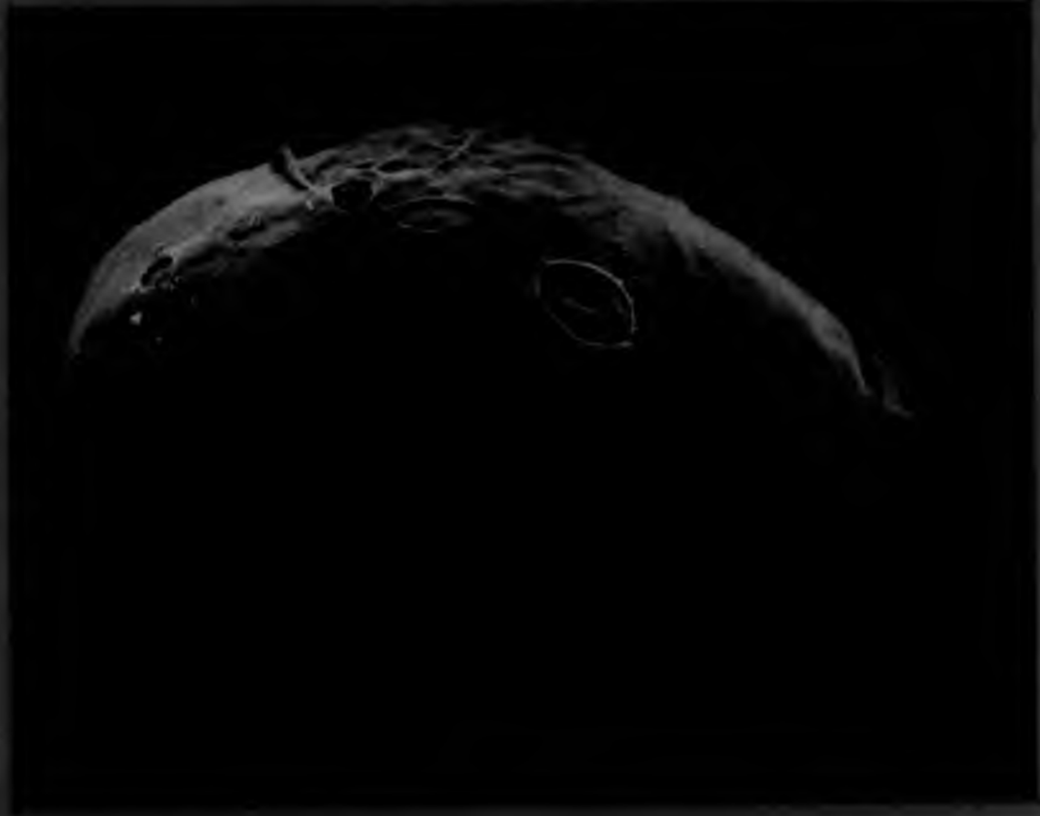


TABLE 9. Water quality parameters measured in incubation system A during the SEM study.

Parameter	Mean	Range
Temperature 'C	27.5	27.0 - 28.0
DO mg/l.	7.4	7.20 - 7.60
pH	7.75	7.67 - 7.84
Total NH3-N mg/l.	0.59	0.34 - 0.84
Total NO2-N mg/l.	0.013	0.081 - 0.126
TVHB cells/ml.	6.25×10^3	$3.60 - 8.94 \times 10^3$
TVFS spores/l.	34,000	18,400 - 49,600
TVSS spores/l.	3750	1200 - 6300

that the eggs (viable and non viable) freshly removed from the mothers buccal cavity between 12 and 24 hours post spawning did not show any microbial activity on the chorionic membrane. It was also evident that the egg surfaces were clean and without any foreign material. Also at that stage, no mechanical aberrations of the eggs were observed. It would seem possible therefore that the oral incubation of the eggs by the female parent protects the eggs from mechanical injuries and keeps the surfaces clean, at least to this stage.

Rimmer (1985) working on early development and buccal incubation in the fork-tailed catfish _Arius graeffei_ Kner & Steindachner showed that the branchial region of the brooding males became distended to accommodate the eggs and larvae, and the oral epithelium thickened to cover the palatine tooth patches. Ntheketha (1984) working on _O.niloticus_ reported that the pharyngeal region of the females became enlarged and thickened prior to spawning and this was maintained throughout brooding. It seems likely therefore, that the morphological changes taking place in the brooding parent's buccal cavity help in some way to provide the spawn with an ideal environment with particular attention to minimizing the possible mechanical injury.

The mechanical injuries to the chorionic membrane were first evident after 12 hours of incubation in

the artificial incubation system. These injuries may have been caused by the surface properties of the incubator. The actual pattern of the artificial churning process might also increase the mechanical stress on eggs.

In an artificial incubation system it is not possible to reproduce all the necessary requirements for the successful incubation of eggs which are to be found in the brooding parent's buccal cavity. Incidental observations of brooding females showed that in O.mossambicus, the rate of churning of eggs within the buccal cavity decreases as the incubation proceeds. Rana (1986a) made investigations into this feature and reported that the natural churning frequency of the eggs at day 1 post spawning is approximately four times higher than that at day 3. In the present investigation, the incubation system used did not provide for such changes in churning frequency during the course of the incubation reflecting those in the nature ie. the actual rate of churning which is employed by the mother may not have been provided by the artificial incubator in the present study.

Rana (1986a) compared the efficiency of two artificial incubation systems designed for the incubation of Oreochromis eggs and found that the frequency of yolk bleb formation was higher in an upright "zugar jar" type incubation system when compared to a similar system used in the present study. He suggested that the increased pressure

exerted on the chorionic membrane due to the upward thrust of the water current may have resulted in weakening of the membrane, hence yolk bleb formation. Though the system used in the present study does not generate a considerable upward thrust, the contact between the surface of the incubator and the eggs seems to be sufficient to result in chorionic damage.

An area of damage on the chorionic membrane seems to be an ideal site to trap foreign material ie. organic debris, in the hatchery water as it appears to be comparatively rough. In an artificial system where a considerable amount of dead and decaying eggs and early fry accumulate, it is reasonable to believe that the water is rich enough with particulate material for such an event to take place. If this is the case, it could well result in attracting potentially pathogenic bacteria onto the damaged site which is already occupied by the organic debris. This phenomenon was clearly visible in the SEM examinations made after 12 hours of incubation, where localised bacterial plaques on the egg surface were centered around what appeared to be organic debris. The water in the incubation system was found to harbour fish pathogenic bacteria such as A.hydrophila and P.fluorescens (Chapter 3).

Gram-negative, facultative, fish pathogenic bacteria such as A.hydrophila are known to produce various kinds of toxic substances and enzymes, some of which have

been thought to play a role in their pathogenicity (Kanai and Wakabayashi 1984). Species such as P. aeroginosa and some fluorescent pseudomonads have also been found to produce toxic protease enzymes which were suspected to be important virulent factors (Kawaharajo, Homma, Aoyama, Okada, and Marihara 1975). According to Kanai and Wakabayashi (1984), if a protease is produced at the site of infection, the destruction of surrounding tissue induced by the protease will support bacterial growth. The bacteria observed to be almost completely covering the eggs after 24 hours of incubation may have occurred as a result of a similar process. The small, localised bacterial plaques may have produced proteases and destroyed the surrounding tissue which supported the bacterial growth.

The increased bacterial activity on the chorionic membrane followed by possible production of toxic proteases appeared to have resulted in the destruction of chorionic integrity. Damage to the chorionic membrane evidently exposes the developing embryo to the outer environment. Therefore, it is possible that once the chorionic membrane is damaged the bacteria become invasive to the embryo and cause mortality. It is also possible that if the eggs are constantly in contact with large numbers of pathogenic bacteria, then contamination of sacfry results in early sac fry mortality. This was found to be the case by McCarthy (1977) who experimentally infected fish ova

with A.salmonicida and found that the resulting yolksac fry were heavily contaminated with the bacteria.

The fungi isolated from the same incubation system in a previous study described in Chapter 3 were found to consist, in part, of the members of the family Saprolegniaceae *Saprolegnia* and *Achlya*. It was also found that the predominant genus was *Achlya* (Chapter 3). During the present investigation there were no eggs observed with fungal spores or hyphae on the egg surfaces which were not in association with bacteria. It seems probable that the water mould population, including the members of family Saprolegniaceae, were only acting as saprophytes, secondarily invading the dead or dying eggs.

Manton, Clarke and Greenwood (1951) and Meier and Webster (1954) in their electron microscopic studies of saprolegniaceae cysts reported that the bifurcated hooked hairs on the secondary cysts of *Saprolegnia* species are concerned with attachment of the cysts to a substrate. They also suggested that these hairs were an adaptation to a parasitic existence. Beakes and Ford (1983) showed that there were no hairs or hooks on the secondary cyst wall of *Achlya flagellata* when compared to that of *Saprolegnia* species. If the presence of hairs and hooks are considered as an adaptation towards a parasitic existence, lack of hairs and hooks on *Achlya* species may explain their saprophytic nature. As the abundance of *Achlya* species

observed in the present study was very much higher than that of Saprolegnia, it is possible that this species only acted as a saprophyte invading dead or dying eggs under artificial incubation. This hypothesis of fungi such as Achlya species acting as secondary saprophytes is supported by the fact that eggs with fungal hyphae were only observed 24 hours or more after incubation. The observations made over the years on the artificial incubation of Oreochromis eggs have clearly shown that if a nonviable egg is freshly removed from the mother's mouth and placed in an artificial incubation system, even with a low count of Saprolegniaceae spores in the water, it is attacked by fungi wihin 12 hours.

The lack of bacterial activity on the surface of eggs freshly removed from the female's buccal cavity suggests the possibility of the presence of some antimicrobial properties in the buccal cavity of brooding females. Further investigations on this aspect have been carried out and are described in Chapter 7.

In summary therefore, it appears that mechanical injury to the chorionic membrane, potentially pathogenic bacterial action and secondary fungal attack is a series of consequent events occurring on the O.mossambicus eggs under artificial incubation. The observation of the formation of bacterial plaques on the eggs even after 48 hours of incubation suggest the

possibility that this phenomenon may occur any time between the introduction of eggs into the incubation system and hatching. Depending on the number of eggs that undergo this process, the hatchability of a clutch could vary.

These results together with the probable explanation put forward raise a further question. Would it be possible to improve the hatchability and survival of eggs by eliminating the pathogens ie. disinfection? This aspect was also investigated and described in Chapter 6.

CHAPTER 6.

DISINFECTION OF *O.MOSSAMBICUS* EGGS AGAINST POTENTIALLY PATHOGENIC BACTERIA AND FUNGI.

6.1. INTRODUCTION

The practice of disinfection of fish eggs against pathogens is well established amongst fish culturists, especially in well developed intensive culture operations where it is often a routine procedure. In such intensive hatchery operations where artificial environments have been created for the rearing of eggs and fry, the capital and operational costs are substantial and it is necessary to minimize egg and fry mortality for a viable industry. As an indication of the severity of the damage which can occur unless precautions are taken, Colesante *et al* (1981) reported that rearing muskelunge (*Esox masquinongy*) at Chautanqua hatchery in New York was a total failure due to the massive and sudden mortalities of eggs and fry. A variety of causes have been reported for such mortalities.

The association of bacterial and fungal infections with the mortality of developing eggs under artificial hatchery conditions has been well documented (Agersborg 1933, Tiffny and Wolf 1937, Wright and Snow 1975 and Wright 1976). These reports include both cold and warm

water fish species such as salmon (S. salar), rainbow trout (S. gairdneri) and common carp (C. carpio). However, the available literature on Orechromis species are very scarce. One of the few studies reported was that of Rothbard and Pruginin (1975) who stated that the presence of spoiled eggs may cause infection and loss of the whole spawn under artificial incubation. Further it was already observed that O. mossambicus eggs suffer severe mortalities of bacterial and fungal origin under artificial incubation and it was considered disinfection would be an appropriate step towards improving hatchability of eggs (Chapter 3).

There are many reports available on disinfection of fish eggs against the bacterial and fungal pathogens. Wright and Snow (1975) used the chemical disinfectants, formalin and acriflavin, to disinfect largemouth bass (Micropterus salmoides) eggs against bacterial pathogens such as A. liquefaciens. Another chemical disinfectant, malachite green is reported to have been used successfully to disinfect brown trout (Salmo trutta) eggs against Saprolegnia parasitica infections by Cline and Post (1972). McFadden (1969) found that an organic iodine compound, Povidone iodine (an iodophore), was effective for disinfecting rainbow trout eggs against A. liquefaciens. Ultraviolet light was employed by Kimura, Yoshimizu, Tagima, Ezura and Sakai (1976) and Kimura, Yoshimizu, Tagima and Ezura (1980) to disinfect a hatchery

water supply contaminated with fish pathogenic bacteria and fungi. Though there are many such reports available on the disinfection of warm and cold water fish eggs none of them deal with cichlid eggs including O.mossambicus.

In the fisheries note issued by the Ministry of Agriculture, Fisheries and Food, U.K., on "Disinfectants in Fish Farming" (1978), Finlay stated "..... Disinfectants used in fisheries should be aimed at destroying all types of infectious agents including bacteria, fungi, viruses and protozoans. However, it may be desirable under certain circumstances to use a treatment selectively effective against specific agents ...". In the present study the suspected causative agents associated with the mortality of O.mossambicus were previously identified (Chapter 3). The suspected primary causative agents were bacteria such as A.hydrophila, P.fluorescens and Flavobacterium species. Also, it was evident that certain fungi isolated such as Saprolegnia species, Achlya species and Fusarium species, act as secondary pathogens (Chapter 5). Hence, this experiment was designed to assess the efficacy of selected chemicals and ultraviolet light as disinfectants against those specific agents on O.mossambicus eggs.

During the present investigation four commonly available chemical disinfectants together with ultraviolet irradiation were adapted to disinfect O.mossambicus eggs.

6.2 MATERIALS AND METHODS.

6.2.1 Source and handling of eggs

A batch of twenty broodstock females were used as the source of eggs. They weighed between 156.7 and 193.8g ($\bar{x}$=178.4g) and measured between 15.8 and 17.9cm ($\bar{x}$=16.8cm) in standard length. The brood stock were maintained in fibreglass aquaria with recirculated water as described in Chapter 2 section 2.2. The collection and handling of eggs was carried out as described in Chapter 2 section 2.3. All the eggs used in the experiment were aged between 12 and 24 hours post spawning.

6.2.2 Incubation system

The incubation system described as System A in Chapter 2 section 2.4 was used throughout the investigation of the chemical disinfection. Where ultraviolet light was used as a measure of disinfection the incubation system A was modified by coupling an ultraviolet sterilization unit (Aquatic Services International Limited, Hants SO4 7WF, U.K.) and a flowmeter. The modified system A will be referred to as System C (Figure 41). The disinfection trials using UV light were carried out in this system. The controls for the UV trials were held in system A prior to modification.

Figure 41.

Diagram showing the side elevation of the incubation system C used for UV water sterilisation trial.

P : Submersible water pump

S : Sump tank

BF : Biological filter

H : header tank

UV : UV water sterilisation unit

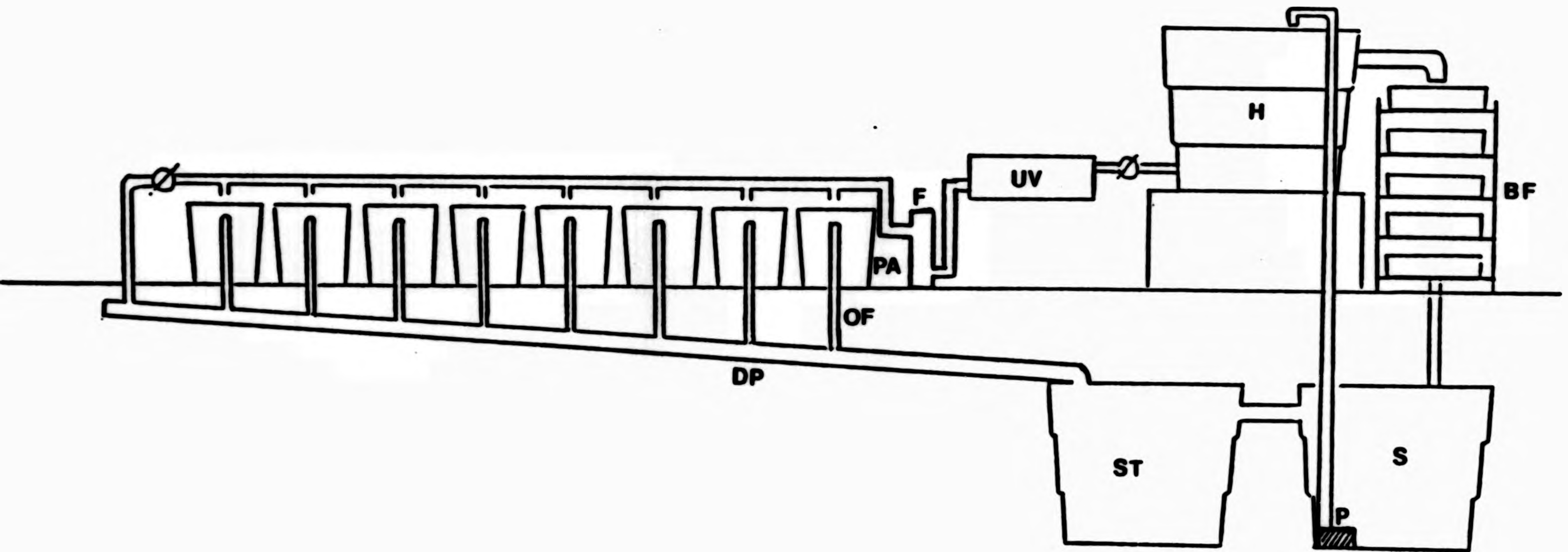
F : Flow meter

PA : Perspex aquaria

OF : Out flow

DP : Drain pipe

ST : Settling tank



H
UV
F
BF
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OF
DP
ST
S
P

6.2.3 Disinfectants

6.2.3.1 Chemical disinfectants

Four widely used chemical disinfectants namely formalin (40% formaldehyde from Lothian Chemicals Limited, Edinburgh EH5 IQD, U.K.); acriflavin (acriflavin, neutral, 100% active powder from Sigma Chemical Company, P.O. Box 14508, St Louis, Missouri 63178, U.S.A.); malachite green (malachite green oxalate powder from Sigma Chemical Company, P.O.Box 14508, St Louis, Missouri 63178, U.S.A.) and buffodine (buffodine 1% active iodine from Evans Vanodine International Limited, Preston PR5 8AH, U.K.) were used during the investigation.

6.2.3.2 Ultraviolet light

For the ultraviolet treatment a 15 watt UV water sterilization unit was employed. The calculated power of the unit was 25061 micro watt per square cm. The dose levels used are given in Table 10.

6.2.4 Experimental procedure

The eggs were removed from the brooding females. A random sample of 50 eggs from each spawn was fixed in Bouins' fixative for the assessment of fertility. The remaining eggs were divided into groups of 50 and used

TABLE 10.

Calculated percentage hatchebility of viable fertile eggs + standard deviation disinfected at different dose levels.

Disinfectant and dose level	% hatchebility of fertile eggs
Formalin	
Control	57.8 + 3.3b
100 ppm	55.5 + 5.7b
500	69.8 + 5.3d
1000	70.2 + 6.5d
2000	75.6 + 2.9de
Malachite green	
Control	58.5 + 8.7b
0.10 ppm	56.7 + 8.3b
1.00	78.7 + 2.1e
5.00	79.5 + 3.1e
10.0	81.7 + 2.0ef
Acriflavine	
Control	55.0 + 1.1b
250 ppm	56.9 + 3.7b
750	81.5 + 5.0ef
1500	83.0 + 3.0ef
3000	81.9 + 3.5ef
Buffodine	
Control	62.0 + 4.4bc
10 ppm	68.6 + 5.4cd
50	79.2 + 5.7e
100	86.6 + 2.5ef
200	0a
Ultraviolet irradiation	
Control	58.5 + 4.0b
UV I	88.4 + 6.9f
UV II	90.3 + 3.6f

Values which bear similar subscripts are not significantly different at $P<0.05$.

(Duncan's multiple range test was used)

in the trials. A single trial for a particular chemical disinfectant consisted of a range of four different concentrations and a control (Table 10). Each trial was duplicated and was repeated five times.

The solutions of the appropriate concentrations of the chemical disinfectants used were freshly prepared for each trial. The concentrations used (Table 10) were based on recommended dose levels, as commonly used by the fish culturists with a range of concentrations below and above these levels. In the case of buffodine the dose levels were calculated for the amount of active ingredient present assuming that it contains 1% active iodine (personal communication with Vanodine International).

The chemical solutions were placed in a water bath at a temperature of 27±1'C, one hour before the treatment to raise the treatment temperature. The batches of 50 eggs were transferred to plastic dishes (7x7x2.5cm) which had been modified by replacing the base with 1 mm nylon mesh. The dishes containing eggs were then placed in the appropriate disinfectant solutions. The treatment time was standardized to 15 minutes for each dose level. After 15 minutes the dishes containing eggs were removed from the disinfectant solution and washed gently by rinsing five times in distilled water. Each disinfected egg batch was placed in a separate incubator. During each trial, a

control was carried out using distilled water. The number of fry which hatched after four days were counted within twelve hours of hatching.

For the UV treatment trials, two dose levels were used, calculated on the basis of the rate of flow of water through the sterilisation unit. The two flow rates used were 6 and 12 l per minute. Those flow rates gave a UV dose level of 43556 and 87112 micro watt seconds per square cm respectively.

To evaluate the UV treatment trials, the same procedures were used as for the chemical treatments. The trials were carried out in duplicates for each dose level. Each trial, including the control, was repeated five times. The number of hatched fry were counted within 12 hours of hatching.

6.2.5 Water quality analysis.

6.2.5.1 Physical parameters

The pH, dissolved oxygen concentration and the temperature of the water in the incubation system were monitored daily as a routine practice. The methods described in Chapter 2 section 2.5 were employed.

6.2.5.2 Chemical parameters

At the beginning and the end of each

experiment, the water from the incubation systems A and C was analysed for total NH3-N and total NO2-N as described in Chapter 2 section 2.5.

6.2.5.3 Microbiological parameters

The total viable heterotrophic bacteria (TVHB), total viable fungal spores (TVFS) and total viable Saprolegniaceae spores (TVSS) in the water were estimated for both incubation systems A and C, at the beginning and the end of each trial, using the methods described in Chapter 2 section 2.6.

6.2.5.4 Isolation and identification of bacteria and fungi

During the course of study, the potentially pathogenic bacteria were once isolated and identified. The fungi observed were also isolated and identified once. The methods described in Chapter 2 section 2.6 were used.

6.3 RESULTS

Analysis of the physicochemical qualities of the water observed in the incubation systems A and C during the course of the study is given in the Table 11. No difference in these water qualities was observed between the two systems. The microbiological qualities of the water measured in the two systems are also given in the

Table 11.

Water quality measurements in the incubation systems A and C during disinfection trials.

Parameter	Incubation system	Mean	Range
Temperature 'C	A/C	27.5	27-28
DO mg/l.	A/C	7.4	7.2-7.6
pH	A/C	7.75	7.67-7.84
NO2-N mg/l.	A/C	0.013	0.018-0.126
NH3-N mg/l	A/C	0.59	0.34-0.84
TVHB No/ml	A	6.25×10^{3}	$4\times10^{3}-8\times10^{3}$
	C	Nil	Nil
TVFS No l^{-1}	A	4.56×10^{3}	$2\times10^{3}-7\times10^{3}$
	C UV1	26.55×10^{3}	$18\times10^{3}-24\times10^{3}$
	C UV 11	Not measurable	
TVSS Nol^{-1}	A	3.43×10^{4}	$2\times10^{4}-5\times10^{4}$
	C UV1	1.32×10^{2}	$1\times10^{2}-2\times10^{2}$
	C UV11	<250	

Table 11. It is clear that both UV dose levels, 43556 micro watt seconds per square cm (UV 1) and 87112 micro watt seconds per square cm (UV 2), reduced the TVHB counts from 6.25×10^3 to nil. However, the TVFS counts were different between the two UV dose levels. The low dose level UV 1 only reduced the counts by 21.9% whereas UV 2 reduced it by 98.8% (Table 11). The TVSS counts were also reduced to 1.32×10^2 at UV 1 whilst UV 2 resulted in negative counts (the sensitivity of the technique used could only detect spores above 250/1).

The potentially pathogenic bacterial flora identified in the system A consisted of two species of Pseudomonas, including Pseudomonas fluorescens, Flavobacterium sp., Chromobacterium violaceum, Micrococcus sp., and Bacillus sp. The pathogenic fungal flora consisted of Saprolegnia sp., three species of Achlya, including Achlya prolifera and Achlya flagellata, Pythium sp., two species of Fusarium and Allomyces sp. The major contributor to the large numbers of TVFS was Trichoderma sp. which was also abundant in the air.

The hatchability of fertile eggs for each treatment and for the controls is given in Table 10. The hatchability in all control groups was between 55.0±1.1% and 62.0+4.4%. One hundred ppm formalin, 0.1 ppm malachite green and 250 ppm acriflavin did not improve the hatchability significantly (P>0.05). The manufacturers

recommended dose of 100 ppm buffodine gave the highest hatchability (86.6±2.5%) and of the four chemical disinfectants used, acriflavin and malachite green gave a good hatchability (above 80%) at higher concentrations (Table 10). Formalin showed the least improvement out of the chemical disinfectants used (Table 10).

Both UV dose levels gave higher hatchability than chemical disinfectants. The UV2 gave an improved hatchability (90.2±3.6%) over UV1 (88.4±6.09%) but the difference was not significant (P<0.05).

6.4 DISCUSSION

In the present study the hatchability of fertile eggs observed in the controls was less than 62%. This is comparable with the hatchabilities studied in the same system during the previous experiments described in Chapter 3.

All four chemical disinfectants used in the present study are commonly available and widely used in aquaculture. Ross and Smith (1972) reported that betadine and wescodine, two related iodophores, killed some aeromonads, pseudomonads and *S. parasitica* mycelium after 5 minutes exposure to 25 ppm active ingredient. Buffodine, a related iodophore used in the present study gave significantly improved hatchability (P>0.05) at the 10,50 and 100 ppm levels. The improvement at 50 and 100 ppm

levels was significantly higher than that at 10 ppm level (P>0.05). In contrast the hatchability at the dose of 200 ppm was nil. This supports the results of Wright and Snow (1975), who reported only 3% hatchability of largemouth bass eggs following a 200 ppm wescodine treatment. The toxicity may vary with fish species and care should therefore be taken not to use the dose levels above manufacturers' recommendations under any circumstances.

There is no recommended dose level for acriflavin as an egg disinfectant as it is not marketed as such, but there are previous reports of efficacy of the chemical against the bacteria (Elson 1983, Wright and Snow 1975). The latter authors found that 750-1000 ppm acriflavin for 15 minutes was effective in disinfecting largemouth bass eggs against A. liquefaciens. In the present study the dose levels 750, 1500 and 3000 ppm gave significantly improved (P>0.05) hatchabilities. In contrast, the improvement observed at their doubled dose rates was not found to be significantly different (P<0.05). This suggests the high margin of safety of the chemical as an egg disinfectant.

Malachite green at 1, 5 and 10 ppm dose levels improved the hatchability (P>0.05) when compared with the control. However, the percentage hatch at 10 ppm was only slightly above 80% and this was no better than 100ppm buffodine or 750, 1500 and 3000 ppm acriflavin.

Though malachite green is the conventional fisheries fungicide, there is a large diversity in concentrations and exposure times suggested by various workers. Alderman (1982a) demonstrated zero growth of S.*parasitica* *in vitro* at 1 ppm malachite green. In the same study he found that different dye lots had varying anhydrous dye concentrations ranging from 34-98%. Though the recommended dose of malachite green as a disinfectant of fish eggs is 1 ppm for 15 minutes (Alderman 1982b), the increased hatchability and lack of toxicity observed at 1 to 10 ppm level in this study suggests the possibility of low anhydrous malachite green oxalate concentration in the dye used.

The hatchability obtained with formalin was the lowest of all the chemicals. The hatchability results following treatment with 500, 1000 and 2000 ppm formalin were found to be significantly different (P>0.05) from those of the control. Though these dose levels gave improved hatchabilities, the highest percentage hatch at 2000 ppm was less than 76%. In contrast malachite green at 10 ppm, acriflavin at 750 onwards and buffodine at 100 ppm gave more than 80% hatchability. This indicates the relative inefficiency of formalin as an egg disinfectant. Formalin up to 2000 ppm level may not have been adequate enough in preventing settlement and killing of bacteria and fungi on the egg surface. This is supported by the fact that Wright and Snow (1975) recovered A.*liquefaciens* from

largemouth bass eggs following disinfection with 2000 ppm formalin for 15 minutes. Incidental observations of a treatment of 10000 ppm formalin slightly improved hatchability to 76.35% but the survival of the fry up to 6 days post hatch was only 21%. Increasing the dose level of formalin does not seem to reduce the hatchability of O.mossambicus eggs but appears to affect the post hatching survival of yolk sac fry. Perhaps this might be a result of low retention time of the chemical on the egg surface.

Kimura et al (1976) examined the disinfectant effect of UV on cell suspensions (10^6 to 10^8 cells/ml) of Aeromonas hydrophila and P.fluorescens. They found a 99.9% or more reduction of viable bacterial cells by UV treatment at more than 22100 micro watt seconds per square cm dosage. In the present study both UV levels used reduced total viable heterotrophic bacterial cell counts to nil, this is in agreement with Kimura et al (1976). Vlasenko (1969) described a minimum lethal dose of 39564 micro watt seconds for the zoospores of unspecified Saprolegnia The dose level was described in a different unit ie. micro watt per second, rather than micro watt second per square cm as in the present study. However, it appears that the calculation of dose level in units of micro watt second is higher than that from micro watt second per square cm. In that respect the dose level is obviously higher than that used in the UV1 in the present

study. This explains the fact that UV1 only reduced the total viable fungal spore counts by 21.9%. It also explains the presence of 2.31×10^2/l spores/l level of total viable Saprolegniaceae spores in the water at the UV1 level. However, the UV2 level used in this study was higher than that used by Vlasenko (1969) and reduced the total viable saprolegniaceae spore counts to a minimum ie. <250 spores/litre. The hatchability observed between UV1 and UV2 was not found to be significantly different ($P>0.05$). But the mean hatchability observed was higher at both UV dose levels than for the chemically disinfected eggs (Table 10). Though reduction of the total viable fungal spore counts and the total viable saprolegniaceae spore counts were different between two treatments (Table 11) the hatchability of the eggs was not. This further supports the suggestion that bacteria are the major contributors towards egg mortality (Chapter 3 and 5).

Considering the overall performance of each chemical disinfectant tested, formalin appeared to be the least effective. Although malachite green gave good hatchability at 10 ppm level, the risk associated with the higher concentrations is unknown as the actual anhydrous malachite green oxalate concentration of the dye is unpredictable. Acriflavin above 750ppm did improve the hatchability against the commonly occurring potentially pathogenic bacteria and fungi, though this is not generally

used as an egg disinfectant. This also has a very high margin of safety as well as being cheap. Buffodine appeared to be very effective at 100 ppm active ingredient level, but it has a very high toxicity to O.mossambicus eggs above that level. The ultraviolet light at 87112 micro watt second per suqare cm level gave the best improvement in terms of hatchability and showed no harmful effects to eggs and yolk sac fry. It is therefore, appropriate to state that the O.mossambicus egg mortalities under artificial incubation could be effectively reduced by disinfection with chemical hatchery disinfectants and sterilizing the hatchery water by UV irradiation.

CHAPTER 7

INVESTIGATION INTO THE POSSIBLE ANTIBACTERIAL PROPERTIES OF THE BUCCAL AND SKIN MUCUS OF BREEDING FEMALES.

7.1 INTRODUCTION

There is convincing evidence that mouth brooding tilapine species evolved from substrate spawning species (Fishelson 1966; Fryer and Iles 1972; Trewavas 1973; Barlow 1974; Balon 1975). The mouth brooders have a relatively lower fecundity and larger ova, and lack the larval period in their ontogeny. The substrate spawners have significantly higher fecundity with correspondingly less energy-rich eggs and include an abbreviated but distinct larval period in development.(Noakes and Balon 1982). It is believed that these differences have evolved alongside the mouthbrooding habit. Ensuring the protection of the progeny from adverse environmental conditions and predation, presumably contributed to enhanced survival.

It is obvious that mouth brooding facilitates a physical protection from environmental hazards to the eggs and fry simply by containing them in the buccal cavity; a confined environment. It is still not clear whether the brooding parents (females amongst Oreochromids) posses any specialised anatomical or biochemical adaptation in the buccal cavity which favours survival of the eggs and

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fry in addition to the simple physical protection.

Histochemistry of the surface mucus of various species of fish has been studied (Enomoto and Tomiyasu 1961, 1962; Jones and Reid 1973; Fletcher, Jones and Reid 1976). However, the oral mucus of fish, including cichlids has been neglected and very little is known of its specialised nature. The fascinating behaviour of parental care, including mouthbrooding in Oreochromids, together with the potential importance of this group as cultured fish brings cosiderable interest to the investigation of the protective mechanism of mouthbrooding.

Of the few who have dealt with mouthbrooding cichlids and their oral incubation, Shaw and Aronson (1954) were the first to look into the possible protection by means of mucus in *S. macrocephalus*, a paternal mouthbrooder. They reported that the fish has specialised glands in the buccal cavity which they suggested might secrete an anti-bacterial agent to help in survival of the eggs and fry. Their methods were rather crude compared to the methods currently available and they were unable to come to any positive conclusions in their study.

A few years later, Hildermann (1962), discussing immunogenetic studies of poikilothermic animals, noted the observations of Frishold, an aquarist. He mentioned that discus fish (*Symphysodon discus*), when reared in the absence of parents, are susceptible to fatal

infections by microorganisms which may be eliminated by adding antibiotics into the rearing water. Hildermann suggested that newly hatched fry may receive something passively from the parents. He hypothesised that these newly hatched discus fry obtain antibodies against important pathogens from the parental mucus which is equivalent in function to mammalian colostrum. This they obtain when apparently feeding on parental skin mucus. Evidence of this phenomenon in fish is somewhat rare.

Harrel, Etlinger and Hodgins (1976) demonstrated vibrio-static activity in rainbow trout skin mucus of fish immunised against vibriosis. No investigations in Oreochromids have been carried out with the exception of the work by Ntheketha (1984). However, the techniques used and the number of samples examined in this study were inadequate, such that the conclusions reached should be regarded with caution.

The results of some of the previous experiments carried out during the present investigation have demonstrated that the survival of _O. mossambicus_ eggs outside the buccal cavity can be improved by disinfecting against microbial pathogens found in the aquatic environment (Chapter 6). This suggests that, in addition to the physical protection, the buccal cavity may offer a protection from microoganisms in the water. Also, the existing knowledge indicates that, if there is any such

protection, it might be manifested through the qualities of the buccal mucus, since this changes according to the breeding cycle of the brooding parent (Varute and Jirge 1971). Therefore the present study was designed to investigate the possible antimicrobial qualities of the skin and buccal mucus of O. mossambicus at different stages of their breeding cycle.

7.2 MATERIALS AND METHODS

7.2.1 Experimental fish

The O. mossambicus used had a mean weight of 164.6g; range 146.4-184.3g and a mean standard length of 17.4cm; range 17.0-18.2cm. The fish were maintained in square glassfibre aquaria with recirculated water as described in Chapter 2 section 2.2.

A total of twelve females were used in the experiment to collect mucus from the skin and the buccal epithelium. Females were selected in three different categories for the purpose of the investigation. The category I, prespawning; category II, egg carrying and category III being fry carrying females. The prespawning females were selected on the basis of their courtship behaviour and the appearance of the genital papilae. They were considered to be not more than 24 hours prior to spawning. All the egg carrying females were within 72-96 hours post spawning and the fry carrying females were

between 9 and 10 days post spawning.

7.2.2 Collection of mucus

The methods used by Harris (1972) and Fletcher and White (1973) for the collection of mucus were modified and utilised during the present study. When a female of a particular category was ready for sampling, the fish was gently removed from the holding aquarium and anaesthetised using Benzocaine at 100 mg/l level. The fish was then rinsed with sterile Phospate Buffered Saline (PSB) and placed in a clean plastic tray (60x30x8cm), with 5 ml of PBS at pH 7.4. The mucus was gently scraped off from both sides of the fish into the tray by means of a clean plastic spatula. Extreme care was taken to avoid contamination of mucus scrapings with urine, faecal matter or blood.

Once scraping of the skin mucus was completed the fish was killed by a neck incision. The mouth was opened widely by cutting through the lower and upper jaw articulation. The superficial skin layer of the upper palate was gently peeled off using a sharp scalpel blade. The upper palate was selected for sampling as the pharyngeal glands are found to be located in this region (Shaw and Aronson 1954). The peeled tissue was rinsed in PBS and placed in 5ml of teleost saline (Lockwood 1963) for six hours at 4'C which causes mucin to be expelled into the saline as

described by Harris (1972). After six hours the tissues were removed. This procedure was repeated for each fish used in this investigation.

7.2.3 Preparation of mucus extracts

The buccal and skin mucus saline washings, collected as described in section 7.2.2, were centrifuged separately at 8000g for 30 minutes in a refrigerated centrifuge at 4'C. The supernatants were dialysed against distilled water for 24 hours at 4'C. The dialised material was then stored in sterile plastic stoppered ampules at -70'C. This procedure was repeated for each fish used.

Once the mucus collection and extraction was completed, the skin and buccal mucus extracts from the fish belonging to the same category were pooled. The final pooled mucus extracts were then freeze-dried in an Edwards Pirani II freeze drier.

7.2.4 Preparation of bacterial cultures.

Five gram-negative bacterial species were used in the antibacterial assay. The species P.fluorescens, A.hydrophila and Flavobacterium sp. used were original isolates from the aquarium water (Chapter 3 section 3.2.5). The species Yersinia ruckeri (isolate NCMB 1316) and Edwardsiella tarda (isolate NCMB 2034) were obtained from the reference collection at the Institute of Aquaculture,

University of Stirling. All these species have been recognised as fish pathogens (Richards and Roberts 1978; Frerichs 1984).

The pure cultures were transferred to TSA slopes prepared in sterile 25ml glass universal bottles. The slopes were incubated at room temperature (18-20'C) for a period of 6 days before the assay.

For the antibacterial assay antibiotic medium No.1 (Oxoid Ltd.) was used as it was recommended for microbiological assay. The agar medium was prepared in portions of 100ml in 125ml glass bottles and sterilised at 15lb pressure for 15 minutes. The bottles containing sterile liquid agar medium were kept in a water bath at 47'C. Solidification of the medium was prevented by this.

Bacterial suspensions were prepared in sterile glass bottles (25ml) by suspending a loopful of bacteria from a culture slope into 10ml of sterile PBS. The bacterial concentration of the suspensions was individually estimated by measuring the optical density using a spectrophotometer (WPA S 105) using PBS as the blank. The suspensions were prepared to give a close range of optical densities ensuring somewhat uniform concentrations. The suspensions were then placed in the same water bath as the agar medium at 47'C.

Five ml of bacterial suspension was added to 100ml of agar medium (preliminary trials showed that this

concentration produced a uniform visible growth on the agar plates) and mixed by gentle shaking whilst partially under water in the bath to prevent solidification of the agar medium. Aliquots of 12ml were placed in 7.0cm diameter sterile plastic petridishes (Sterilin Ltd). Using this procedure ensured that the level of medium was the same in each petridish. This was adapted from the technique known as the "pour plate method".

7.2.5 Antibacterial assay

After aproximately two hours, once the culture plates were solidified, four wells were cut in each culture plate using a sterile gel punch. The freezedried mucus extracts were resuspended in 5ml portions of sterile PBS. Aliquots of 0.2-0.3ml were then placed in the wells. Separate plates were used for skin and buccal mucus extracts and aquarium water from the tanks where the fish had been kept was used as a control. After innoculation of plates with mucus and control samples, an antibiotic assay disc containing 30ug of Oxytetracycline was placed in the centre of each culture plate as a positive control. The precise pattern of the inoculation of the culture plates is given in Figure 42. The plates were then incubated at 26'C for 48 hours.

7.3 RESULTS

Figure 42.

The pattern of innoculation of buccal and skin mucus in bacterial culture plates used for antibacterial assay.

A : Pooled mucus (buccal or skin) of pre spawnwers

B : " " egg carriers

C : " " fry carriers

D : Control (aquarium water)

E : Positive control (30ug Oxytetracycline disk)

Figure 43.

Photograph of A. hydrophila plate showing the result of the anti-bacterial assay (buccal mucus).

Note the lack of antibacterial activity around the mucus wells.

A : Pooled buccal mucus of pre spawners

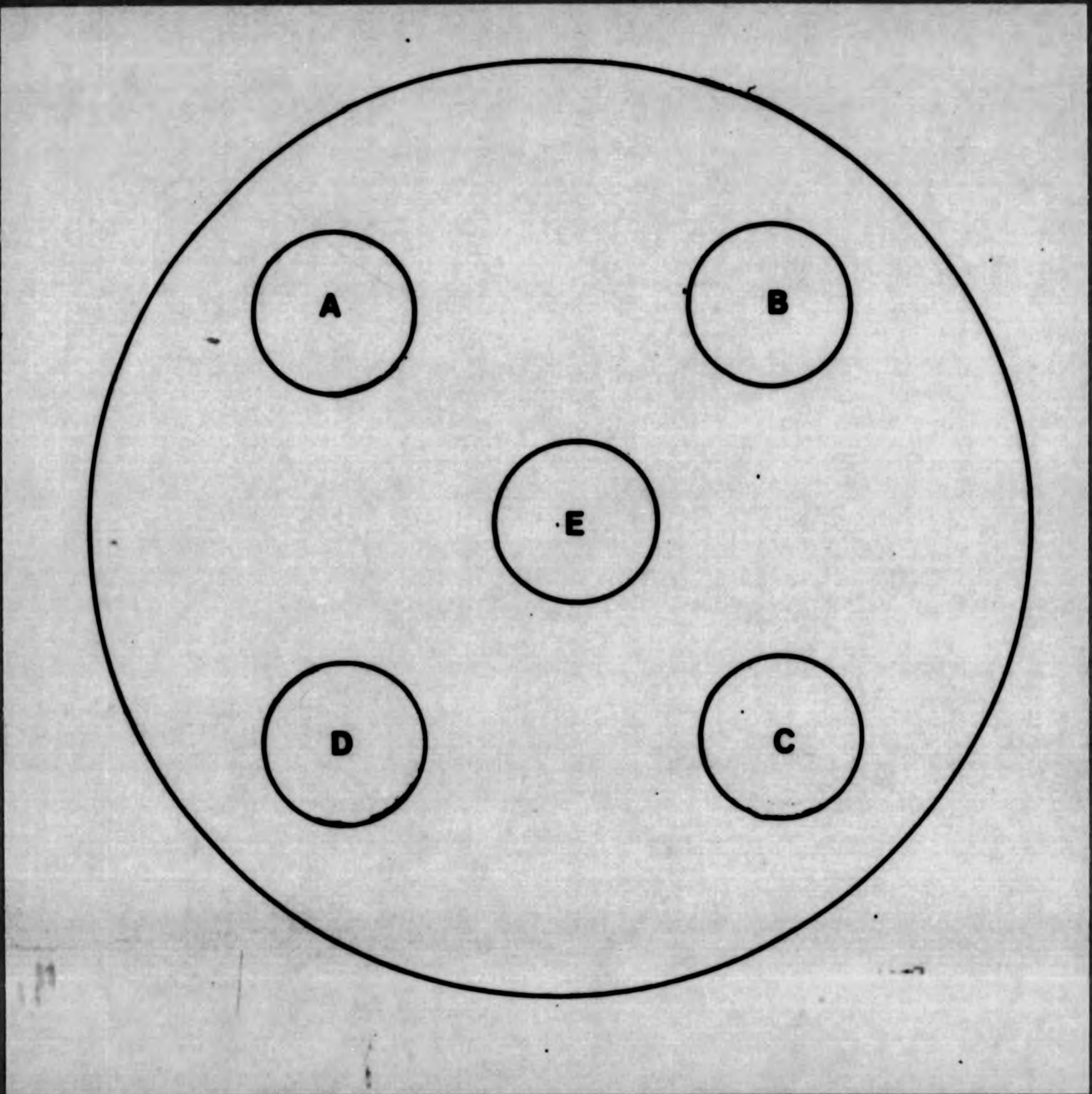
B : " egg carriers

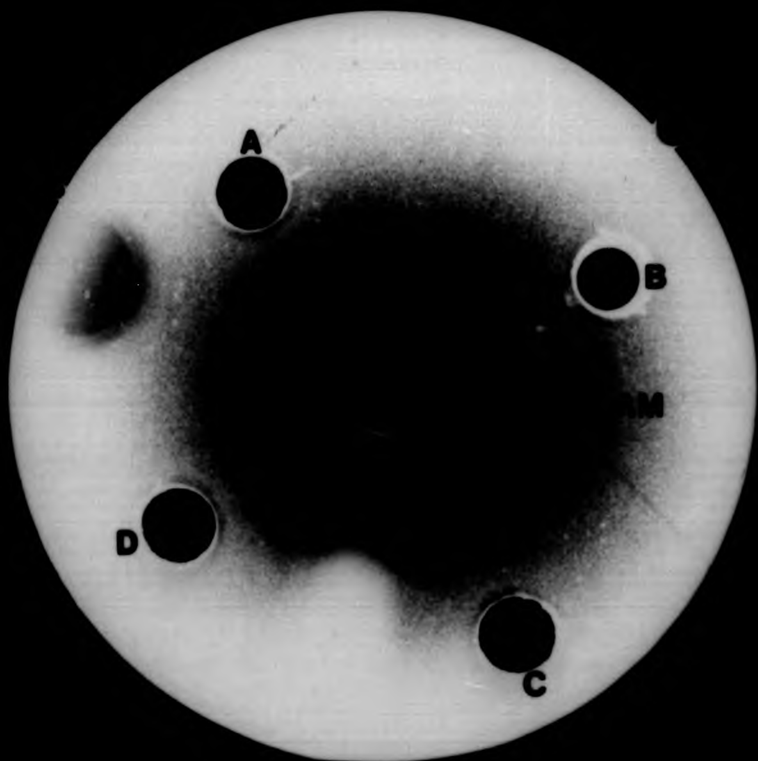
C : " fry carriers

D : Control (aquarium water)

E : Possitive control (30ug Oxytetracycline)

AM : Anti-bacterial activity of the positive control.


A
B
E
D
C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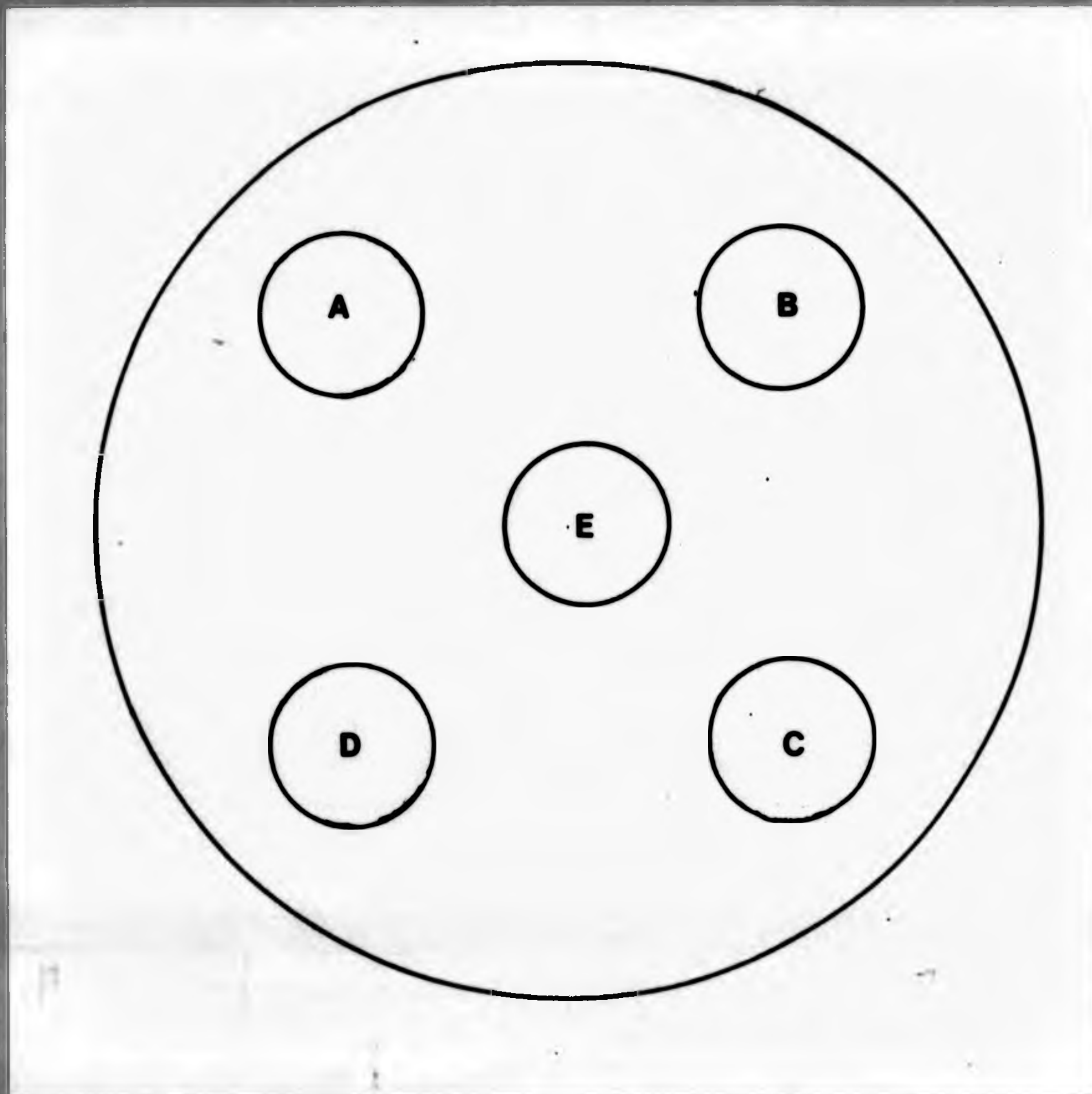
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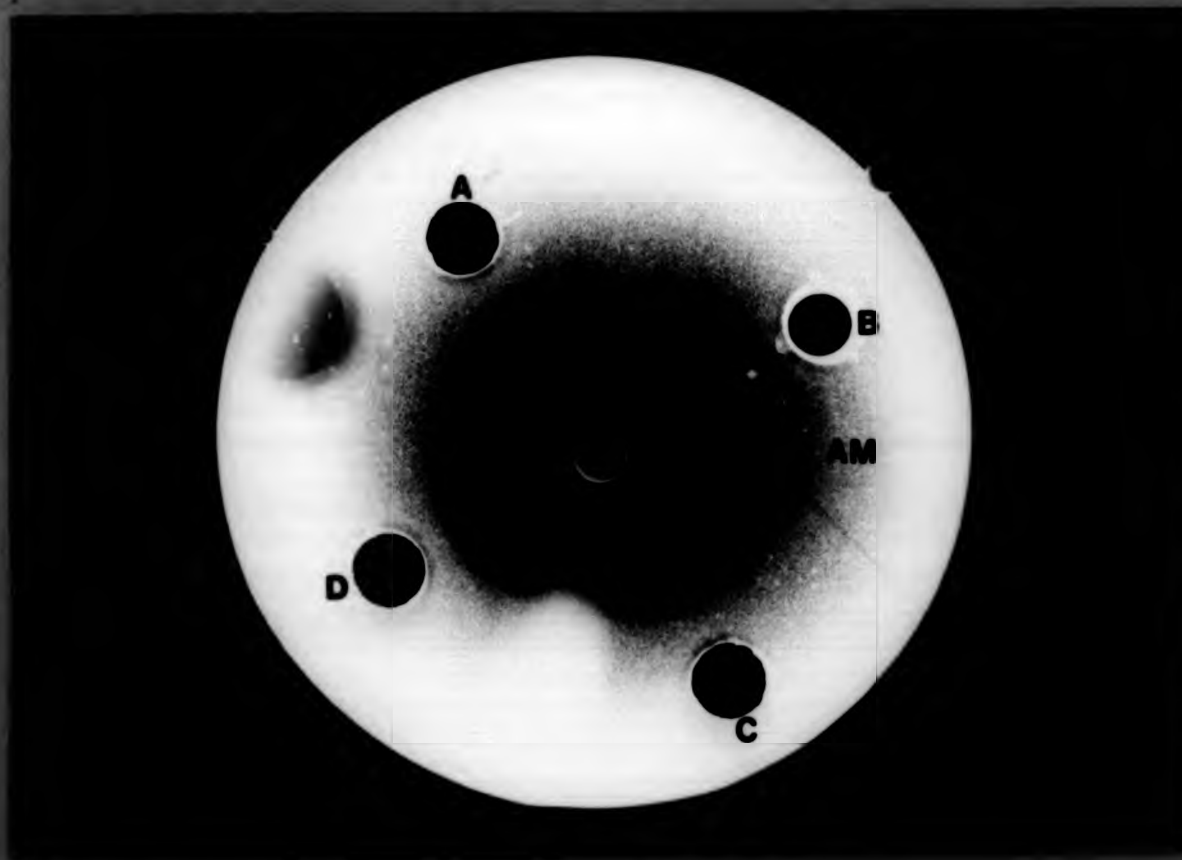
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Visual observation of the lining of the buccal cavity of the fish before and after spawning showed certain morphological differences. The tissue lining over the posterior dorsum of the mouth of the females ie. posterior palate, showed an extremely mucoid, spongy, thick and whitish appearance. This was more prominent in females carrying eggs or fry than in the females prior to spawning. Apart from this, no distinct differences were observed in the general appearence of the buccal cavity between pre spawning, egg carrying and fry carrying females.

During the antibacterial assay, a definite, clear, bacteria free zone was observed around the positive control antibiotic assay discs placed in all five types of bacterial culture plates, indicating the effect of the antibiotic on the bacteria. It was expected that if antibacterial activity was present in the mucus, this would diffuse from the wells out into the surrounding medium and inhibit the bacteria in the medium resulting in a clear zone around the mucus wells within 48 hours of incubation. Although the assay was repeated twice, no antibacterial activity was noted around the wells containing either buccal or skin mucus as was observed around the positive control antibiotic discs. The negative control wells containing aquarium water were also found free of a clear zone around, as expected. Figures 43-45 show some of the results obtained during the assay.

Figure 44.

Photograph of P. fluorescens plate showing the result of the anti-bacterial assay (buccal mucus).

Note the lack of antibacterial activity around the mucus wells.

A : Pooled buccal mucus of pre spawners

B : " egg carriers

C : " fry carriers

D : Control (aquarium water)

E : Positive control (30ug Oxytetracycline)

AM : Anti-bacterial activity of the positive control.

Figure 45.

Photograph of E. tarda plate showing the result of the anti-bacterial assay (skin mucus)

Note the lack of antibacterial activity around the mucus wells.

A : Pooled skin mucus of pre spawners

B : " egg carriers

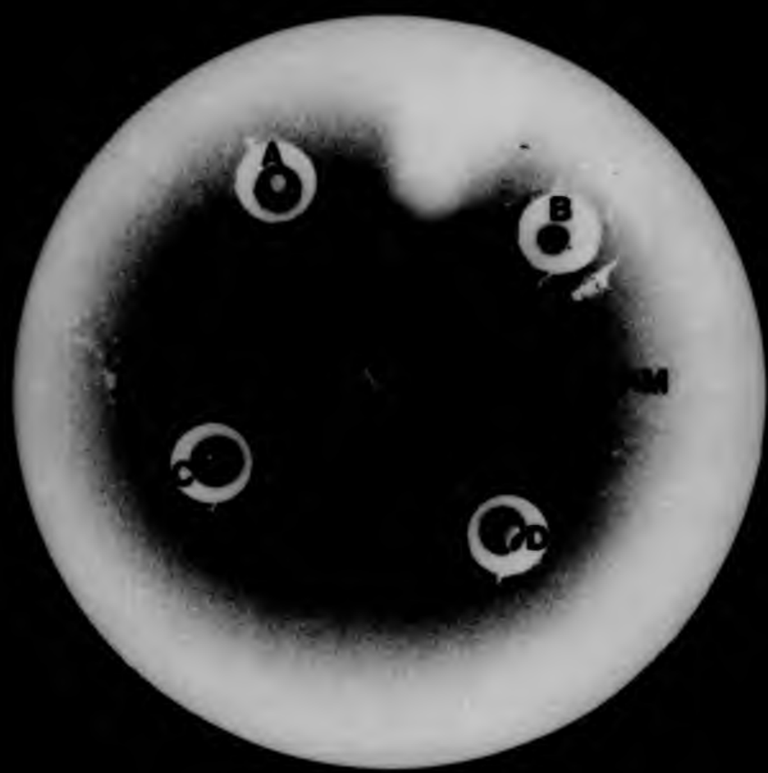
C : " fry carriers

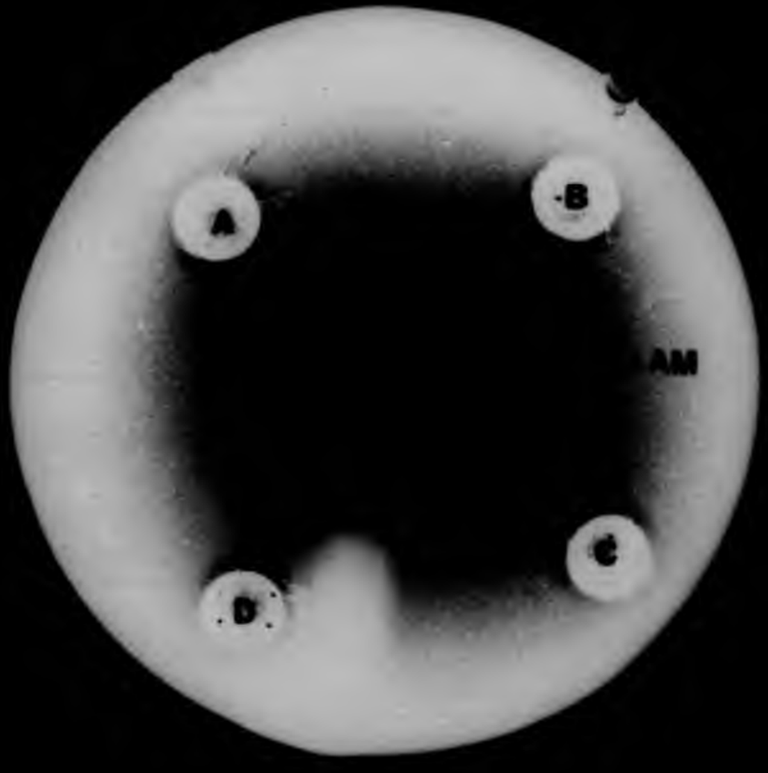
D : Control (aquarium water)

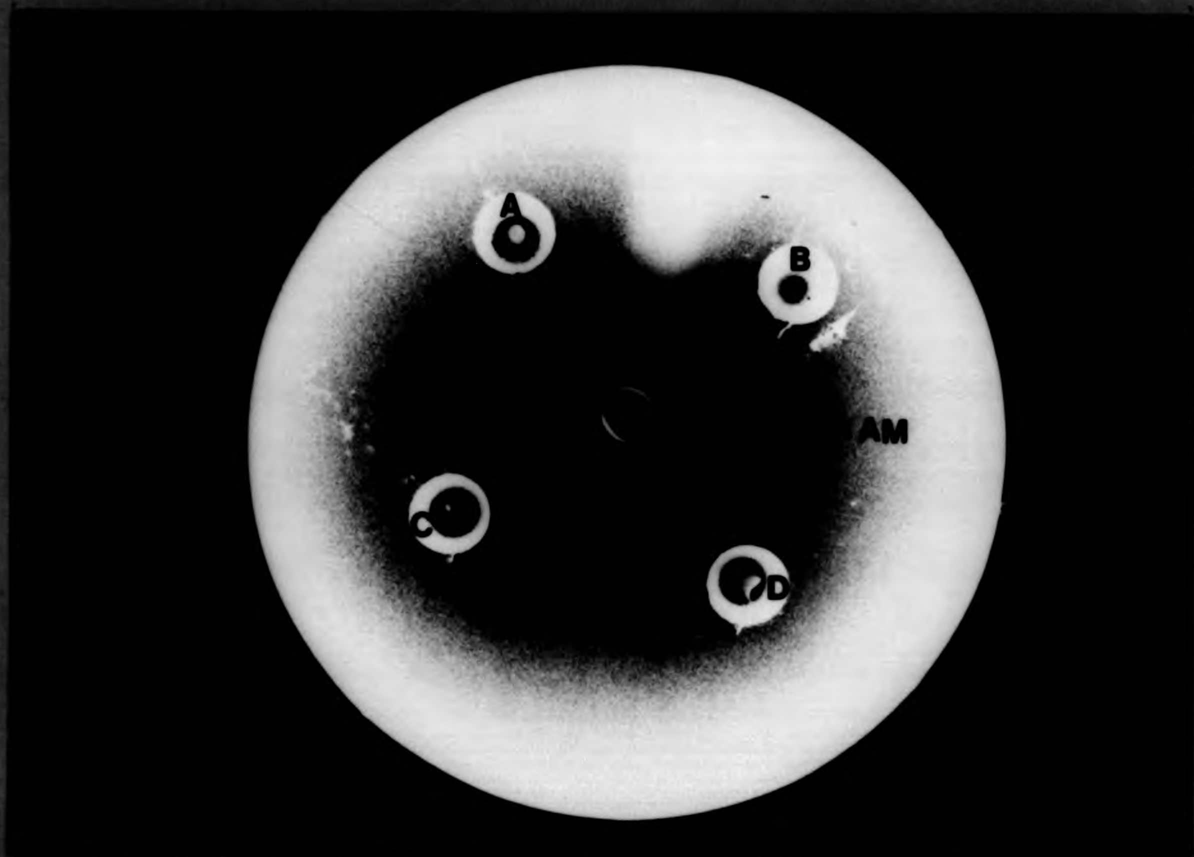
E : Positive control (30ug Oxytetracycline)

AM : Anti-bacterial activity of the positive control

Figure 44.
Photograph o
anti-bacteri
Note the lac
wells
A : Pooled
B :
C :
D : Control
E : Positiv
Ab : Anti-ba






A
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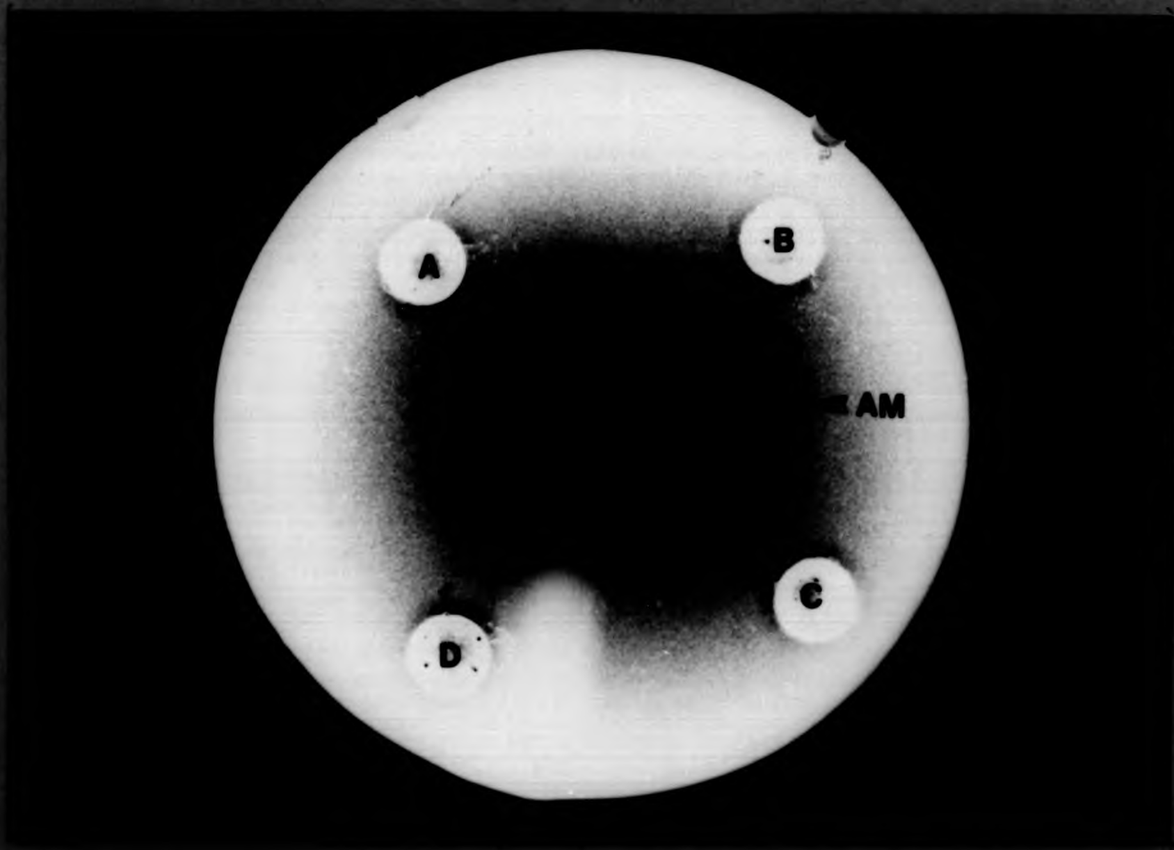
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7.4 DISCUSSION

Mucus is a secretory product which occurs almost universally in the animal kingdom and is involved in various life processes. In fish the skin mucus is said to have a possible osmoregulatory function (Van Oostren 1957) and a lubricating function which reduces the turbulence generated by swimming (Rosen and Cornford 1971). It has also been said that the skin mucus of fishes may be important in the natural defence against parasites and pathogenic micro organisms (Fletcher 1978; Ingram 1980).

A histological investigation of the buccal epithelium of the mouth brooding and substrate spawning tilapias carried out by Ntheketha (1984) reported that there was an increase in number and size of mucous cells in breeding mouth brooders, whilst there was no noticeable change in epithelial thickness and the number and size of mucous cells in breeding and nonbreedng substrate spawners. Though he did not include O.mossambicus in his study and also did not examine a satisfactory number of fish, the circumstantial evidence suggests that the mucus is secreted in large quantities during mouth brooding. This would support the suggestion that it plays a role in the survival of eggs and fry under oral incubation.

Shaw and Aronson (1954) investigated the oral incubation of Sarotherodon macrocephalus, a paternal mouth

brooder and reported that a pair of unusual pharyngeal glands were found on the dorsal surface of the palate. During the present study the extremely mucoid, spongy, thick whitish area observed in the posterior palate of breeding O.mossambicus appeared to be similar to the description given by Shaw and Aronson (1954). Areas of a similar appearance were also identified in breeding O.spilurus, O.macrochir and O.niloticus (Ntheketha 1984). Incidental observations made on the buccal cavities of substrate spawners revealed no such glands. Therefore it appears that the presence of these glands coincides with the breeding method and cycle of the fish. This mucus secretion may be under hormonal control in these species.

Physical contact with the surface of fish seems to result in an increased mucus production. Noakes (1972) found a positive correlation between the mucous cell concentration in the epidermis of parent Cichlosoma citrinellum (Linnaeus) and the degree of physical contact between the parent fishes and young. These reports, together with the observations made in the present study, suggest that the presence of eggs and/or fry in the buccal cavity may act as a trigger mechanism for the production of large quantities of mucus in the buccal cavity during mouth brooding. The stimulation for the production of mucus may be a mechanical process as reported by Pickering and Macey (1977). However, the actual role in which the mucus helps

in protection of eggs and fry, if it does, is still not clear since the results showed that, under the experimental conditions, there was no specific antibacterial activity.

Antibacterial activity of the fish skin mucus has only been positively demonstrated by a few people. Liguori, Rugieri, Baslow, Stempien and Nigrelli (1963) found antibacterial activity against Escherichia coli in the mucus of Pacific golden striped bass (Grammistes sexlineatus whilst Nigrelli, cited by Jakowska (1963), experimentally demonstrated the antibacterial action of the skin mucus of a small tropical marine teleost against Staphylococcus aureus and Pseudomonas pyocyanea using the paper disc method. Shaw and Aronson (1954) failed to demonstrate such antibacterial activity in the buccal and skin mucus of S. macrocephalus whereas Harrel et al (1976) demonstrated positive anti Vibrio anguillarum activity in rainbow trout mucus and stated that complement exists not only in serum but also in mucus of fish and it can have an anti microbial function, at least in vitro. However the method used in the assay by Harrel et al (1976) is different from that by the other workers mentioned because they used concentrated mucus instead.

Hjelmeland, Christie and Raa (1983) using nonconcentrated rainbow trout mucus also demonstrated a positive anti V. anguillarum activity and attributed it to the presence of protease, a proteolitic enzyme. However, in

the present study, neither the preliminary trials carried out using nonconcentrated mucus nor the experimental trials carried out using an approximately ten-fold concentration buccal mucus of *O. mossambicus*, regardless of their breeding conditions, demonstrated any antibacterial activity against *A. hydrophila*, *P. fluorescens*, *Flavobacterium* species, *Y. ruckeri* or *E. tarda*. All the previous positive reports on antibacterial activity were based on skin mucus of fish whereas all the attempts on buccal mucus were unsuccessful. In this study neither skin nor buccal mucus were effective.

Blood serum could be one of the contaminants of mucus during the handling of fish (Smith and Ramos 1976). According to Hjelmeland *et al* (1983) blood serum is a likely source of protease inhibitors and could be present in mucus as a result of the mode of handling during collection. Though the method employed during the present study for the collection of mucus was not the same as that described by Hjelmeland *et al* (1983), the extreme care taken during the collection of mucus is most unlikely to result in contamination with blood serum.

Varute and Jirge (1971) on their histochemical investigation of mucosubstances in the oral mucosa of *O. mossambicus*, showed the presence of sulphate carbohydrate sulfomucines, sialo mucin along with some neutral mucosubstances, and their cyclic variations in concentration during the breeding cycle. They also

reported that these sulphomucins, sialomucins and glycogens increase, reaching a maximum throughout the oral gestation. They concluded that the cyclic behaviour of the mucosubstances occurs concomitantly with the seasonal breeding cycle. This seems to be a biochemical adaptation of the oral mucosa of the mouth brooding cichlids for oral gestation. However, the role of these mucosubstances in such oral gestation still remains unclear.

The skin mucus of fish has been shown to contain lysozyme (Fletcher and Grant 1968; Fletcher and White 1973), complement components (Harrel *et al* 1976) and C-reactive proteins (Ramos and Smith 1978). According to Ellis (1978) lysozymes probably provide an important defence against many microbial pathogens. Lysozymes cause lysis of gram-positive bacteria by hydrolysing B1-4 glycosidic linkages in the murein component of the cell wall. In gram-negative bacteria, lysis by lysozymes may be mediated by other factors which can disrupt the outer lipid-protein-polysacharide complex of the cell wall and unmask the inner murein layer (Wardlaw 1963). However, Shaw and Aronson (1954) failed to maintain *S. macrocephalus* developing eggs after treating with synthetic lysozyme and suggested that the various bactericidal solutions, in addition to their various toxic properties, may not be sufficiently bactericidal to the particular species that attack the *Sarotherodon* eggs. If lysozyme were the active

antibacterial property of O. mossambicus buccal and skin mucus, the negative results might be explained by their lack of stability as suggested by Pickering and Richards (1980) who also failed to demonstrate any anti-bacterial activity in salmonid mucus. Even so, neither lysozymes nor proteases, as shown to be contained in skin mucus of fish, have been positively demonstrated in the buccal mucosa of mouth brooding cichlids.

During the mouth brooding process the eggs and fry are constantly "churned" within the buccal cavity by the brooding parent. This may result in the formation of mucus coating over the eggs and fry surface. By continous sloughing and replacement of this mucus coat, pathogens could be removed together with the mucus before gaining access to the egg membrane or the skin of fry. Pickering (1974) also suggested that, in fish, continous replacement of mucus prevents colonization by parasites, fungi and bacteria. Hjelmeland et al (1983) reported that V. anguillarum was not inhibited when exposed to protease extracted from rainbow trout skin mucus in a standard nutrient medium, however, the bacteria lost their viability faster than normal. This suggests the possibility that the presence of proteases, lysozymes, sialomucins, sulfomucins etc. in the buccal mucus may reduce the viability of bacteria when in contact with the eggs and fry. This may result in the mucus acting as an agent which delays the

access of bacteria to the eggs and fry surface rather than an antibacterial or bactericidal agent. This explanation is also supported by the fact that the surfaces of the nonviable or dead eggs in the buccal cavity did not show any sign of bacterial activity until a few days after death (Chapter 5).

These explanations however do not disregard the possibility that the buccal mucus of mouth brooding cichlids possesses antibacterial properties against the naturally occurring potentially pathogenic bacteria, but durng the present investigation the collection and assay methods used did not reveal any positive antibacterial activity of the skin or buccal mucus of breeding or nonbreeding O. mossambicus.

CHAPTER 8

EXPERIMENTAL INFECTIONS OF O.MOSSAMBICUS WITH I.MULTIFILIIS AND ITS PATHOLOGY

8.1 INTRODUCTION

The host-parasite relationship varies with the species, age and the size of the host. As a result of a long standing relationship, both the parasite and the host evolve to a state of equilibrium where there is little or no damage caused to the host population by the parasite. However, at the individual level, the extent of the damage caused by the parasite to its host can be variable. Nigrelli, Pokorny and Ruggieri (1976) observed that a few I.multifiliis individuals are always present on the skin and gills of "some" fish in any given aquarium; in these instances the parasite produces little effect on the host. They further stated that some fish species are highly resistant, others develop an acquired immunity, while still other species show a "natural immunity" to the disease. However, according to Bauer (1958), all pond fishes are susceptible to Ichthyophthiriasis and that fry and under yearlings are most susceptible to the disease. More so, even brood-stock and spawners may become infected and die when the intensity of infection is high.

The extent of the damage caused by

I.multifiliis to cultured and wild fish has been well documented and is discussed in Chapter 1. A considerable amount of literature is available on experimental infections of various species of fish (Hines and Spira 1973a, 1973b, 1974a, 1974b, 1974c; Beckert 1975; Parker 1965; Dickerson, Dawe, Gratzek, Brown and Pyle 1981) but they mainly deal with the acquired immunity. Surprisingly, only Hines and Spira (1973a, 1973b, 1974a, 1974b, 1974c) carried out a series of investigations to study the pathological effects of parasitism, including the course of the infecton, the leucocyte response, physiological dysfunction as well as acquired immunity in a particular host.

The pathology of *I.multifiliis* infections has gained considerable attention more recently. Since the critical study of the sequential pathology carried out by Hines and Spira (1974a), McLay (1985) has investigated the ultrastuctural pathology in experimentally infected *S.gairdneri* and Ventura and Paperna (1985) studied the histopathology in a wide range of host fishes, both naturally and experimentally infected, which they obtained from diverse geographical regions. Apart from a few naturally infected fingerlings of *O.mossambicus* which they examined, there seems to be no other reports on pathology of *I.multifiliis* infections in *O.mossambicus*. The fry of this species appeared to be highly susceptible to

I.multifiliis infections (C.Sommerville pers. com.). Thus a better understanding of the effects of the parasite and the pathology in fry would be a useful way to advance the knowledge of Ichthyophthiriasis in this important cultured species.

Therefore, the present study was designed to elucidate the following.

1. The effects of I.multifiliis infections on O.mossambicus fry of different age groups.

2. To calculate lethal and sublethal levels of infections on these various age groups of fry and adult females.

3. To study the histopathology of I.multifiliis infections in O.mossambicus fry ie. one of the most vulnerable stages.

8.2 MATERIALS AND METHODS

8.2.1 Source of fish

Fish of four different age groups were used in the investigation carried out for the estimation of the lethal levels of I.multifiliis infections. The first three groups consisted of 4 day, 8 day and 30 day post hatch O.mossambicus fry. The fourth group was comprised of 12 to 18 month old O.mossambicus

breeding females. They weighed between 157.4g and 174.8g ($\bar{x}$=168.5g) and measured 16.4cm and 17.1cm standard length with a mean of 16.8cm.

The first three groups of fry were obtained from the broodstock maintained in the broodstock system described in Chapter 2 section 2.2. The fish in the fourth group were maintained in the same system.

8.2.2 Source of parasite

The isolate denoted RS/CM/84 (isolated from a male firemouth cichlid, Cichlasoma meeki, (Günther) kept at 27'C in March 1984) was used for the study. Only tomites of age 6-12 hours were used for the infection trials. The infection procedure is described separately.

8.2.3 Experimental procedure

The aim of the study was to establish the lethal levels of infections of I.multifiliis in O.mossambicus of different age groups. For this purpose it was necessary to find as accurate a method as possible to infect the fish with a predetermined number of parasites. There are several methods reported for the induction of I.multifiliis infections. These can be grouped into three categories; the addition of infected fish to a population of noninfected fish (Goven et al 1980; Hines and Spira

1973a); the addition of a standard number of trophozoites to a population of fish (Areerat 1974; Subasinghe 1982) and the exposure of test fish to tha tomites released by a known number of trophozoites (Beckert 1976). Due to the variation in the number of parasites on any individual fish and the variation in the number of tomites released by trophozoites, all these methods were found to result in variation in infection rates on test fish. This was found to be the case, particularly where the fish had not been previously exposed to the parasite. Dickerson _et al_ (1981) developed a method to overcome these problems using a standardised procedure. This procedure was slightly modified and adopted to the requirements of the present study and is described below.

8.2.3.1 Preparation of tomites

I.multifiliis (isolate RS/CM/84) infected _O.mossambicus_ (30-40g) were used to obtain parasites. Trophozoites, five days post invasion, were dislodged undamaged from the host skin by placing an infected fish in a glass beaker with a small volume of water, just sufficient to cover the fish. The subsequent erratic movement of the fish within the confined area resulted in effective dislodging of the trophozoites.

A large number of trophozoites were collected in this way and were placed in several 500ml

beakers with a known volume of well aerated water (pH 7.1-7.3). The beakers containing trophozoites were then incubated at 27±1'C for a period of 18 hours. It had previously been observed that at 27+1'C the trophozoites completed their reproductive phase and produced active tomites within 12-18 hours of incubation. All the tomite suspensions were then pooled. Three 5ml samples were withdrawn from the tomite suspension and to each sample 1ml of 0.1% Neutral Red was added in order to stain the parasites. The number of tomites present in two 1.0ml aliquots from each sample was counted using a Sedgwick-Rafter counting chamber, and the mean number of tomites present in a 1.0ml of original suspension was estimated. By serial dilutions of the original suspension, the required number of tomites for the infection trials were prepared.

8.2.3.2 Infection procedure

The dose levels (numbers of tomites per fish) used in the infection trials are given in the Table 12. In the case of 4 day, 8 day and 30 day old fry, the infection trials were carried out in one litre glass beakers covered with with black polythene. The number of fish used in each container was 20. The exposure time was standardised to 3 hours. After exposure each group of fish was removed from the infection beakers and was placed

TABLE 12. The dose levels of parasites used in the experimental infections of various age groups of <u>O. mossambicus</u>

Dose level of parasites tomites/fish	Age class of the fish (Days)			
	4 day	8 day	30 day	1 year+
5	*	*		
10	*	*		
20	*	*		
40	*	*		
80	*	*		
160	*	*		
320	*	*		
500			*	
640	*	*		
1000			*	
1280	*	*		
2000			*	
4000			*	
5000				*
8000			*	
10000				*
16000			*	
20000				*
30000				*

separately in 12 litre, perspex aquaria containing 8 litres of aerated water. The aquaria were individually aerated and a controlled temperature of 27±1'C was maintained. The infected fish aged 4, 8 and 30 days post hatch were fed twice a day with ground commercial trout pellets (Ewos Baker Ltd, Bathgate, Scotland) at a particle size of 200-300um, 300-500um and 500-1000um respectively. They were observed daily for a period of 15 days for survival. At days 4, 8 and 12 of the course of experiment, half of the water from each aquarium was changed with aerated water.

For group four, the one year+ females, the infection trials were carried out in 40l glass aquaria with 30l of aerated water. A number of 5 fish per aquarium was maintained and the tanks were covered with black polythene. After 3 hours of exposure the individual groups of fish were transferred to 40l glass aquaria with 30l of aerated water. The aquaria were individually aerated and a temperature of 27±1'C was maintained. The fish were fed twice a day with commercial trout pellets (Ewos Baker Ltd) at 1% of their body weight per day. The fish were observed daily over a period of 15 days. Partial water changes were carried out at days 4,8 and 12 of the experiment.

Throughout the investigation duplicate infection trials were carried out for each dose level of tomites exposed within the four groups of fish. An

identical uninfected duplicated group was observed as a control for all four age groups.

8.2.3.3 Histology

For the histopathological studies O.mossambicus fry of 30 days post hatch were employed. Thirty fry were exposed to a sublethal dose of 200-300 tomites per fish for 3 hours. The same exposure procedures were used as described in section 8.2.3.2. A random sample of four fish was removed after 12 hours, 1, 2, 4, 6, 8 and 12 days post exposure. They were killed by a neck incision and were fixed in 10% buffered formalin. Histological sections were prepared according to the procedures given in Appendix 4. They were stained routinely with H&E.

8.3 RESULTS

8.3.1 Experimental infections

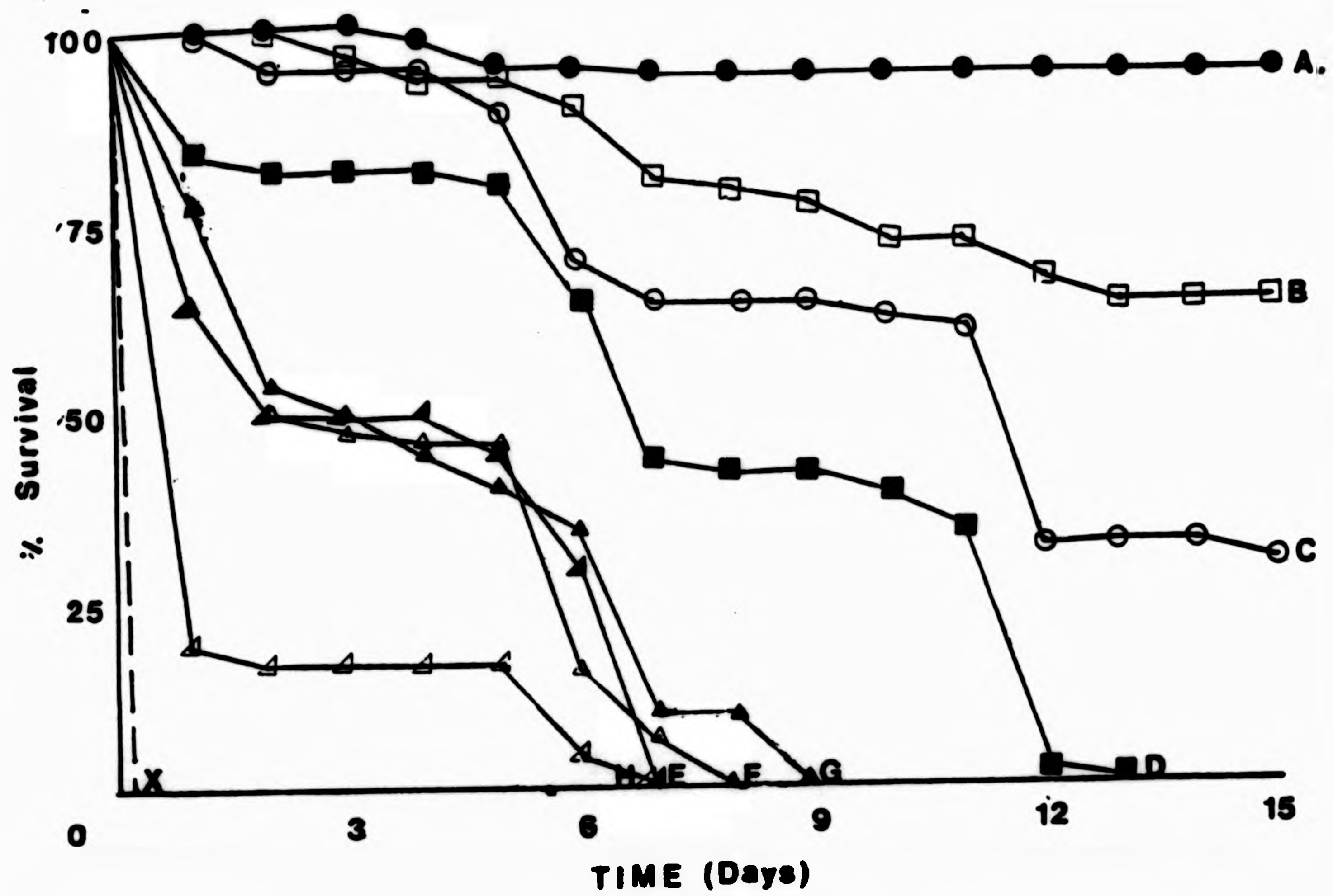
Figure 46 describes the relationship between the pattern of survival of 4 day old fry exposed to varying dose levels of I.multifiliis and time. The dose levels 640 tomites/fish and above resulted in 100% mortality of fry within the 3 hour period of exposure. In contrast, the levels of 10 tomites/fish and below did not cause any mortality within 24 hours of exposure. The exposure levels 20, 40, 80, 160 and 320 tomites/fish

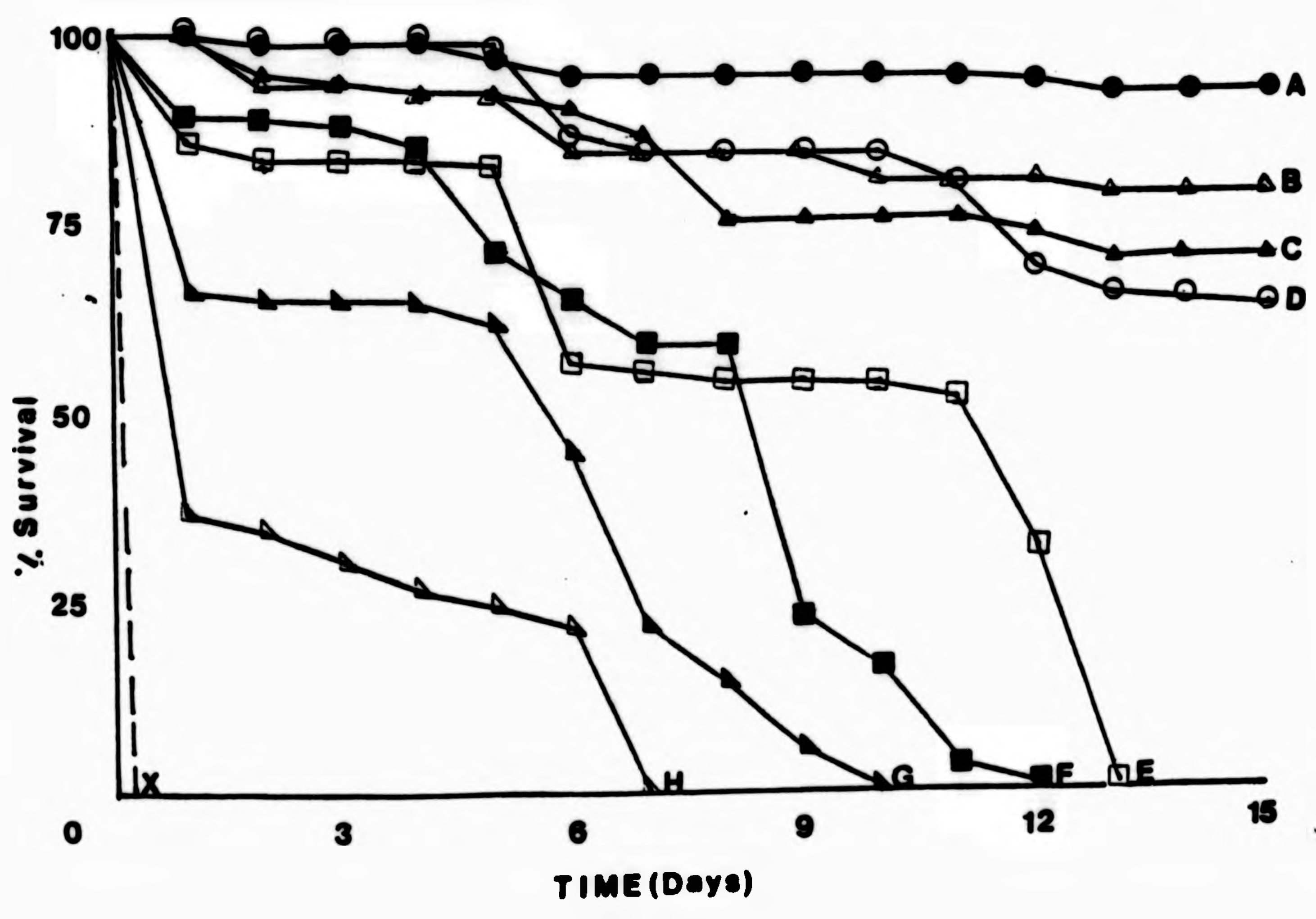
Figure 46 and 47

Graphs showing the pattern of survival of four day old (Fig. 46) and eight day old (Fig. 47) fry exposed to varying dose levels of I.multifiliis.

A : Control
B : 5 tomites/fish
C : 10 "
D : 20 "
E : 40 tomites/fish
F : 80 "
G : 160 "
H : 320 "
X : 640 and 1280 tomites/fish

(Both initial and subsequant reinfections were considered)


100
75
50
25
0
% Survival
3
6
9
12
15
TIME (Days)
A.
B
C
D
H
E
F
G
X


100
75
50
25
0
% Survival
3
6
9
12
15
TIME(Days)
A
B
C
D
E
F
G
H
X

brought about 100% fry mortality by days 13, 7, 8, 9 and 7 post exposure respectively and showed a certain degree of dose independent variability. The lowest dose level of 5 tomites/fish caused only 36.7% fry mortality over the 15 days of the trial. The mortality of the fry in the control group was only 6.7%.

The 8 day old fry also showed 100% mortality during the course of a 3 hour exposure, at dose levels 640 tomites/fish and above (Figure 47). In contrast, the mortality of the fry exposed to dose levels 20 tomites/fish and below was less than 40% over the fifteen day period. The dose levels 40, 80, 160 and 320 tomites/fish exhibited a somewhat similar pattern of mortality showing a rapid drop in survival around days 5 and 6. All those four dose levels resulted in 100% fry mortality. The pattern of mortality was evidently dose dependant.

Figure 48 describes the mortality pattern of 30 day old fry exposed to varying dose levels of I.multifiliis. The fish exposed to the two highest dose levels, ie 8000 and 16,000 tomites/fish showed a similarity to that observed in 4 and 8 day old fry resulting in 100% fry mortality during the course of exposure. The pattern of dose dependant mortality with rapid drops in survival around days 5 and 6 of exposure was evident in the fry exposed to dose levels between 1000 and

Figure 48.

Graphs showing the pattern of survival of thirty day old fry exposed to varying dose levels of I.multifiliis.

A : Control

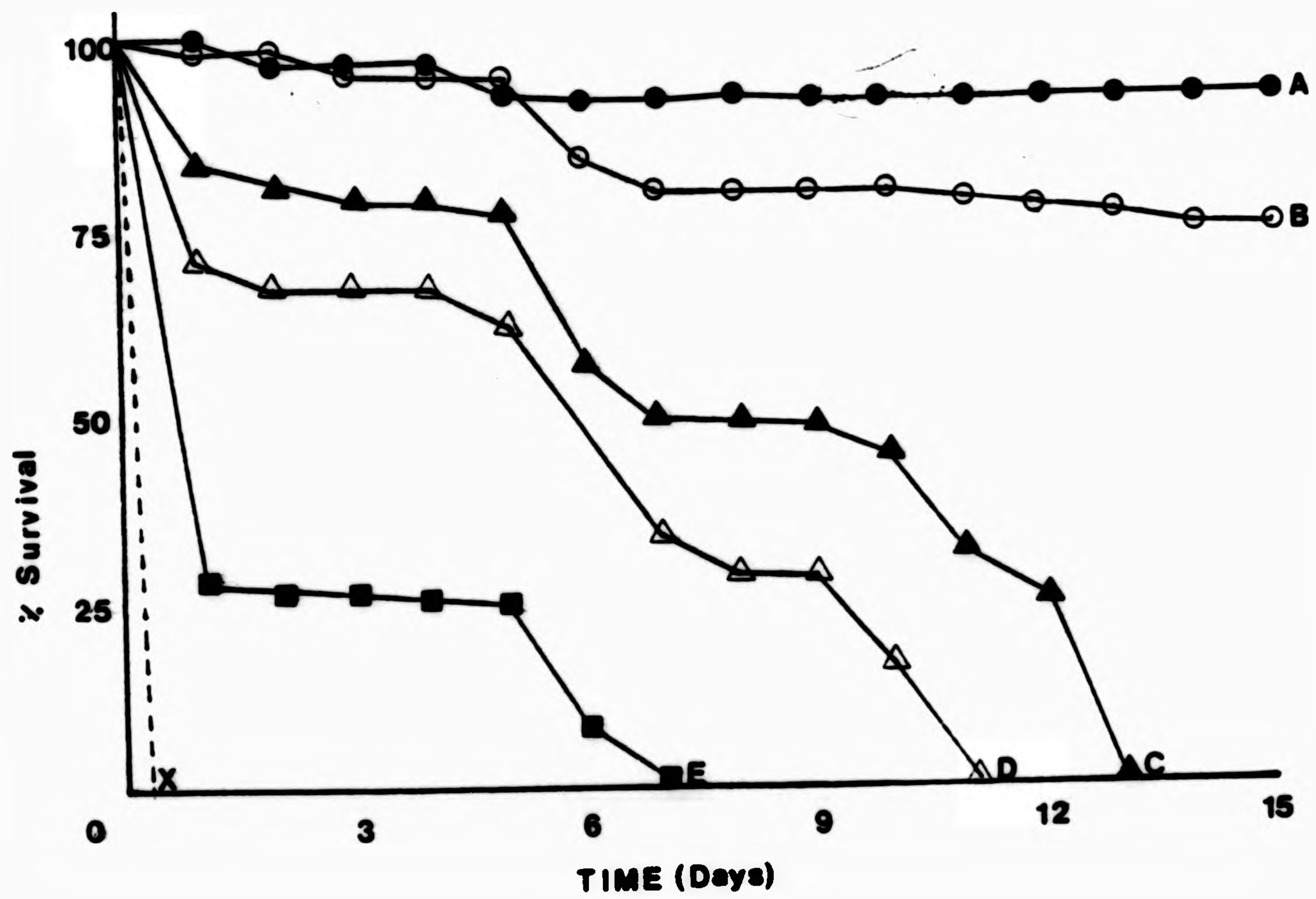
B : 500 tomites/fish

C : 1000 "

D : 2000 "

E : 4000 "

X : 8000 and 16000 tomites/fish.



100
75
50
25
0
% Survival
3
6
9
12
15
TIME (Days)
A
B
C
D
E
X

4000 tomites/fish. Almost 75% of the fry exposed to 500 tomites/fish survived until the experiment was terminated. The survival of the control fish was above 90%.

None of the dose levels of parasites used for the infection of one year+ old female *O. mossambicus* resulted in mortality of the fish. The fish exposed to the highest dose level of 30,000 tomites/fish were found to be very heavily infected and stressed (Figure 49). Their feeding and swimming activities were abnormal and distinctly different from that of the controls. Fish exposed up to 10,000 tomites/fish were never observed to be severely stressed.

8.3.2 Pathology

8.3.2.1 Clinical signs

All the fish exposed to a dose level of 200 tomites/fish in this experiment were found to be visibly infected by day 4 post exposure. The fish were never observed to be severely stressed, ie the extreme flashing behaviour, lethargy and avoidance of feeding, as shown by the heavily infected fish, was absent. Their feeding and swimming behaviour was normal and was similar to the controls.

By the end of day 4 and the beginning of day 5 post exposure, the number of visible white spots

Figure 49.

Photograph of an year+ O.mossambicus exposed to a dose level of 30.000 tomites/fish.

Note the numerous parasites on the body

P : Parasites

(trophonts) on the fry decreased to a minimum as the trophonts escaped from the fish and encysted on the substrate. During this period, and the following 24 hour period, the fish became comparatively less active than the controls. This may have correlated with the tomite emergence from the developed cysts and the beginning of the next phase of the second infection. During the course of infection only 3 fish died in the infected group and the control group had no mortalities.

8.3.2.2 Dermal histopathology

The thickness of the epidermis of the control fish was variable depending on the area of the body. In the dorsal and ventral regions of the body, including the head region, the thickness varied between 6-10 cells, whereas in the lateral regions it was less than 6 cells thick; sometimes only 2-3 layers of cells were observed. Goblet-shaped mucous cells were evident within the upper layers of the epidermis. The density of the mucous cells was visibly higher in the buccal epithelium.

The tightly arranged epidermis lay above the thin basement membrane and the dermis, comprising the stratum spongiosum and stratum compactum, was usually 3-4 times thicker than the epidermis (Figure 50).

After 12 hours of exposure the parasites were found to be located only a few cells below

Figure 50.

Photomicrograph of a histological section of epidermis of an uninfected fish. H&E X 250.

E : Epidermis

B : Basement membrane

SS : Stratum spongiosum

SC : Stratum compactum.

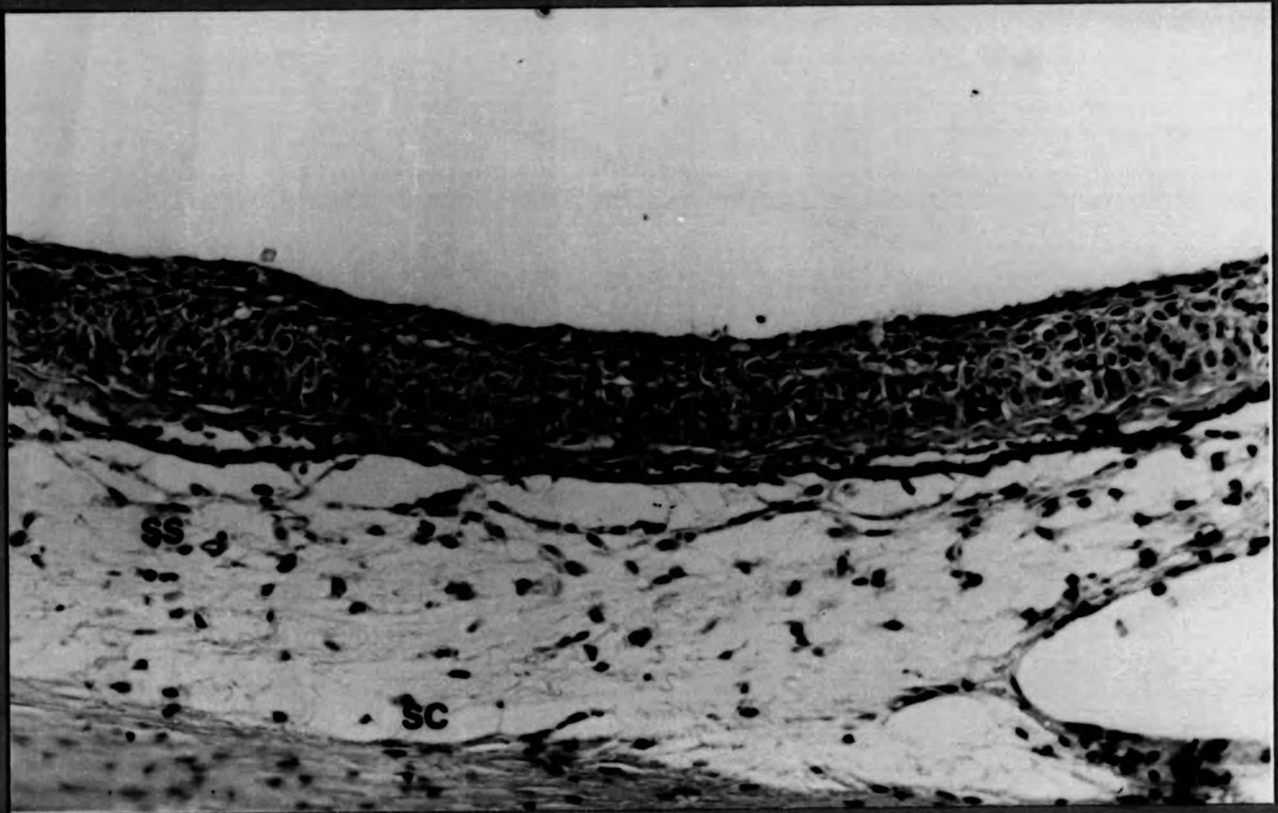
Figure 51.

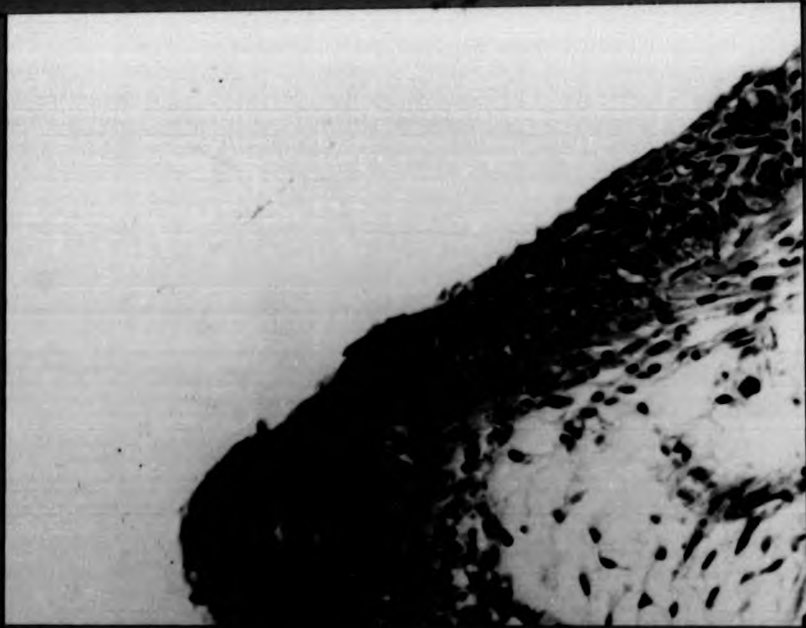
Photomicrograph of a histological section of epidermis of a fish 12 hours post exposure to *I. multifiliis*. H&E X 250.

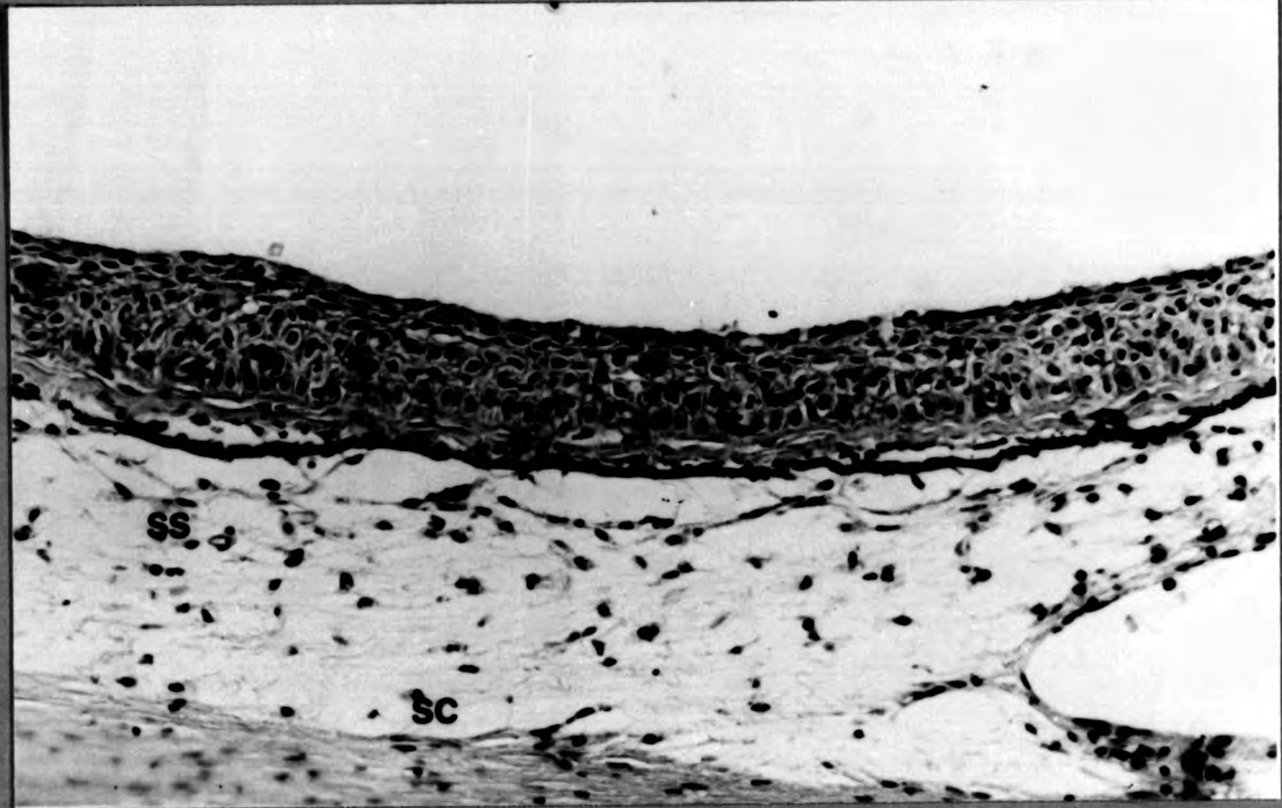
E : epidermis

P : Parasite

B : Basement membrane


SS
SC




SS
SC

the surface of the thicker layers of epidermis (Figure 51). In the areas where the epidermis was thin, the parasites were found to lie above the basement membrane (Figure 52). In some specimens where the invasion by large numbers of tomites was evident, the superficial epithelium was observed to have the appearance of an abraded surface. No host tissue response was evident in the perimeters of the parasites nor was there any visible sign of a path of entry into the inner epidermal layers. An empty space around the parasites was not visible as has been reported by some workers.

By 24 hours post exposure, the parasites were more frequently found to be located on the basement membrane surrounded by an empty space. In these cases the ciliature of the parasites was clearly visible. No sign of any inflammatory response or host tissue reaction was evident.

After 48 hours of exposure,individual fish showed varying degrees of response to infection. Mild hyperplasia was evident in some samples whilst some still showed no sign of any tissue response. Generally, the overlying epidermal cells directly above the growing trophonts were somewhat compressed and flattened (Figure 53). No sign of extensive tissue changes were evident.

By 4 days post infection the growing parasites were found to protrude out into the overlying

Figure 52.

Photomicrograph showing I.multifiliis lying above the basement membrane 12 hours post exposure. H&E X 250.

E : Epidermis

B : Basement membrane

P : Parasite

Figure 53.

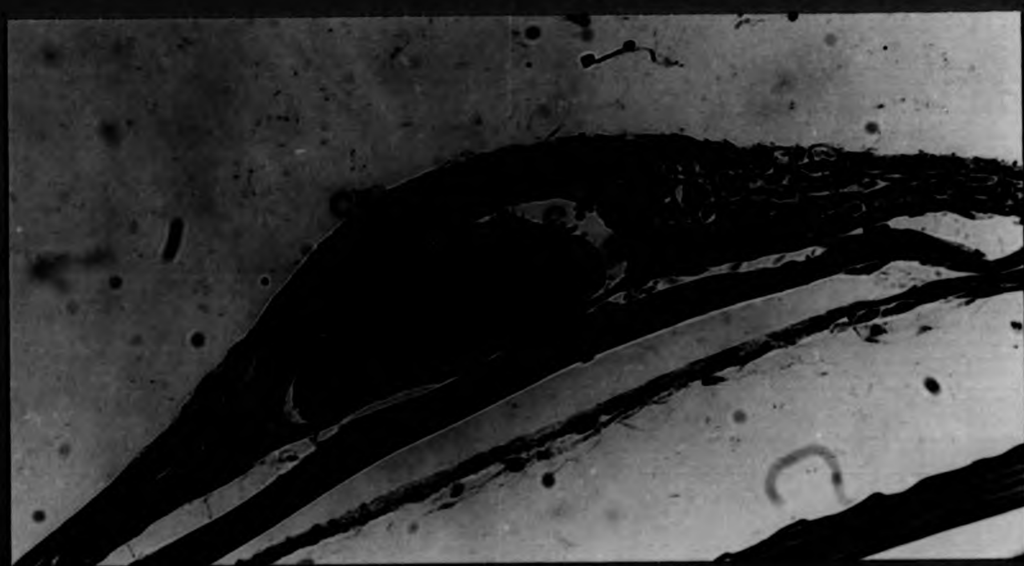
Photomicrograph of a histological section of epidermis of a fish 48 hours post exposure to I.multifiliis showing compression of overlying epithelial cells. H&E X 250.

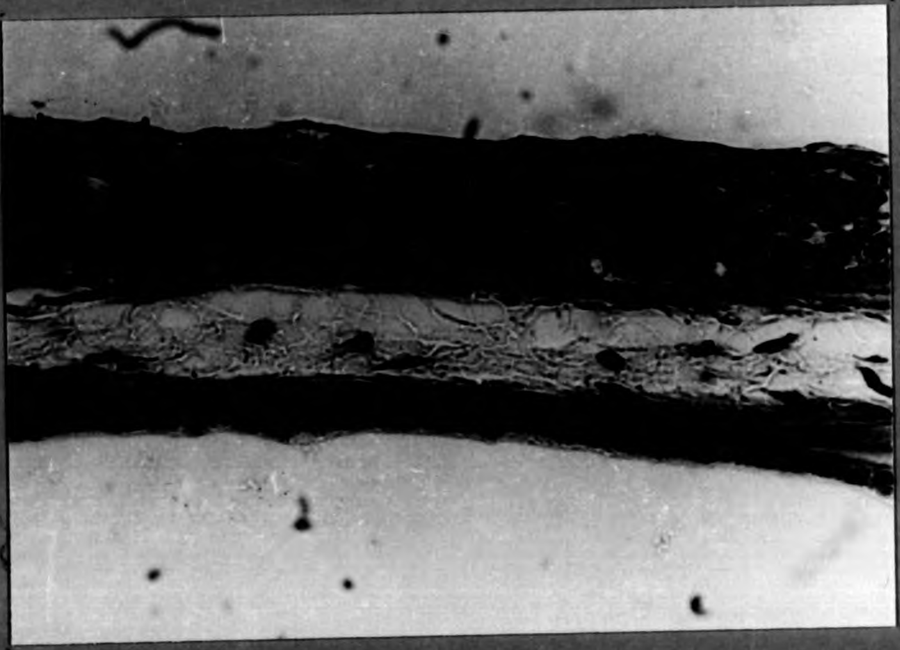
CE : Compressed epidermal cells

P : Parasite

C : Cilia

S : Space around the parasite





epithelium resulting in a nodular-like projection at the site of the parasite. As a result, the displacement of the overlying epidermal layers was fre quently visible. Moderate hyperplasia of the epithelial cells was also evident near the perimeter of the parasites.

The expansion of the growing trophonts into the epithelial layers resulted in flattening and stretching of the overlying epithelial cells to accommodate the parasites. However, in areas where the thickness of the epithelium was high the flattened and the stretched appearence was not very obvious (Figure 54).

In the 8 and 12 day post exposure samples, even in the areas where the parasite invasion was high, generalised hyperplasia was the commonest feature of the epithelial histopathology. However, these hyperplastic areas were confined to the perimeter of the settlement sites of the parasites. Moderate cellular necrotic changes such as pyknosis and karyolysis were evident around the perimeter of the parasites in some samples (Figures 55 and 56). General desquamation and degeneration of the superficial layers of the epidermis was evident to a lesser degree.

8.3.2.3 Branchial histopathology

The epithelium of the primary lamellae in control fish was squamose, especially at the

Figure 54.

Photomicrograph of a histological section of epidermis of a fish 96 hours post exposure to I.multifiliis showing histopathology. H&E X. 400.

CE : Relatively less compressed epidermal cells

P : Parasite

C : Cilia

S : Space around the parasite

G : Goblet cells

Note relatively less histopathological changes

Figure 55 and 56.

Photomicrograph of a hstological section of epidermis of a fish 8 days post exposure to I.multifiliis showing dermal histopathology.

Fig. 55 H&E X 400.

Fig. 56 H&E X 400.

P : Parasite

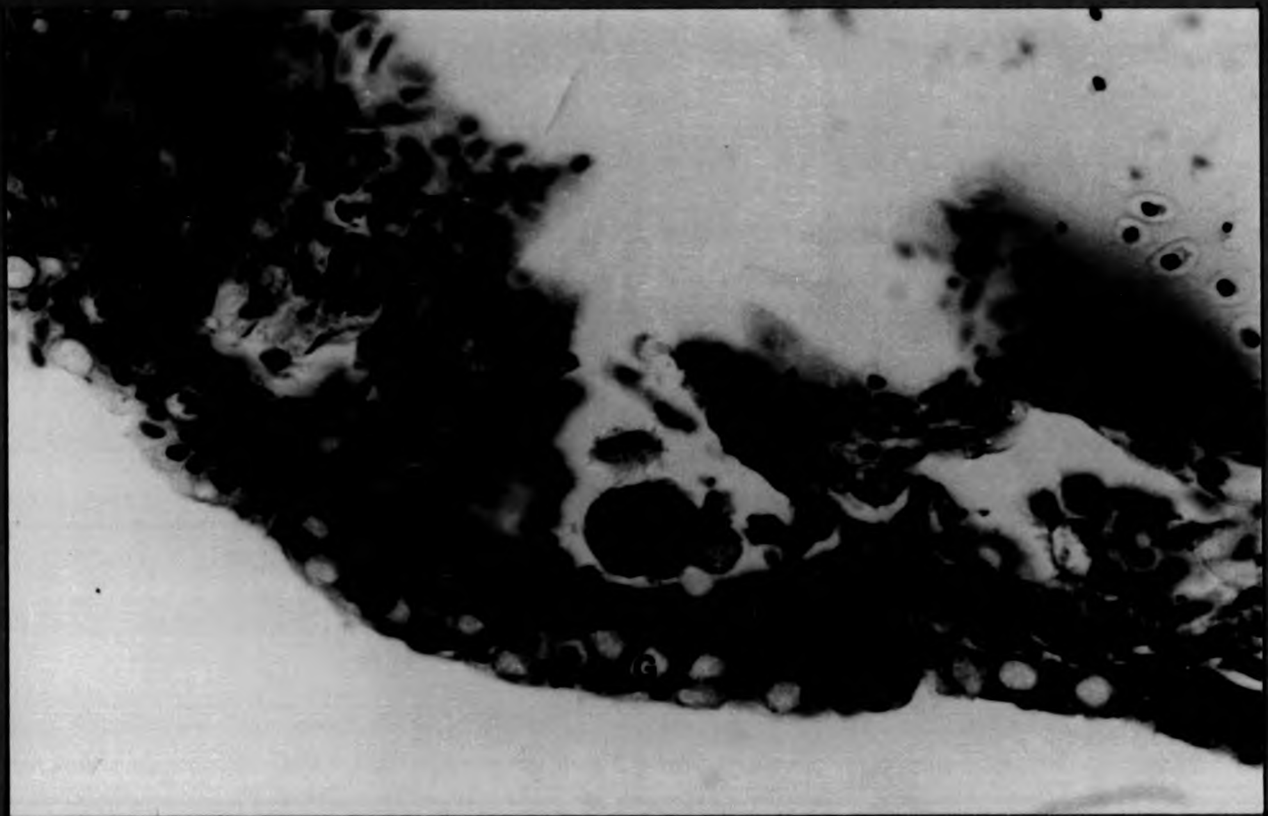
PN : Piknotic neuclei

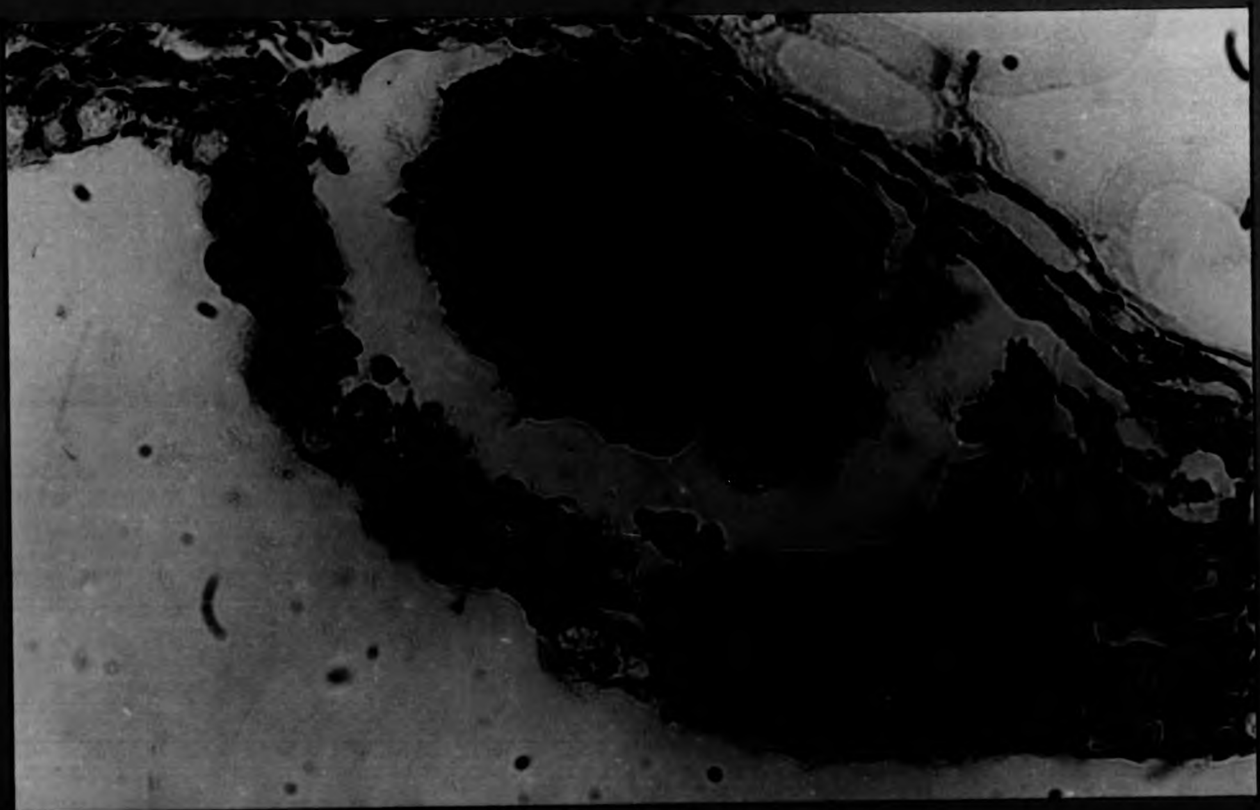
KL : Karyolysis

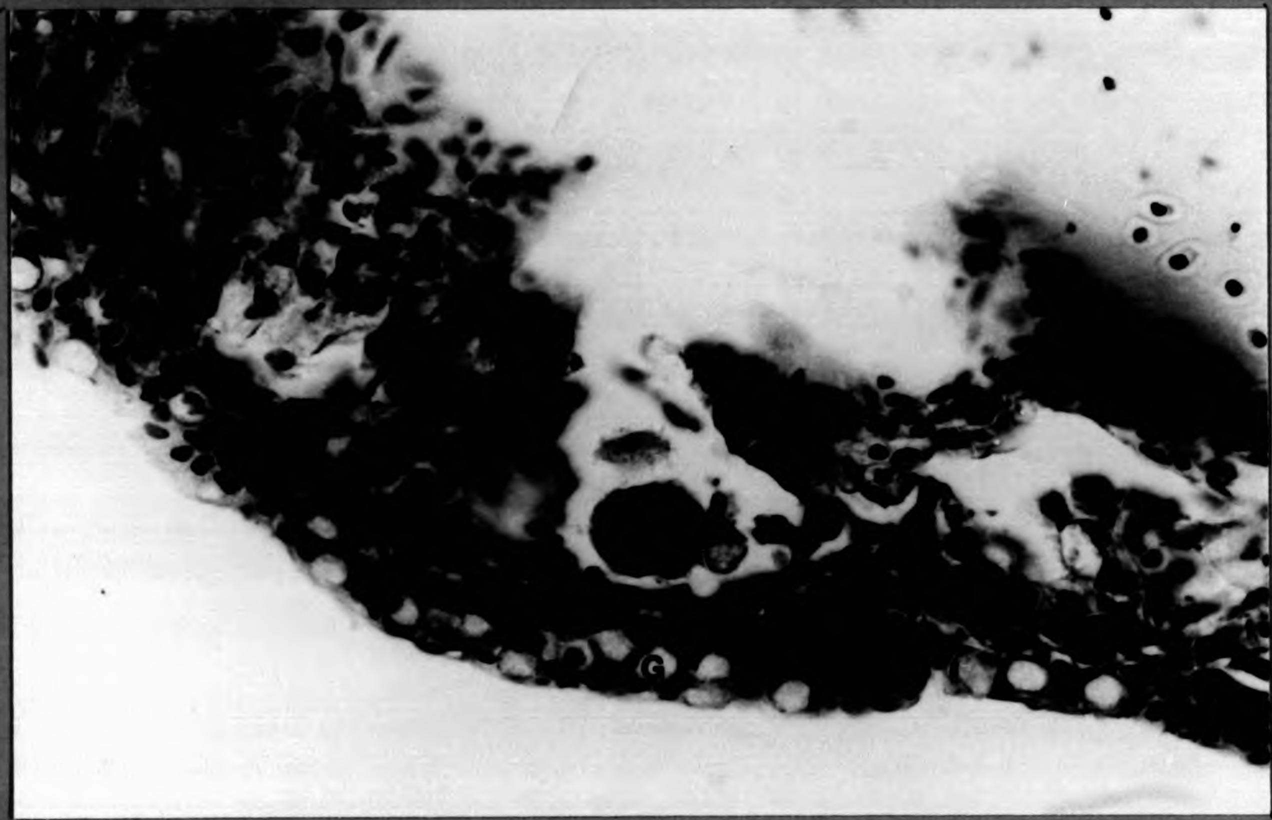
KR : Karyorrhexis

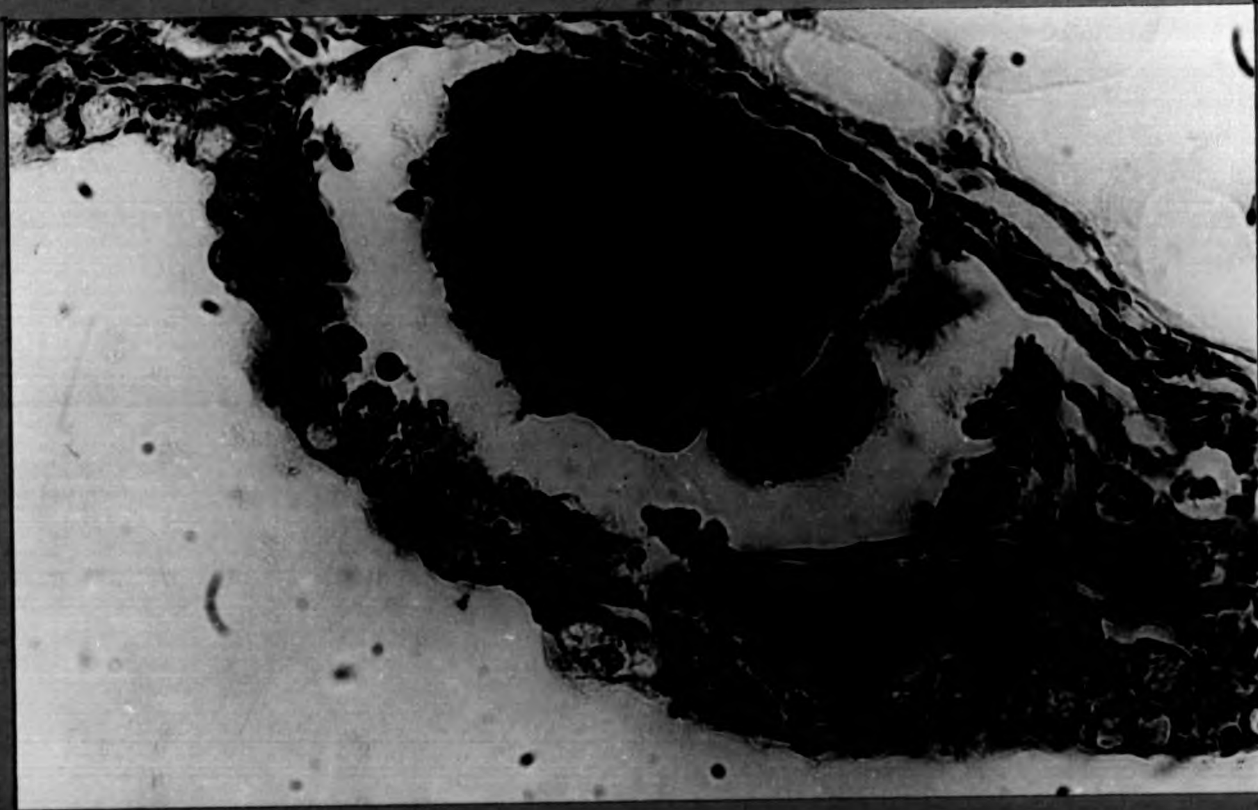
NC : Necrotic changes

G : Goblet cells









base of the origin of the secondary lamellae and was generally 2-4 cells thick. The secondary lamella consisted of an envelope of epithelial cells usually one layer thick supported and separated by pillar cells which are arranged in rows (Figure 57).

Twelve hours post exposure, small parasites were visible within the epithelial layers of the primary lamellae. No sign of host tissue disruption was evident.

After 24 hours of exposure the parasites were found on the basement membrane within an empty space. However, no app arent pathological changes were noted histologically.

By 2-4 days post exposure, generalised hyperplasia, together with hypertrophy of primary and secondary lamella epithelial cells, was visible amongst the individuals which carried heavy infections (Figure 58). In contrast, mildly infected individuals showed no sign of tissue damage. The parasites were found to be settled between two adjacent secondary lamellae and here there was evidence of gradual fusion of the adjacent secondary lamellae to form a cellular enclosure for the growing parasite (Figures 59 and 60). In extreme cases, this enclosure appeared to be multilayered as a result of fusion of more than one secondary lamella.

Eight to twelve days post exposure,

Figure 57.

Gill section of an unexposed fish. H&E X 250.

P : Primary lamella

S : Secondary lamellae

Figure 58.

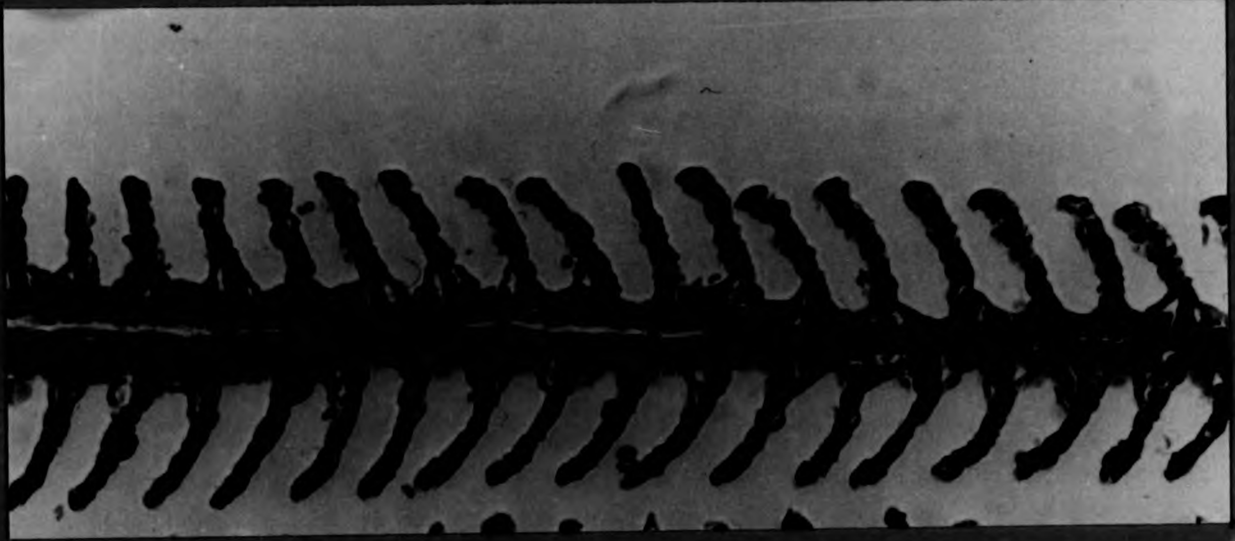
Gill section of a fish 2 days post exposure to *I.multifiliis*. H&E X 250.

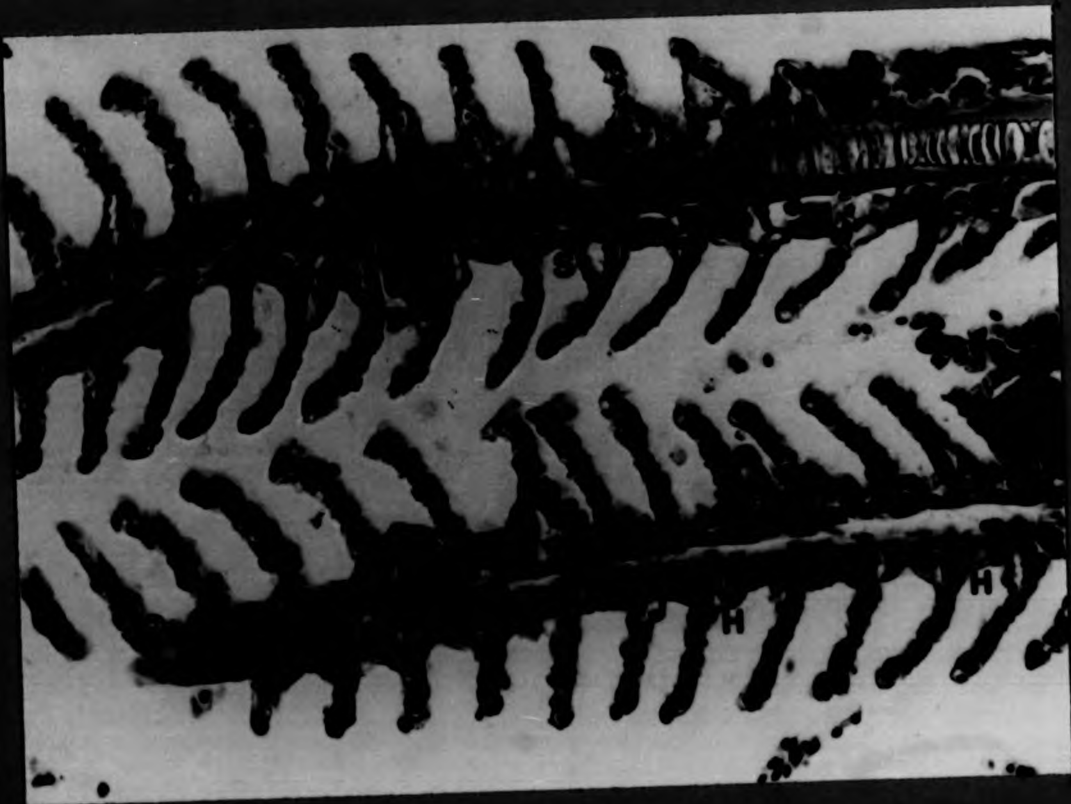
Note separation of secondary lamellae epithelium, hypertrophy of primary lamellae epithelial cell

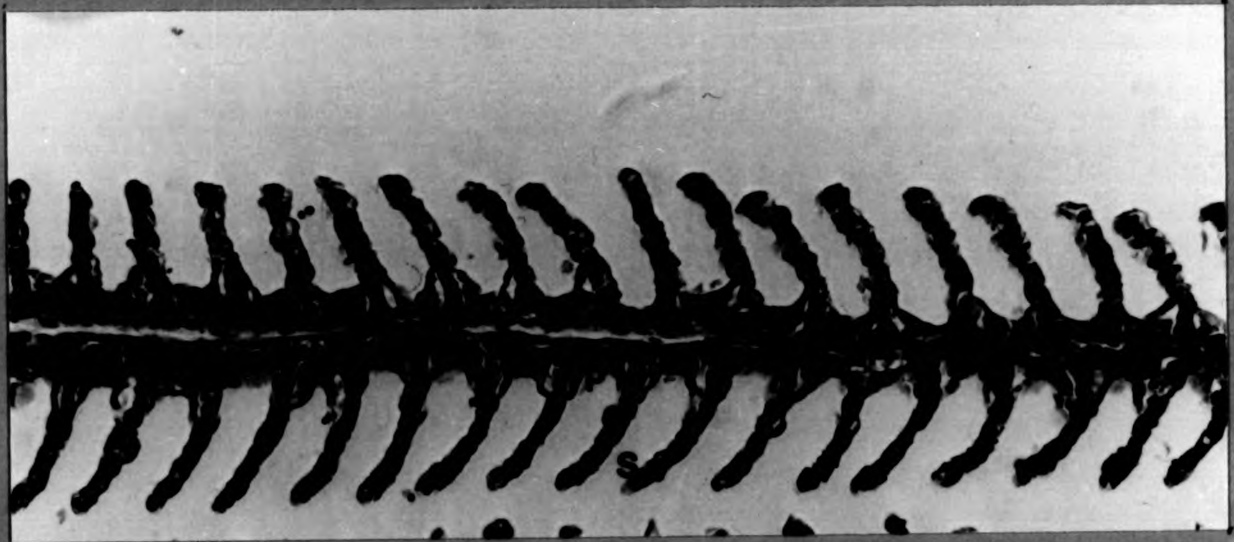
H : Hypertrophic epithelial cells

S : Separation of epithelium

P : Parasite







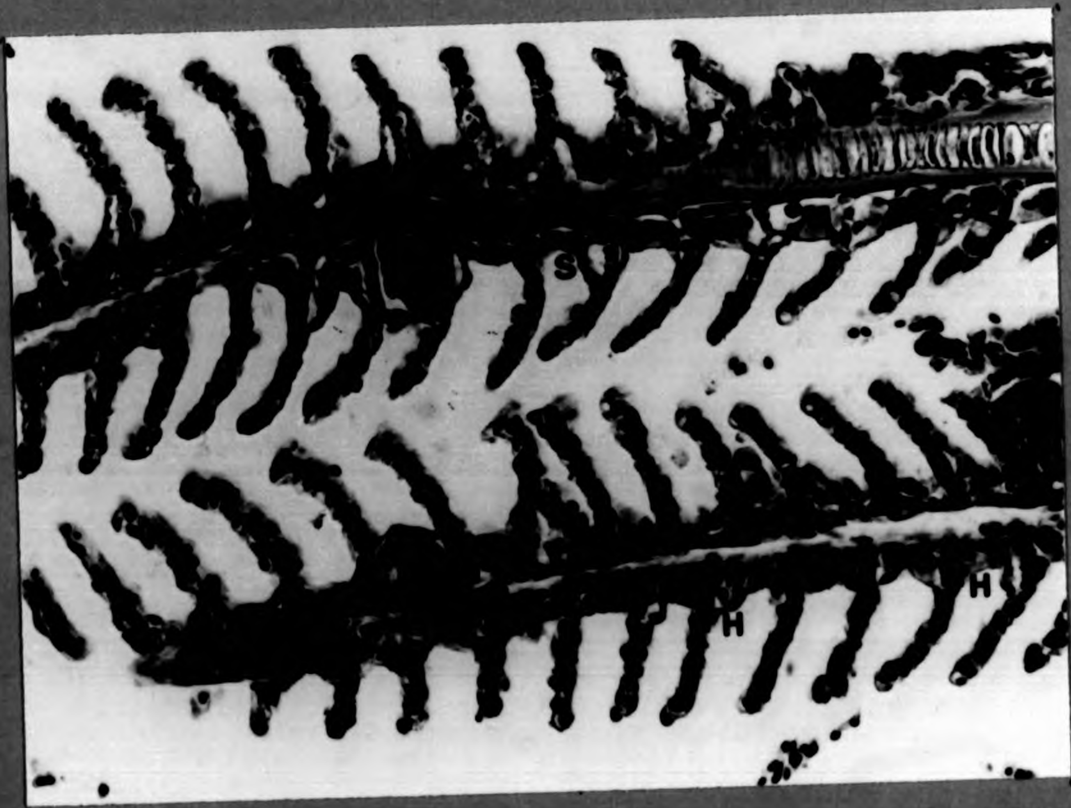


Figure 59.

Gill section of a fish 3 days post exposure to I.multifiliis. H&E X 400.

Note separation of secondary lamellae epithelium, hyperplasia of primary lamellae epithelial cells and fusion of adjacent secondary lamellae.

H : Hyperplastic epithelial cells

S : Separation of epithelium

P : Parasite

F : Fusion of secondary lamellae

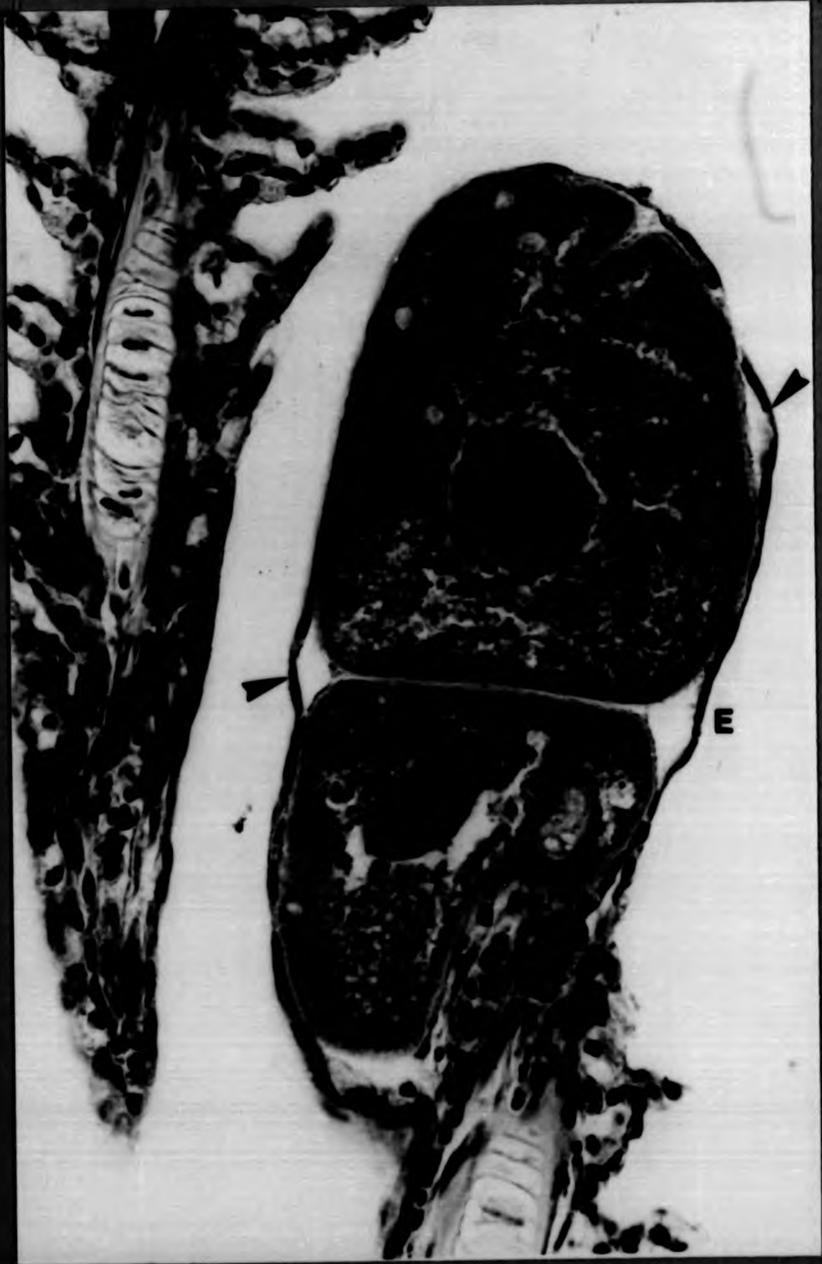
Figure 60.

Gill section of a fish 4 days post exposure to I.multifiliis showing complete fusion of lamellae to form an "enclosure" for the growing parasite. H&E X 400.

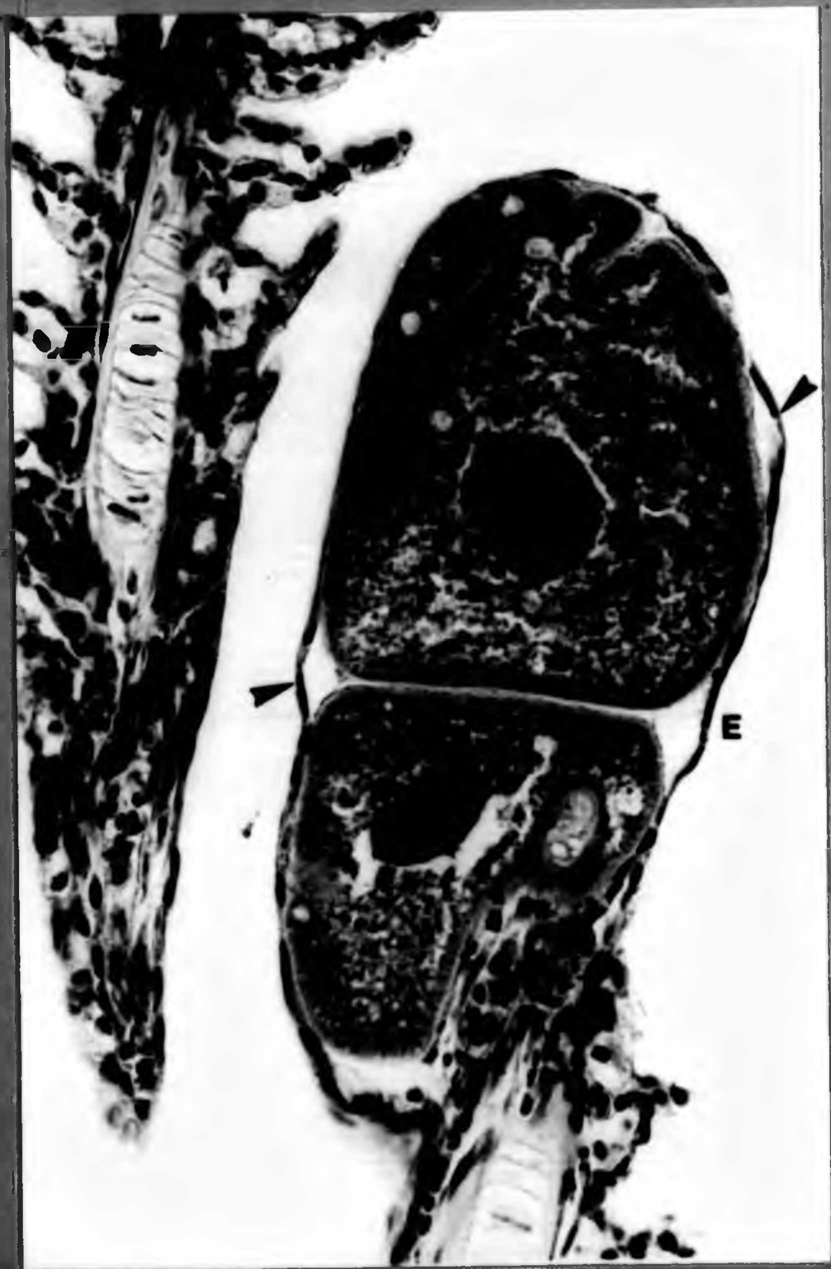
P : Parasite (Note two individuals)

E : Epithelial enclosure

F


E

F


E

extensive hyperplasia and fusion of secondary lamellae was more frequently observed and eosinophilic granular cells were present in such areas. Cellular degeneration, as shown by nuclear pyknosis and karyorrhexis was clear in certain samples. However, these cellular changes were not common. Trophonts were sometimes observed immediately above the basement membrane of the lamellae or, in some specimens, the parasites were found above the basement membrane leaving an empty space underneath.

No histopathological changes were observed in any other organs.

8.4 DISCUSSION

The results of the present investigation indicate an age dependent susceptibility to I.multifiliis infections in O.mossambicus. Similar age dependent infection and susceptibility to Ichthyobodo necator in S.gairdneri was demonstrated by Robertson (1979). The fry used in the present study were very small, especially the four and eight day old fry, and have an approximate growth rate of 13% per day at 24'C (Chapter 4). They grow rapidly in size from the yolksac stage, the surface area increases tremendously with time as fry increase in size. It appears that this size difference, reflected by the age, may have contributed to the observed differences in susceptibility to the parasite.

The larger the surface area the greater the number of parasites which can establish on an

individual fish. At a constant dose level and exposure time the number of parasites which establish on a unit surface area should be lower in larger fish than in smaller fish. On the other hand, the number of parasites established on hosts subjected to different numbers of tomites was linearly dependent on the number of tomites added to the infection arena (McCallum 1982). Similar linear relationship was also found for the digenean Transversotrema patialense and its fish host (Anderson, Whitfield and Dobson 1978). The results of the present investigation supports this relationship in parasite establishment as reflected in dose dependent mortality in the eight and thirty day old fry. However, the irregularity in the mortality pattern observed in the four day old fry needs further explanation.

Generally, four day old O. mossambicus fry are very small (approximately 4mm in total length). It is possible that small size may limit the parasite burden. Anderson (1978) stated that a host has only a finite capacity for infective stages, which will eventually limit the parasite burden possible. I. multifiliis tomites are so small (approximately 20-30um) that for any but the smallest fish, the capacity would be extremely large. However, the four day old O. mossambicus fry are very small and this phenomenon may have operated during these infections and may account for the uneven parasite burden as reflected by

the irregular pattern of mortality amongst the group.

Four day old fry are different from the rest of the fry used as they are incapable of swimming. Their swim bladders are not inflated and they generally stay on the bottom of the aquarium showing only a little twitching movement. If the host finding mechanism of the tomites is entirely by chance, the possibility of establishment of the parasite in four day old fry would not have been different from the other groups of fry used in the infection trials. However, these differences in behaviour of the four day old fry should be the same for all the dose levels tested. Therefore, it seems likely that the heterogeneity of the mortality pattern observed in the four day old group of fry is related to the poor swimming and escape capabilities.

Hyperplasia, epithelial cell hypertrophy and mild degenerative changes were observed in the histological material examined in the present study. Though these features were described by Hines and Spira (1974a) and McLay (1985) the extent of the damage observed during the present study was not as severe as that described by them. Ventura and Paperna (1985) reported that the pathological conditions described by Hines and Spira (1974a) occur only in extremely heavy infections induced experimentally or in particularly severe epizootics. Hines and Spira (1974a) in their study used a 24 day lethal dose level of 400 trophonts/fish whilst McLay (1985) employed a

30 day lethal infection level of 5000 tomites/l. In contrast the present study utilised a dose level of 200-300 tomites/fish, a fifteen day sublethal infection level. Though the direct comparison of the dose levels used is not possible as a result of the differences in measures employed, it is clear that the dose level used in the present study was considerably lower than that by Hines and Spira (1974a) and McLay (1985). The absence of several histopathological changes such as spongeosis and inflammation of underlying musculature, as observed by Hines and Spira (1974a) and MaLay (1985), is likely to be associated with the differences in dose levels.

As noted earlier a long-standing host parasite relationship could evolve to a state of equilibrium where there is little or no damage caused to the host by the parasite. Nigrelli _et al_ (1976) referred to a similar situation as "neutral parasitism". This could facilitate the maintenance of a high degree of compatibility between the host and the parasite, especially in primary and secondary infections (Ventura and Paperna 1985). However, such an event would operate normally under natural conditions; in artificial, experimental situations, this compatibility could be lowered. If the initial infection is mild, this compatibility might be maintained even under artificial, experimental conditions. If this kind of interaction operates then the effect of

parasitism would not be detrimental to otherwise healthy fish.

Roberts and Bullock (1976) and Pickering and Richards (1980) in their reviews on fish epidermis reported that conditions such as inadequate water quality, hormonal stimuli and microbial infections are associated with hyperplastic responses in teleost fish. Ellis (1981) considered hyperplasia as a nonspecific protective response, which minimises the chances of epidermal disruption in the presence of irritant conditions. In Ichthyophthiriasis where the parasites are generally located on the basement membrane, this hyperplastic response could be of extra importance to the parasite. Especially in the epidermal areas where the thickness of the epithelial layers is small (eg. 1-3 cells thick), the growth of the parasite could cause rupture of the epidermis, which eventually exposes the parasite to the external environment. This would be detrimental to the parasite. Therefore, even though one considers the hyperplasia as a nonspecific protective response by the fish, in Ichthyophthiriasis, the parasites seem to benefit from this nonspecific host response. Perhaps this may indicate an advanced nature of parasitism which is capable of taking advantage of the host response.

Robertson, Roberts and Bullock (1981) speculated that the first response to *I. necator* by

S.gairdneri was the exhaustion of the mucus from the goblet cells and their subsequent sloughing. However, this phenomenon was only evident in extremely heavy infections in O.mossambicus. Therefore, it may be a feature of an intense, heavy infection.

The lack of extensive dermal and branchial histopathology in the present investigation gave rise to the question "Is this low level of response a feature of tilapines ie. O.mossambicus or could it be just a reflection of the level of infection employed?". In order to try to answer this question histopathological examination of two 125g female O.mossambicus exposed to a dose level of 20,000 tomites/fish was carried out 15 days after initial exposure. Almost all of the extreme histopathological changes observed by previous workers such as Hines and Spira (1974a) and McLay (1985) were convincingly evident, including sloughing of epithelium, spongeosis and oedema. However, no underlying muscular hypertrophy as described by Hines and Spira (1974a) was noted. Therefore, it was considered that the degree of histopathological changes observed during the present study was merely a result of a low dose infection rather than any specific differences which could be attributed to the tilapine fishes. However, it was evident that, to produce similar hisopathologcal changes in the dermal and branchial epithelium of O.mossambicus, a considerably higher dose

level of infection would be needed than for C.carpio and S.gairdneri.

Hines and Spira (1974a) observed pathological changes in the liver such as gradual devacuolization of cytoplasm of the hepatocytes and focal areas of of early necrosis. They attributed these changes to the mobilisation of fat reserves, perhaps as a result of starvation. In contrast McLay (1985) attributed the observed degenerative necrosis of hepatic tissues to an increase in secondary lysosomal bodies in the hepatocytes resulting in impaired lysosomal function. The present study did not reveal any histopathological changes in the liver tissue of experimentally infected fry. No mobilisation of fat or glycogen reserves was necessary as all the fish continued feeding until the experiment was terminated. If the level of infection used had been very high, similar histopathological changes as described by Hines and Spira (1974a) and McLay (1985) might have been expected in O.mossambicus fry.

CHAPTER 9

IMMUNOLOGICAL STUDIES OF *I.multifiliis* INFECTED *O.mossambicus*.

9.1 INTRODUCTION

Fish have been shown to develop immunity to lethal *I.multifiliis* infections following sublethal exposure to the parasite (Beckert and Allison 1964; Parker 1965; Hines and Spira 1974c; Beckert 1975). Fish vaccinated by the injection of killed *I.multifiliis* antigen have also been found to be protected from infection (Parker 1965; Beckert 1975). Recently Goven, Dawe and Gratzek (1980) showed that not only *I.multifiliis* antigen but also the antigen prepared from killed *Tetrahymena pyriformis* (Lwoff), a related but free living ciliate, can also protect fish from fatal *I.multifiliis* infections. They indicated that the ciliary properties of this organism were involved in the initiation of the antibody production. Later they investigated the antigenic relation between *T.pyriformis* and *I.multifiliis* using *invitro* immunological tests (Dickerson, Brown, Dawe and Gratzek 1984). They concluded that an antigenic relationship does exist between these two organisms and the cross reacting antigens appeared to be localised in the cilia. The advantage of this heterologous system for the protection of fish from

I.multifiliis by immunisation was soon realized, since T.pyriformis is readily cultivable. Wolf and Markiw (1982) successfully immunised S.gairdneri against I.multifiliis by immersion immunisation using Tetrahymena thermophila. This observed success in immunising fish against I.multifiliis was considered an important advance relevant to the growing tilapia culture industry.

In general, tilapia appear to be more resistant to ectoparasitic diseases than some other warm and cold water cultured fish (C. Sommerville pers. com.). However, very little is known about the ability of cichlids, including tilapias to mount an immune response to pathogens.

Sailendri and Muthukaruppan (1975) studied the immune response of O.mossambicus to cellular and soluble antigens using bovine serum albumin and sheep red blood cells. Mohan (1977) investigated the mechanisms of cell mediated and humoral immune response in O.mossambicus. Later Jayaraman, Mohan and Muthukaruppan (1979) assessed the cell mediated and humoral immunity to sheep erythrocytes in O.mossambicus using in vitro capillary migration inhibition (CMI) and plaque forming cell assay (PFCA) respectively. However, there seems to be no reports on the ability of tilapias to acquire immunity to specific pathogens. Therefore, it was considered important to investigate whether tilapias are able to mount an immune

response to pathogens such as I. multifiliis, which they confront under both natural and artificial conditions.

When considering the mouth brooding species of tilapias which carry their young in the buccal cavity, it is particularly interesting to investigate to what extent, if any, this method of brooding might contribute to the protection of the progeny. I. multifiliis appeared to be a convenient model system to investigate this possibility. The information generated from such an investigation would initiate an interesting area of applied research with implications for the developing industry of aquaculture. The present study was therefore, designed to elucidate

I. the ability of O. mossambicus to acquire immunity to I. multifiliis and

II. to investigate the possibility of conferred protection against I. multifiliis by the immunised mothers to the progeny whilst brooding.

9.2 MATERIALS AND METHODS

9.2.1 Source of fish.

Male and female O. mossambicus of 1 year+ and measuring 16.3cm (range 16.1-16.7cm) in standard length and weighing 168.4g (range 158.3-179.6g) were used during the present investigation. These fish belonged to the brood

stock described in Chapter 2 section 2.1. They had not been previously infected with I.multifiliis. All the females used were continuous and reliable spawners and carried complete spawning records including the date of spawning, clutch size and fertility rate. The fish were maintained in square glassfibre tanks prior to the experimental infections as described in Chapter 2 section 2.2.

9.2.2 Source of parasite

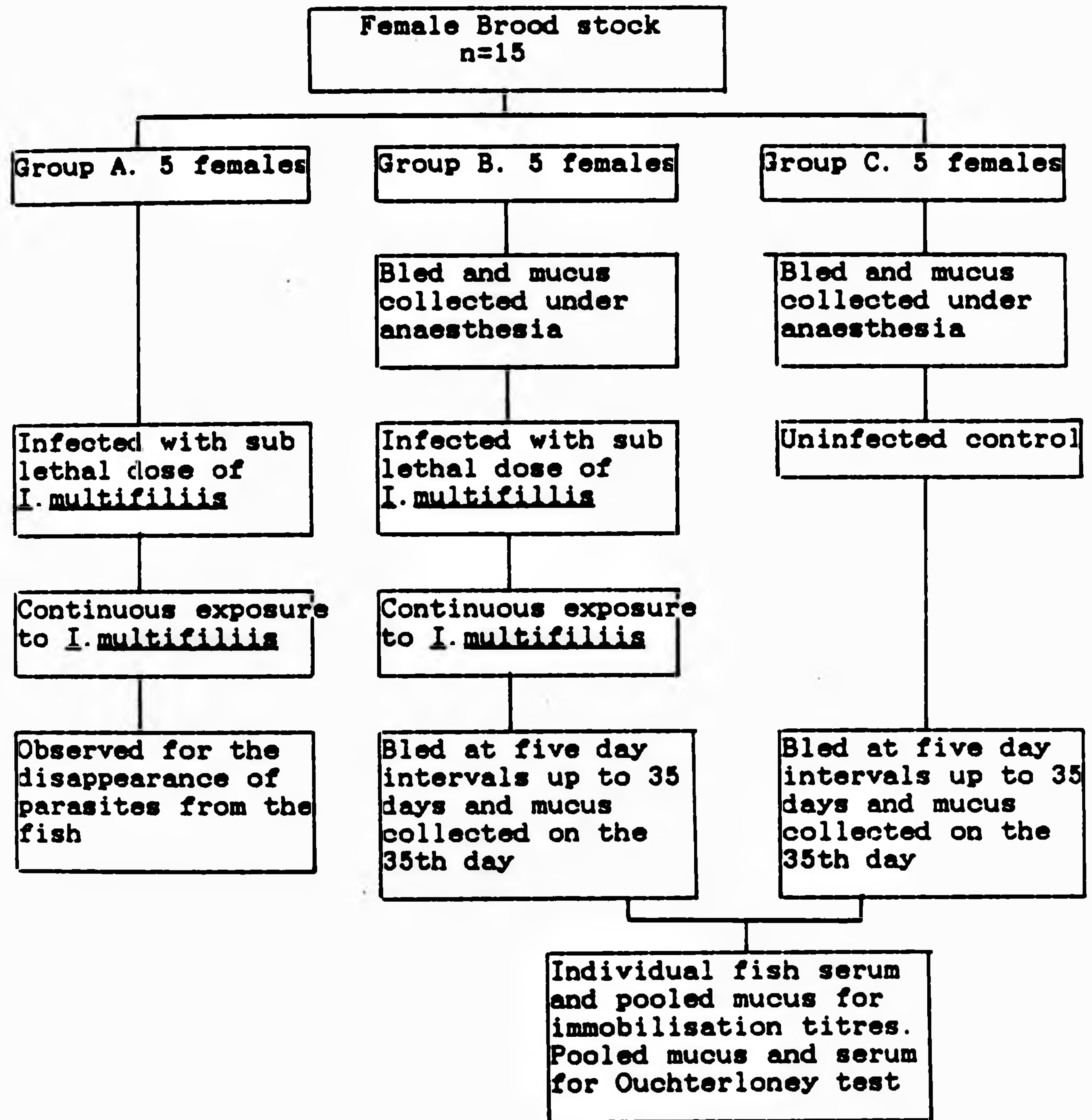
The parasite isolate denoted RS/CM/84 (chapter 8) was utilised throughout the experiment. All the experimental infections, including challenge infections were carried out using tomites aged 6-12 hours. The procedure used for the collection of parasites and the preparation of tomite suspensions were described in chapter 8 section 8.2.3.1. The infection procedures are described under the relevant sections below.

9.2.3 Experimental procedure

The experimental procedure is illustrated in Figures 61 and 62. The experiment was divided into three major components namely, the investigation of acquired immunity, the investigation of possible fry protection by nonimmunised females and by immunised females which are described in sections 9.2.3.1, 9.2.3.2 and 9.2.3.3.

Figure 61.

Flow chart showing the experimental procedure used for the investigation of acquired immunity.



Female Brood stock n=15
Group A. 5 females
Group B. 5 females
Group C. 5 females
Bled and mucus collected under anaesthesia
Bled and mucus collected under anaesthesia
Infected with sub lethal dose of I. multifiliis
Infected with sub lethal dose of I. multifillis
Uninfected control
Continuous exposure to I. multifiliis
Continuous exposure to I. multifiliis
Observed for the disappearance of parasites from the fish
Bled at five day intervals up to 35 days and mucus collected on the 35th day
Bled at five day intervals up to 35 days and mucus collected on the 35th day
Individual fish serum and pooled mucus for immobilisation titres. Pooled mucus and serum for Ouchterloney test

9.2.3.1 Experimental infections of the females for the investigation of acquired immunity.

Fifteen brood fish were used in this study. They were divided equally into three groups A,B and C. Group A was used for experimental infections. Group B was used for the experimental infections and the collection of mucus and blood. Group C was used as an uninfected control and was also used for collection of mucus and blood.

Four days before the groups A and B were experimentally infected, skin mucus was collected from the fish in the groups B and C. The mucus collection and concentration procedures are described in Chapter 7 section 7.2.2 and 7.2.3. Once the mucus was collected, the fish were also bled by means of cardiac puncture and 0.3-0.5 ml of blood was removed from each fish. The procedure used for the bleeding and collection of serum is described in section 9.2.4.

The three groups of fish were then placed in separate glass aquaria (5'x1.5'x1.5') with 60 litres of water. Each aquarium was aerated individually and a constant temperature of 27+1'C was maintained. Groups A and B were then exposed to a dose level of 10,000 tomites/fish. The dose level was sublethal and was obtained from the experiments described in chapter 8. Group C remained uninfected. All three groups of fish were fed at 1% body weight/day with commercial trout pellets. At the 12th,

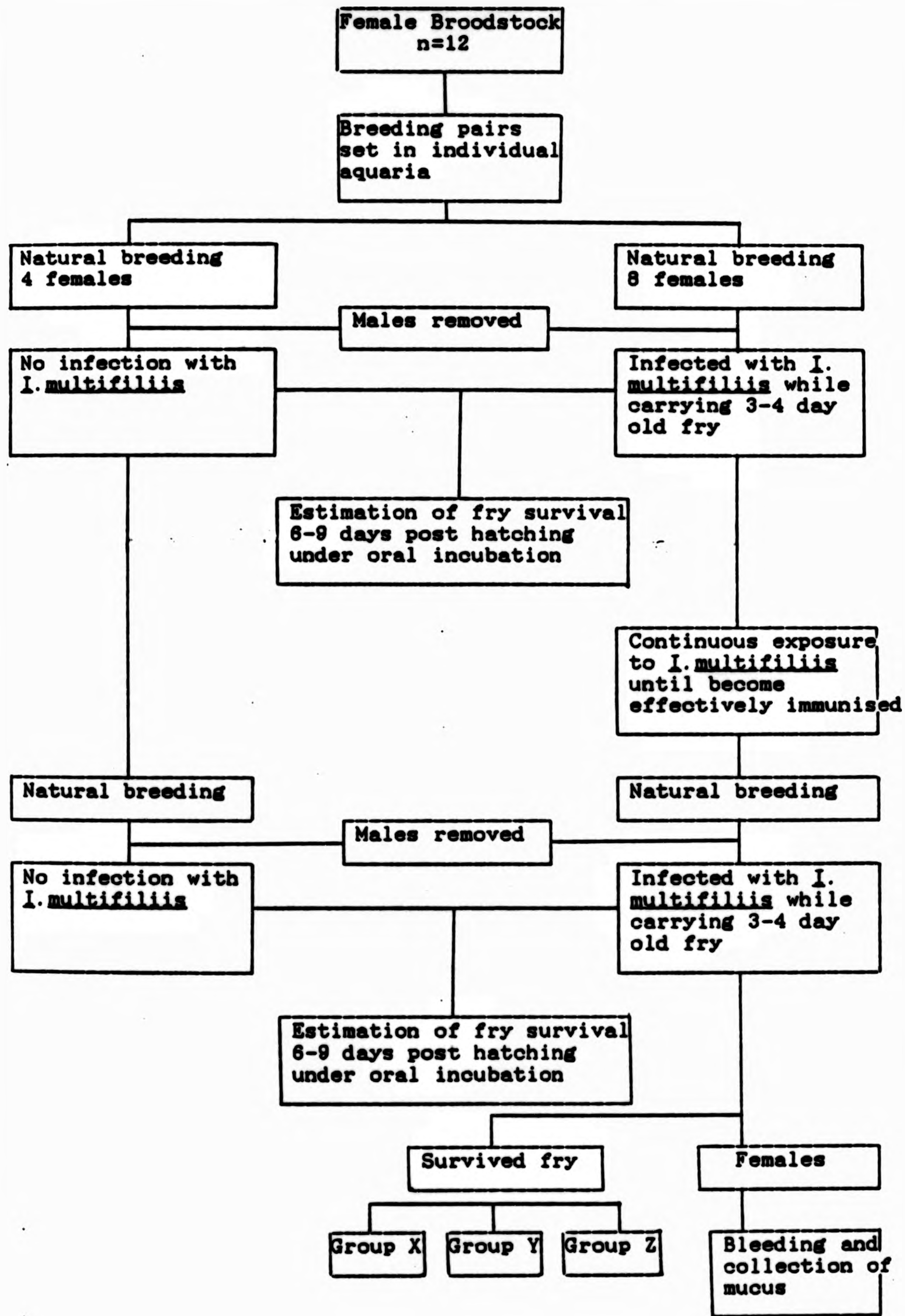
24th and 30th days after the initial infection, a tomite suspension calculated to produce a dose level of 10,000 tomites/fish, was added to the aquaria containing groups A and B fish. This was carried out to maintain a standardized continuously infectious environment. Partial water changes were performed in all tanks when necessary. Possible removal of tomite stages from the tanks was minimised by monitoring the parasite life cycle and coordinating the timing of the partial water changes with the stages at which the parasite was either encysted on the substrate or embedded in the fish. Fish in groups A and B were closely observed on a daily basis for the presence of visible I.multifiliis on the skin and fins. Once there was no visible evidence of parasites, five daily skin scrapings were carried out on individual fish to confirm the absence of the parasite.

The fish in group B were individually bled at 5, 10, 15, 20, 25, 30 and 35 days after the initial infection and serum was collected. The skin mucus was also collected and concentrated on day 35 post infection. The uninfected control fish in the group C were also bled on the same days as those in group B and the skin mucus was collected and concentrated on day 35.

The experiment was terminated after 35 days. Two individual fish from group B were maintained in an infectious environment for a further 9 months to examine

Figure 62.

Flow chart showing the experimental procedure used for the investigation of the possible protection of fry.



Gp. X. Observed for the presence of parasites
Gp. Y. Preparation of fry homogenate
Gp. Z. Challenge infection with *I. multifiliis*

the long term acquired immunity.

9.2.3.2. Infection of nonimmunised mouth brooding females to investigate the possible protection of fry.

Twelve broodstock females were used in this experiment. When the females were ready to spawn (as determined by the external appearance of the genitalia and the behaviour of the female), they were removed from the broodstock tanks and placed separately in square glass aquaria containing 40 l of water. Each aquarium was individually aerated and held at a constant temperature of 27+1'C. A fertile male was also introduced into each aquarium. When the females had spawned and were carrying eggs, the males were removed. When the eggs were hatched and the fry were 4-6 days old the individual mouth brooding females were infected with I.multifiliis by adding a suspension of tomites calculated to produce a dose level of 500 tomites/fish. The preparation of the tomite suspensions was carried out as described in Chapter 8 section 8.2.3.1. The dose level used was sublethal to the female but lethal to 4-6 day old fry. The levels were determined from the results of experimental infection trials carried out previously and are described in Chapter 8. The fish were observed daily. The entire tank was also observed closely for the presence of living or dead fry. Three days after infection the females were removed and the buccal cavity

was examined for the presence of fry.

This procedure was repeated for eight females. The remaining four females were subjected to the same experimental procedures but no tomite suspensions were added to the tanks. When the fry were eight days old ie. 12-13 days post spawning, the buccal cavities of the control females were also examined for the presence of fry.

9.2.3.3. Infection of immunised mouth brooding females to investigate the possible protection of fry.

The same twelve fish used in the section 9.2.3.2 were used in this experiment. The eight previously infected females were individually maintained in 40 l glass aquaria for six weeks. During this period at the 12th, 24th and 30th days of the initial infection a heavily infected 50-60 g O. mossambicus carrying 4-5 days old parasites was added to each aquarium, so as to ensure that the fish were kept continuously in an infectious environment. It was considered that a strictly controlled level of infection as used in the experiment to examine the development of the immune response was not necessary in this case since the aim of the infection was to ensure a continuous infectious environment. Experimental females were closely observed for the presence of I. multifiliis in the skin and fins and when no obvious parasites were visible random skin scrapings were regularly examined to confirm the absence of

I.multifiliis. The water quality of the tanks was closely monitored with particular attention being paid to pH and ammonia since these appeared to affect the fecundity and the development of the parasite (personal observations). Partial water changes were exercised when necessary.

During the course of this investigation the fish were fed with commercial trout pellets at 1% body weight per day. When there were no *I.multifiliis* found on the skin or fins of females maintained in an infectious environment they were considered to be "effectively immunised".

At the end of the six week period, the eight "effectively immunised" females were subjected to the same experimental procedures described in section 9.2.3.2. When the females were carrying 4-6 days old fry they were infected with a dose level of 500 tomites/fish (the same level as used in section 9.2.3.2). Three days after the infection the fish were removed and the buccal cavity was examined for the presence of fry.

The four uninfected control fish used in the previous experiment (section 9.2.3.2), were also used as the control group for this section. They were subjected to the same procedures but were not infected with *I.multifiliis*. Once they were carrying eight day old fry the buccal cavities were examined for the presence of fry.

9.2.3.4. Challenge infection of fry

O.mossambicus fry used in this section of the investigation were obtained from the fry collected at the end of the previous experiment described in section 9.2.3.3. The fry obtained from an individual female were divided into three groups X,Y and Z. Group X fry were killed and examined microscopically under high power to determine the presence of I.multifiliis. The group Y fry were used for the preparation of fry homogenates as follows.

Group Y fry from each of the eight females were placed into separate glass test tubes containing 5ml of PBS. They were carefully macerated using a glass rod and the suspension was centrifuged at 8000g for 15 minutes at 4'C. The supernatant from each tube was collected and concentrated as described in Chapter 7 section 7.2.3. These were stored at -70'C for future use.

The group Z fry were used for the challenge infections of I.multifiliis. The fry were subdivided into three groups with a minimum of ten fry per group; the actual group size depended on the number of fry available. The subgroups were subjected to a previously calculated lethal dose level of 640 tomites per fish. The dose level was calculated from the experiments described in Chapter 8. The infection procedure was as described in Chapter 8 section 8.2.3.2. As the fry used here were 7-9 days old,

the lethal level calculated for 8 day old fry was used for the challenge infections. Fry were observed for survival and mortality for three days.

9.2.4. Collection of blood.

The collection of blood was only carried out on anaesthetised live fish. The fish were anaesthetised using Benzocaine (Ethyl P. amino benzoate) at a final concentration of 100 mg/l.

Though it has been reported that the withdrawal of blood from the caudal vein was successful with O. niloticus (Olufemi 1984), preliminary trials carried out did not provide satisfactory results with this technique on O. mossambicus. It was found that using this technique took longer than cardiac puncture, thus causing greater stress to the fish. It was also found that, in some cases, the tail region of the fish posterior to the needle insertion became dark during and after collection of blood. This is most likely due to damage to the central nervous system. Because of these obvious reasons cardiac puncture technique was attempted for the withdrawal of blood and was found to be very successful. Considering the success rate, the accuracy and the consistency of the technique as well as the reduced stress to the fish, it was decided to use the cardiac puncture technique for the withdrawal of blood in the present study.

A one ml disposable tuberculin syringe fitted with either 23 or 25 gauge needle depending on the size of the fish was used. The heart was punctured from the ventral side of the fish. Only 0.3-0.4ml of blood was withdrawn from an individual fish at any one time. The whole process of bleeding was completed within 40-60 seconds after the fish had been immobilised by the anaesthetic. The blood was collected into 2ml plastic vials and was left overnight at 4'C. The serum was aspirated using a micropipette and was stored at -70'C for future use.

9.2.5. Immobilisation titres.

The serum and mucus collected from group B fish described in section 9.2.3.1 were used for the parasite (trophont) immobilisation titres. Twenty four well micro titre plates (Sterilin) were used and the procedures described by Sonneborn (1950) were slighty modified and employed. Appropriate serial dilutions of serum and mucus were prepared using PBS and 0.3ml aliquots were used in the wells. Ten freshly dislodged 4-5 day old trophonts were added to each well containing the serial dilutions of serum and mucus. The parasites were observed for motility over a period of 3 hours. These immobilisation tests were carried out separately for the five females used and each sample was replicated four times.

9.2.6. Preparation of I. multifiliis antigen.

A large number of 5 day old trophonts were dislodged from infected fish. They were placed in one 1 beaker with 500 ml of well aerated water. The beaker containing the trophonts was then incubated at 27'C for 24 hours. After 24 hours a sample of water from the beaker was examined for the presence of tomites. Then, 4-5 drops of 10% formalin was added from a pasteur pipette to the beaker containing tomites to kill them. The water containing formalin-killed tomites was decanted and centrifuged at 8000g for 15 minutes at 4'C. The supernatant was decanted and the residual collected. This tomite concentrate with a small amount of PBS was ultrasonicated and the resultant I. multifiliis whole cell antigen was stored at -70'C for future use.

9.2.7. Ouchterlony immuno-diffusion tests.

The serum and mucus collected from immunised and nonimmunised females was used for the immuno-diffusion tests. Agar gel plates were prepared using Nobel Agar (Difco) in 7cm diameter disposable plastic petridishes. Wells were cut using a hole punch. The double diffusion test was carried out using serum and mucus extracts from immunised and nonimmunised females against I. multifiliis whole cell antigen.

At the end of the experiment described in

section 9.2.3.3. the immunised fish were bled and the serum was separated. Also mucus was collected and concentrated. These serum and mucus concentrates were also used in the immuno diffusion tests. The fry homogenates prepared from the group Y fry described in the section 9.2.3.4 were also used for the Ouchterlony test against I. multifiliis whole cell antigen.

9.3. RESULTS

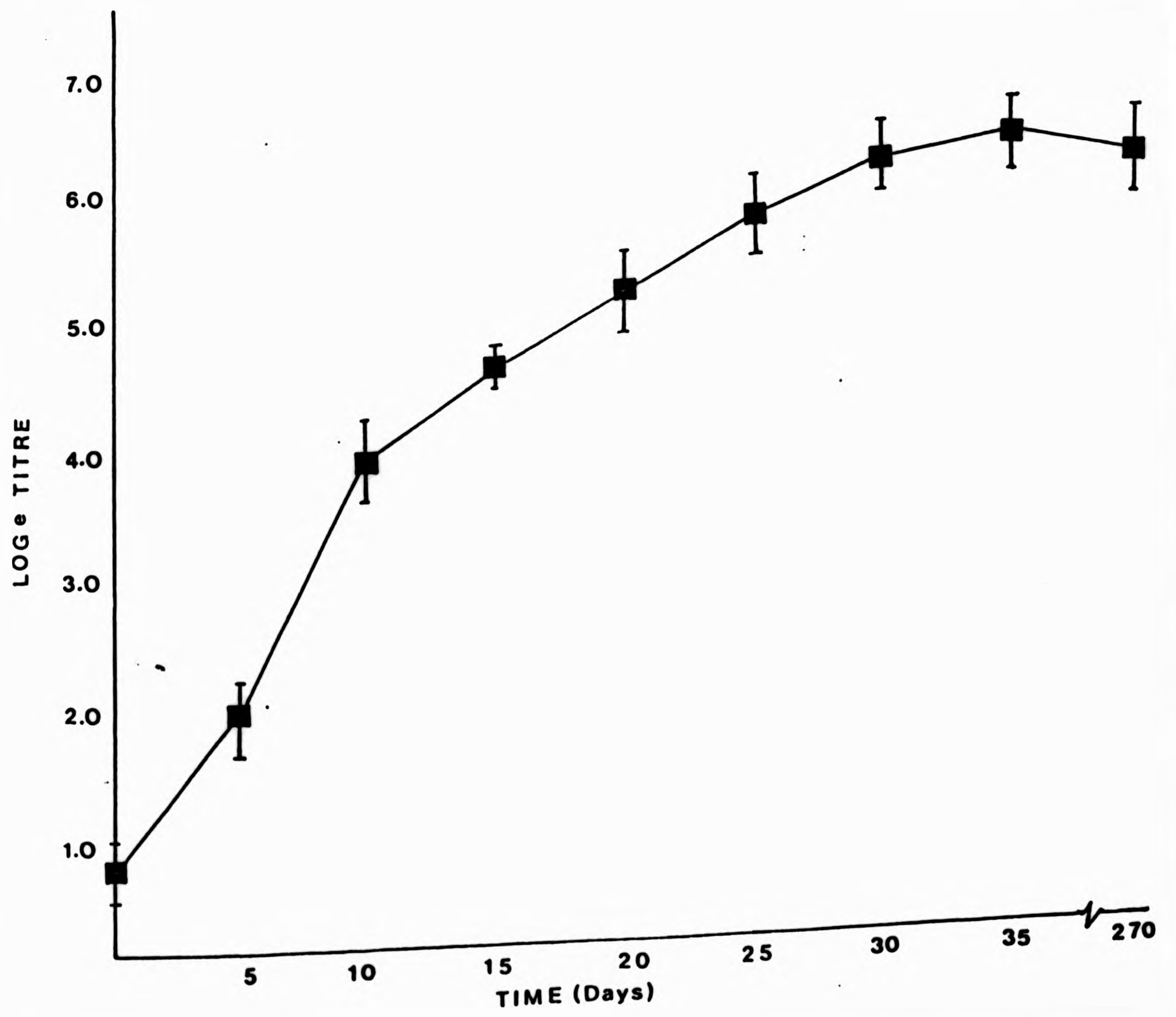
9.3.1. Investigation of acquired immunity in adult females.

All five fish in group A were visibly infected with I. multifiliis 4-5 days post infection. The visibility of the parasites began to decrease rapidly from day 12, and by day 14-15 no parasites were visible. The examination of skin smears from the fish revealed that the parasites had completely disappeared by day 17-19 post infection. All fish remained free of I. multifiliis under infectious conditions until the experiment was terminated.

Figure 63 represents the relationship between the log e mean serum immobilisation titres of the five fish in group B exposed to a sublethal infection of I. multifiliis, and time. The titres rose from day 0 onwards with a maximum rise between day 5 and 10 post infection. The titre reached a maximum of 6.23±0.49 at day 35 post infection. The immobilisation titre estimated from

Figure 63.

Graph showing the relationship between the log'e serum immobilisation titre and time of fish exposed to *I.multifiliis*. Bars indicate the standard deviation.



LOGe TITRE
7.0
6.0
5.0
4.0
3.0
2.0
1.0
5
10
15
20
25
30
35
270
TIME (Days)

the two females from group B, 270 days post infection, kept under infectious conditions gave a log e titre of 6.09±0.30 (Figure 63).

Table 13 describes the parasite immobilisation strength of the mucus and serum from the fish used in groups B and C. The serum and mucus extracted from the group B fish at day 35 post infection and the group C uninfected control fish were used here. The nonimmune control fish serum (group C) resulted in less than 50% immobilisation at all dilutions below 1:2. In contrast, the immune fish (group B) serum showed 50% or more immobilisation at all dilutions below 1:1024. The pooled concentrated mucus from the nonimmune control fish showed no degree of immobilisation whilst pooled concentrated mucus from the immunised fish (group B) gave 50% immobilisation at 1:2 dilution.

Figures 64-65 show the results of the Ouchterlony double diffusion tests carried out using immune and nonimmune pooled serum and pooled mucus against ultrasonicated *I. multifiliis* whole cell antigen. The pooled mucus and serum collected at day 35 post infection from the groups A and B fish and noninfected group C fish were used here. No precipitating lines were observed from serum and mucus of nonimmunised control fish. In contrast, both pooled immune serum and mucus gave positive precipitating lines against the antigen. There was no indication of more

Table 13.

Parasite immobilisation strength of the serum and mucous of five effectively immunised *O. mossambicus*.

Sample	Dilution	Immobilisation				
		Fish1	Fish2	Fish3	Fish4	Fish5
Non-immune serum	1:1	****	***	***	***	***
	1:2	**	**	**	**	**
	1:4	*	*	*	-	-
	1:8	-	-	-	-	-
Immune serum	1:128	****	****	****	****	****
	1:256	****	****	****	****	****
	1:512	****	***	****	****	****
	1:1024	***	**	***	***	***
	1:2048	*	-	*	*	*
Non-immune pooled mucous	1:1	-				
	1:2	-				
Immune pooled mucous	1:1	**				
	1:2	**				
	1:4	*				
	1:8	-				

**** = 100% immobilisation in 3 hrs.

*** = 75% do

** = 50% do

* = 25% do

- = no immobilisation in 3 hrs.

Figure 64 and 65.

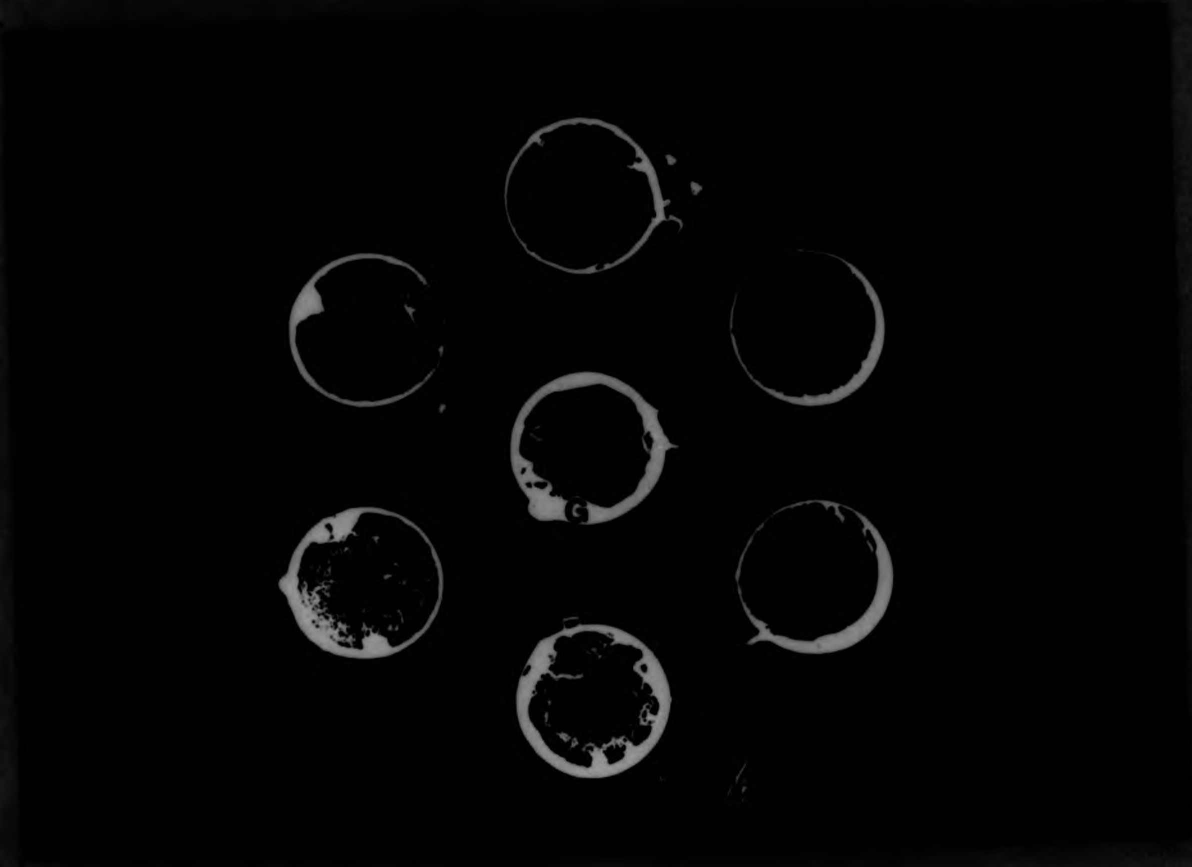
Photographs showing the results of the Ouchterlony immuno-diffusion tests.

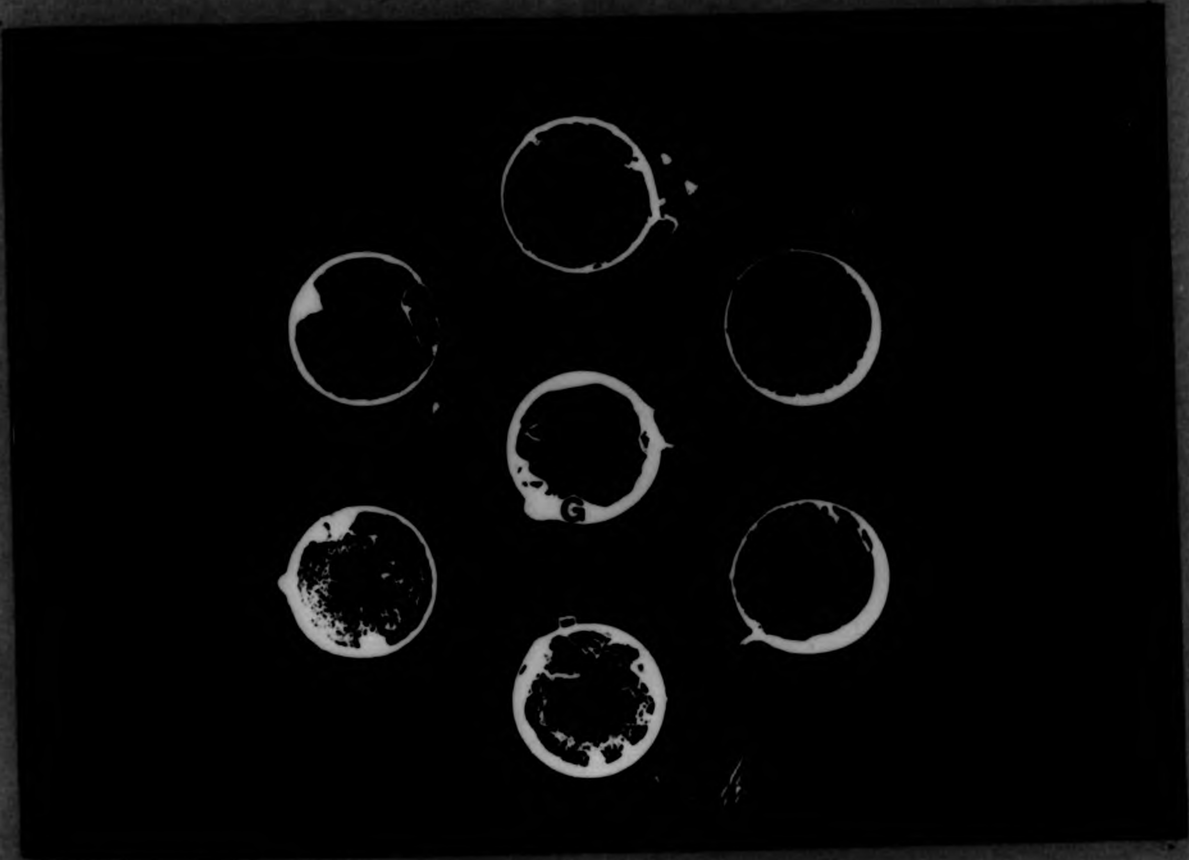
A and B : Pooled immune mucus

C and D : Pooled immune serum

E and F : Pooled fry mucus

G : I.multifiliis whole-cell antigen





than one precipitating line between any serum or mucus sample and *I. multifiliis* antigen. The precipitating lines carried their identity within serum and mucus.

9.3.2. Investigation of the protection of fry.

The history of the last ten spawns from the twelve females used in the investigation is given in Table 14. The clutch size and the number of fry recovered after three days post hatching under artificial incubation, was not significantly different ($P<0.05$) between the twelve females. Therefore during this investigation it was assumed that the individual females would produce a number of fry similar to the mean number of fry observed for their previous ten spawns.

During the course of the investigation each female was spawned twice. The number of fry collected from the buccal cavity of noninfected control females at both spawns, was not significantly different from the calculated mean number of fry collected over the previous ten spawns ($P<0.05$). In contrast, no fry survived from any of the females subjected to the first infection of *I. multifiliis* during the incubation period.

Table 15 records the observed and estimated fry survival from the previous ten spawns in control and infected females. As the difference in fry survival between the control and infected females was self evident,

TABLE 14

Spawning history of the twelve female O.mossambicus used during the investigation of possible fry protection.

The mean values calculated for the 10 previous spawns.

Fish no.	Mean clutch size (SD)	Mean % fertility (SD)	Mean fry survival (SD)
1	493 (76.2)	78.3 (15.9)	307 (59.5)
2	502 (86.1)	84.6 (9.7)	336 (57.0)
3	477 (58.7)	84.6 (10.6)	325 (61.3)
4	460 (73.6)	81.7 (10.4)	311 (70.5)
5	443 (76.3)	79.7 (19.5)	295 (108.9)
6	455 (66.7)	75.7 (10.2)	285 (63.3)
7	464 (113.0)	77.8 (16.8)	297 (118.0)
8	474 (126.8)	77.2 (9.5)	305 (108.8)
9	458 (95.0)	85.7 (9.8)	315 (75.4)
10	463 (46.9)	79.8 (16.1)	296 (64.8)
11	509 (78.0)	74.8 (15.2)	304 (66.7)
12	481 (64.1)	78.0 (12.4)	304 (67.9)

TABLE 15.

Comparison of the observed and expected fry survival between female O. mossambicus used during the investigation of possible fry protection. The results obtained for the two experimental spawns are included.

Fish No.	Expected No. of fry	Observed No. of fry				
		Non-infected control		Non-immunized infected	Immunized infected	% fry survival from immune fish
		SP1	SP2	SP1	SP2	
1	307	328	342			
2	336	369	311			
3	325	297	341			
4	311	346	372			
1	295			0	64	21.6
2	285			0	32	11.2
3	297			0	47	15.8
4	305			0	56	18.3
5	315			0	60	19.0
6	296			0	44	14.8
7	304			0	24	7.89
8	304			0	9	2.90

no statistical analysis was used to examine the significance.

In contrast to the first infection, all eight immunised females subjected to infection of *I. multifiliis* whilst carrying 3 day old fry, resulted in fry survival when observed 3 days post infection. The largest number of fry recovered was 64 whilst the smallest was 9 (Table 15). The highest calculated percentage fry survival from the expected number (mean of ten previous spawns) was 21.6 and the lowest was 2.9%. The number of fry recovered was substantially lower than that of the control fish (Table 15) and therefore no statistical analysis was necessary.

The fry recovered from the immunised females 3 days post infection were 100% infected with *I. multifiliis* (microscopic examination of group X fry).

The challenge infections carried out on the fry from immunised females (group Y fry) at 8 days old, resulted in 100% mortality at a dose level of 640 tomites/fish. This dose level was previously estimated for 8 day old fry as a 24 hour LD100 (Chapter 8). The fry collected from uninfected control fish also resulted in 100% mortality following infection at a similar dose level of *I. multifiliis*.

Ouchterlony double diffusion tests carried out using the homogenate prepared from the fry collected from immunised females (group Z fry) or nonimmunised

control females did not show any precipitating lines against I.multifiliis antigen. In contrast, the serum and mucus concentrates from the immunised females gave positive precipiting lines against the antigen. The serum and mucus from the control females produced negative results.

9.4. DISCUSSION

The post invasion immunity to I.multifiliis has been clearly demonstrated in the cyprinid, C.carpio (Hines and Spira 1974c), the ichtalurid, I.punctatus (Beckert 1975) and the salmonid, S.gairdneri (Wahli and Meier 1985). The present investigation demonstrates the presence of post invasion immunity in the mouth brooding cichlid, O.mossambicus. It is clear that O.mossambicus, recovering from a sublethal level of infection of I.multifiliis, remains refractory to a normal lethal level of infection when subsequently exposed to the infection. The immunity was retained for at least 270 days (the duration of the experiment) when the fish were maintained in the infectious environment.

Hines and Spira (1974c) noted that C.carpio when exposed to a sublethal level of infection, developed a heavy infection which peaked at 16 days after the initial exposure. The parasites had completely disappeared from the skin and gills by 21 days post exposure. Wahli and Meier (1985) in their report on "Ichthyophthiriasis in Trout",

stated that the fish were heavily infected three weeks after the initial infection. However, they did not indicate clearly the time required for the complete disappearance of the parasites from the fish for comparison. In the present study the time required for the complete disappearance of the parasite from O. mossambicus was comparatively shorter ie between 17-19 days. One of the obvious differences between the present study and those of Hines and Spira (1974c) and Wahli and Meier (1985) is the temperature at which the fish were maintained. Wahli and Meier (1985) used a temperature of 11'C, Hines and Spira (1974c) 20-23'C and for the present study 27±1'C. The immune response in all ectothermic vertebrates is temperature dependent and a low temperature delays or completely abolishes antibody production (Ellis 1978). The life cycle and the development of the parasite is also temperature dependent (Subasinghe and Sommerville 1985), thus the level of infection and the effect of the parasite on the fish host could vary according to the temperature, hence contributing in different ways to the immune response of the fish. Therefore, the temperature differences between the experimental systems together with the specific variations between the hosts may have contributed to the differences in the time required for the total disappearance of the parasite from the hosts.

The magnitude of the primary and secondary

immune response has been reported to be dose and route dependent (Ellis 1982). Rijkers, Frederix-Wolters and Van Muiswinkel (1980) using intramuscular priming of C. carpio with sheep red blood cells (SRBC) demonstrated that a high dose resulted in a low primary response but a high secondary response and vice versa. According to Trizio and Cudkowicz (1978) cited by Ellis (1981), the high secondary response resulting from low dose priming may be due to the more effective stimulation of T-helper function. Hines and Spira (1974c) found that there was a rise in serum immobilisation titres between days 10 and 22, the period in which all the parasites disappeared. In the present study the serum immobilisation titres increased from day 0 to day 35 with a rapid increase between days 5 and 10 (Figure 63). The comparatively early immune response indicated by this early rise in serum immobisation titres and visual disappearance of the parasites from the skin of O. mossambicus shows the possibility of differences in priming associated with the initial dose level of infection. The actual dose level of infection used by Hines and Spira (1974c) and that in the present study is not therefore directly comparable due to the differences in the measure of dose levels. However, the difference might be explained if the primary dose level used in the present study was considered to be comparatively lower than that of Hines and Spira (1974c).

In teleosts the average time for the appearance of antibody secreting cells (ASC) following antigen injection is about 8 days with maxima occurring about 16-18 days (Ingram and Alexander 1980). However, the peak production of ASC in O.mossambicus was found to be five days post immunisation (Sailendri and Muthukaruppan 1975); but their fish had been maintained at 30 C. Therefore it is possible that the observed faster rise in serum immobilisation factors in the tilapia was associated with the dose level of infection, the species of fish and the maintenance temperature.

The disappearance of parasites from the host fish coincided with the development of immunity. However, the mechanism by which this protection is made possible, is still not clear. Hines and Spira (1974c) suggested that a crossover of the serum immobilisation factor, most likely an antibody, into the mucus of the resistant fish is responsible for the inability of the parasite to penetrate the mucus layer and infect the fish. Goven et al (1980) hypothesized that immobilising and agglutinating antibodies produced by the fish might be concentrated in external mucus secretions of the resistant fish and be able to immobilise the tomites.

Subasinghe (1982) observed that the tomites can still penetrate the skin of immunised juvenile C.carpio, however the subsequent fate of the parasite was

not understood. It has already been noted that no apparent histopathological changes in the dermal epithelium could be seen until after 24 hours of exposure to the tomites (Chapter 8). It is possible that the parasites penetrating the epithelium of the immunised fish could be killed within the epidermis by the host antibodies within a few hours. Further investigations are necessary to try to elucidate the effective mechanism, such as by a study of the sequential pathology of the dermal epithelium of immunised fish following challenge infection of I. multifiliis.

The presence of precipitating antibodies has been demonstrated in various species of fish, naturally infected with several intestinal parasites. Harris (1972) detected precipitating antibodies in chub (Leuciscus cephalus) infected with the helminth Pomphorhynchus laevis whilst Cottrell (1977) demonstrated precipitating antibodies in the serum of plaice, P. platessa infected with Cryptocotyle lingua. McArthur (1978) found precipitating antibodies in the serum of New Zealand eel, Anguilla australis schmiditii Phyllips, against the trematode Telogaster opisthorchis Macfarlane. Amongst these studies only Harris (1972) detected precipitating antibodies in the gut mucus secretions. This may have been associated with the mode of attachment of this particular acanthocephalan in which considerable penetration and epithelial damage takes place in comparison to the other intestinal

helminths. However, he did not find any evidence of host rejection associated with the presence of these antibodies.

In contrast Hines and Spira (1974c) using immuno-electrophoresis demonstrated that immunised C.carpio serum and mucus contained more high molecular weight proteins than nonimmunised fish. Those immunised fish were completely refractory to reinfection of I.multifiliis. They suggested that these could be antibodies.

Fletcher and Grant (1969) showed the presence of some of the blood proteins in the external mucus of plaice P.platessa. After identification of some of these shared proteins Fletcher and Grant (1969) suggested an active secretory system moving immunoglobulins out of the serum into the mucus. The results of the present study also strongly suggest the presence of specific antibodies in the serum of O.mossambicus immune to I.multifiliis and the possible secretion of these proteins into the mucus. Although no one has demonstrated the presence of specific precipitating antibodies in the serum and mucus of fish immune to I.multifiliis until the present study, Hines and Spira (1974c) suggested that the immobilisation factor which was likely to operate at the host parasite contact point was most likely to be an antibody. The inability of the normal control serum and mucus to produce precipitating lines against the parasite as well as the lack of immobilising capability in the normal control mucus is a

strong indication of the presence of specific antibodies in immunised fish. An effective protection mechanism against reinfection through acquired immunity of a humoral origin is evident.

Though the immunisation of fish by injection of killed I.multifiliis or related ciliate antigens has been demonstrated by Goven, Dawe and Gratzek (1980, 1981), no reports were found on the passive immunity by injection of immune fish serum. However an incidental observation made during the present investigation suggested that passive immunity by injection of immunised serum is possible. It was also noted that the parasite burden on such immunised fish was effectively lower than that on nonimmunised fish when exposed to a similar dose level. Similar protection was demonstrated by Harrel et al (1976) in rainbow trout injected with trout anti-Vibrio angullarum serum, against vibriosis.

The detection of precipitating antibodies in fish serum has been found to be difficult by many workers and it has been suggested that the production of precipitating antibodies in the lower vertebrates may not be particularly well developed (Ellis 1978). However, precipitating antibodies in rainbow trout serum have been found to be easily detectable using gels composed of 1% agarose and 3% NaCl (Ellis 1978). According to Ellis (1985) the formation of a precipitate in Ouchterlony double

diffusion tests does not necessarily mean the presence of an antigen-antibody reaction. False positives may occur due to the possible presence of C-reactive proteins (CRP) and they can be easily distinguished from antigen-antibody precipitates by soaking the gel in 5% sodium citrate (Ellis 1978). Further, Ellis (1982) stated that a positive reaction appearing on a gel plate was dependent upon the host species and the antigen used. However, the positive precipitating lines observed during the present study were insoluble in 5% sodium citrate. This supports the conclusion that they were indeed antigen-antibody precipitates and not CRP.

The fish used during the present study were never observed to be infected with any other protozoan ectoparasites. However, the occasional occurrence of the monogenean *Cichlidogyrus* species was evident on the immunised fish. The acquired immunity by the fish against *I. multifiliis* did not seem to have any effect on this monogenean. Hines and Spira (1974c) also noted occasional moderate infections of *Trichodina* species and *Dactylogyrus* species in immune carp. This evidence also supports the involvement of specific immunity to *I. multifiliis* in the "effectively immunised" tilapias.

An incidental observation made during the present study was the inconsistency in the serial passage of parasites from one fish to another. Similar observation

made by Hines and Spira (1974c) gave rise to the suggestion that this may be due to the factors diffusing out from the recovering fish and protecting the susceptible fish when kept in the same small aquaria. During the present study this problem was overcome by partial water changes in aquaria where the fish were maintained. This supports their suggestion, however, that the effect of physicochemical qualities of water on the parasite's viability and reproductive capability should not be under estimated.

Erratic and low infections of I. multifiliis during experimental investigations were attributed to poor estimation of tomite numbers by Dickerson, Dawe, Gratzek, Brown and Pyle (1981) and to intraspecific physiological strains by Bone (1983). Subasinghe and Sommerville (1985) in a study designed to investigate the possible differences between strains found that two I. multifiliis isolates originating from two distinctly different climatic conditions, behaved similarly in terms of their reproductive capabilities. The lack of erratic or low infection rates experienced during the present investigation may be a result of the more careful estimation of the numbers of tomites propagated from a single isolate of I. multifiliis used.

Fecundity in fish is usually taken as the mean number of ripening eggs in the ovary or the number of eggs spawned (Baganel 1978). Lowe-McConnell (1955)

suggested that a more useful definition of fecundity for tilapias is the number of fry produced during the life time of an individual. For practical purpose Mires (1983) suggested that the fecundity in farm tilapias could be regarded as the number of fry produced in a year beginning from the first spawning.

Tilapia eggs show a pleurimodal development in the ovary: the eggs of more than one spawn ripen in the ovary at the same time (Peters 1959). Also it is not uncommon in tilapias to reabsorb ripe eggs. As a result of these factors the prediction of fry production from egg fecundity values is likely to be misleading. Macintosh and Sampson (1985) in their manual of hatchery methods for *Oreochromis* species defined the fecundity of this species as a direct count of the number of first feeding fry produced by a single female brooder. *O.mossambicus* show a linear relationship between the body weight and the number of eggs produced, and within a given range of body weight the number of eggs produced is not significantly different (Rana 1986b). The female *O.mossambicus* used during the present study had complete individual spawning records including the weight length, clutch size, fertility rate and percentage fry production under artificial incubation. Therefore the prediction of the number of fry produced by an individual female during the present investigation was made on as much relevant data as possible which comprised

the individual spawning history. No more reliable method is known at the present time.

It was assumed that an individual female would produce a number of eggs similar to that of the mean number of eggs produced during the previous ten spawnings. It was also assumed that a single female would produce a number of fry under oral incubation, similar to that of the mean number of fry resulting from her last ten spawns under artificial incubation. The efficiency of the artificial incubation in terms of percentage hatchability was noted to be greater than 90% (Chapter 6). In the results of the present investigation the number of fry recovered from uninfected control females were not significantly different (P>0.05) from the predicted numbers based on their individual spawning histories, thus increasing the confidence in the method of prediction employed.

The observed differences between the number of fry recovered from uninfected control and infected females, exposed to *I. multifiliis* for the first time, were very high. There was a complete loss of brood due to *I. multifiliis* infections in the females carrying 4-6 day old fry following this initial exposure to the parasite. The dose level of infection used was higher than that calculated for 4 day old fry. However, as the surface area of a female is considerably larger than that of her brood, a certain percentage of parasites was expected to be

unavailable for the fry as they would invade the female. This was expected to reduce the actual dose level of infection to the fry. On the other hand the actual exposure time was longer during the present experiment than that used to calculate the lethal levels in fry. This was due to the technical difficulties encountered during the investigation especially in the handling of females while carrying their broods. However, it was expected that the compensation between the reduction in dose level and the increase in exposure time would result in a dose level closer to the lethal dose calculated for 5 day old fry. The results clearly indicate that the 4-6 day old fry did not receive any possible protection against I. multifiliis infections from their naive mothers, whilst they were being orally incubated.

Oral incubation is a delicate process. It was frequently observed that a sudden disturbance of the female resulted in complete rejection of the brood. It was also noted that in crowded conditions dominant males often disturb brooding females which also results in rejection of their broods. However, during this experiment when the carrying females were exposed to tomites they were in individual tanks and no disturbance from other fish was possible. Hence, the complete loss of fry brooded by the previously uninfected females, following exposure to I. multifiliis, would be either due to direct invasion of

the fry by the parasite or as a result of the stress response of the females to the tomite invasion. However, the lethal dose of I.multifiliis to females of that size is considerably higher than that introduced during this experiment. Therefore, it seems unlikely that this dose level would have exerted a sufficently intense amount of stress on the female to have caused rejection of her brood. An incidental observation made on a group of broodstock O.niloticus mildly infected with I.multifiliis showed females carrying fry without any obvious stress. Therefore, the possibility of the loss of fry as a result of the female's stress response was excluded.

The most interesting observation made was the 2.9% to 21.6% survival of the fry brooded by the immunised females three days post exposure to I.multifiliis. The exposure level was sublethal to females but was lethal to fry. The only difference between this brood of fry and the previous brood was the fact that, whilst carrying the first brood the females were not "effectively immunised" against I.multifiliis. This indicates a possible protection of the fry by the mother through oral incubation. The protection was consistent among all the females but to varying degrees. The fact that all the eight individuals tested showed some degree of enhanced survival of fry supports the suggestion of conferment of protection by the immunised female parent. The mechanism of this protection is not

clearly understood from the results obtained and requires some further explanation.

The mucus in higher vertebrates is secreted by goblet cells present in the epidermal layer. The mucus contains both proteins and carbohydrates. In addition to these macromolecular components fish mucus is known to contain antibodies and/or antipathogenic factors (Bradshaw, Richard and S igel 1971, Cameron and Endean 1973 and Harrel *et al* 1976) which would be beneficial to the host (Ingram 1980). Herrel *et al* (1976) concluded that skin and gut mucus of fish may represent a first line of defence in the initiation of antimicrobial activity. Further, lysosomes (Fletcher and White 1973) and CRP like substances (Ramos and Smith 1978) have also been demonstrated in the epidermal mucus. Ingram (1980) stated that it is most likely that the level of mucus secretion reflects the degree of initial resistance to invasion by a variety of potentially infective agents. However, in *O. mossambicus* no antibacterial properties were detected either in buccal or in epidermal mucus (Chapter 7) but the presence of antibodies against *I. multifiliis* in the skin mucus of "effectively immmunised" fish has been demonstrated.

Young fish hatch or are born into a hostile environment when their immunological competence is severely limited (Manning, Grace and Secombes 1982 and Manning, Ruglys, Grace and Botham 1981). As the young hatchlings are

not immunocompetent, they only have a very limited capacity to respond to the environmental antigenic stimulants being unable to either produce effector lymphocytes or circulating antibodies. Thus, they are very susceptible to and have very little resistance to immediate environmental pathogens.

In the present study, the fry carried by immunised females though surviving the initial exposure to I.multifiliis did not show any sign of resistance to the challenge infection. They all resulted in 100% death as did the controls. If the fry had developed any immunocompetence against I.multifiliis as a result of their previous exposure, they would have certainly shown some degree of resistance to the challenge infections. No studies have been made to elucidate the age at which O.mossambicus fry are first able to mount an immune response. It is possible that the period of 3-4 days following initial exposure to the parasite may have been too short for the fry to mount an immune response to the parasite.

In higher vertebrates the lack of immunocompetence is compensated for by the passive immunity derived from their mothers to protect the young during their earliest period of independent life. The earliest reports on passive immunity derived by fry from their parents were made by Hildermann (1959 and 1962), on his classic study on amazonian discus fish, S.discus, details

of which have already been discussed in Chapter 7. Later van Loon, van Oosterman and van Muiskinkel (1980) discovered immunoglobulins in the roe of C.carpio. Bly (1984) made an intensive investigation to determine whether transmission of passive immunity from mother to young occurred in fish. Her experiments with 125 I-labelled homologous immunoglobulins and low molecular weight bovine serum albumin, showed that both were taken up into the young of viviparous species and the eggs of oviparous species. She also demonstrated that in the viviparous bleny, Zoarces viviparous L., the differentiation of thymus and pronephros is earlier than in oviparous species. Based on such evidence she made the suggestion that embryos may be immunologically competent while still remaining in the ovary and therefore capable of mounting an immune response against both maternal and sibling antigens. However, no passive immunity to a particular pathogen was demonstrated in that study.

During the present investigation Ouchterlony double diffusion tests did not show any positive precipitating lines between I.multifiliis whole cell antigen and homogenates of fry derived from immunised mothers. This suggests the lack of specific antibodies against I.multifiliis in fry. If there was any positive immunity against the parasite derived from the immunised mothers it would have been expressed either on gel

diffusion tests or by displaying some degree of resistance to the parasite when challenged outside the buccal cavity. Thus the possibility of passive immunity derived from the mother does not seem to provide an adequate explanation for the observed degree of survival of fry following exposure to a lethal dose of I. multifiliis whilst they were being orally incubated by immunised mothers. However, the idea of passive immunity should not be ruled out. Further investigations are required including quantitative and qualitative analysis of immunoglobulins to elucidate the possible transfer of specific antibodies from mother to eggs and fry.

The histological changes taking place in the buccal epithelium prior to spawning in mouth brooding tilapias has already been discussed in Chapter 7. It is clear that an excessive mucus production occurs in the buccal epithelium while the brooding of the progeny takes place. The presence of parasite immobilising factors such as an antibody in the mucus of effectively immunised females has also been demonstrated. Any hypothesis which is put forward in order to explain the observed degree of protection of yolk sac fry by immunised females during oral incubation needs thoughtful integration of those two factors. The buccal cavity itself is a physically restricted environment for fry. The fry are continuously bathed in the water which flushes across the buccal cavity

due to the active pumping process of the female. Meanwhile the female continously produces mucus and the fry are being coated in a layer of mucus within the buccal cavity. It is therefore possible that this mucus coat secreted by the immunised mother immobilises or reduces the viability of the parasite, at least to a certain degree at their point of contact with the mucus. Although the *in vitro* immobilisation experiments clearly indicated the ability of mucus to immobilise the parasite, the degree of concentration of mucus and the time required for the immobilisation of the parasites in nature are still unknown. However, it is quite possible that given the right concentration of mucus the parasite viability could be reduced in a much shorter time than it takes for complete immobilisation. Therefore it is appropriate to suggest that the continuous sloughing and recoating with mucus produced by the immunised females results in partial immobilisation and/or reduction in viability of the parasite; hence the observed degree of protection.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

The progressive intensification of the culture of tilapias to meet world demand for fish protein and the ways in which it has been constrained by the inability to obtain adequate quantities of quality fry was the stimulus to the present investigation. As a step towards the rectification of this shortfall, Pullin (1982) considered the development of a small number of large hatcheries with well trained personnel to be a better prospect than the development of large numbers of small hatcheries scattered throughout the rural areas of developing countries. However, in areas where the infrastructure (mainly transport and communication) is poor and the fuel prices are high, small scale local hatcheries would be desirable and should be economical. When considering this type of small scale operation, a tank or lake-based hatchery system, together with an artificial incubation facility appears an ideal proposition.

The present investigation showed that incorporation of a trickle gravel biological filtration unit into a small scale artificial incubation system is essential for the maintenance of adequate water quality (physical, chemical and microbiological). This type of system could be expected to support approximately up to 65%

hatchability of viable eggs and about 80% survival of yolk sac fry.

It was also noted that in the design of incubation systems for artificial incubation of *Oreochromis* eggs, considerable importance should be given to the simulation of the natural conditions for the eggs as far as possible. Incorrect flow rates and rapid movement ("churning") of eggs were the most likely cause of the mechanical injuries to the egg surfaces. Therefore, the use of incubators with smooth inner surfaces and simple trickle gravel biological filters in small scale artificial incubation systems should be considered as highly desirable.

The scanning electron microscopic studies demonstrated that the mechanical injuries to the egg surface increase the opportunities for the bacterial settlement on the egg. It was suggested that the mortality of eggs was most likely to be due to the subsequent bacterial action. Though it is commonly accepted that the majority of egg mortalities in a hatchery are a result of fungal infections, it was evident that the fungi played only a secondary role in the egg mortalities observed during the present study. Therefore, when diagnosing the cause of mortality of eggs in hatcheries care must be taken to study the mechanism of the mortality. Proper understanding of the primary cause of egg mortality should

lead to more appropriate measures to improve the survival rates.

It was clearly demonstrated that disinfection of eggs is highly desirable in improving the hatchability. Commonly available chemical disinfectants could be used, however risk of over dosing and human health hazards should be considered. Introducing these types of chemical disinfectants in to rural areas of developing countries where tilapia farming is taking place, needs careful supervision.

One of the most interesting observations made during the present study was the efficiency of ultraviolet light as a measure of disinfecting hatchery water to improve hatchability of eggs. It was found that UV light could improve hatchability to above 90% and also could allow for accommodation of a certain amount of non-fatal mechanical injuries to the chorionic membrane which might otherwise result in bacterial and fungal infections. Therefore, efforts should be made to incorporate UV water sterilisation units into Oreochromis hatcheries. However, the drawbacks would be the need for electricity and the capital and running costs. In this context, it would be desirable to use biological filtration together with chemical disinfection for the small scale rural hatchery operations and to incorporate UV water sterilisation in the larger commercial operations.

The dynamics of the physicochemical and microbiological qualities of the hatchery water observed during the present investigation was interesting. Though there was no organic loading as food introduced into the system, the accumulation of organic waste from excretory products and dead eggs and fry could be detrimental to the survival rates by reducing the water quality.

The observed effects of the chemical qualities of the hatchery water, such as ammonia and nitrite, clearly demonstrated the hardiness of O. mossambicus compared to most of the other cultured species of fish. The eggs and yolk sac fry both displayed a very high level of resistance to levels of ammonia and nitrite which are lethal to some fish species. However, further investigation would be helpful in understanding the median lethal tolerance levels of toxicants for a given exposure time (eg. 72hr LC50) for different developmental stages of fry during the early life history. This is especially important as the susceptibility of fry appeared to increase as they gradually developed their gill secondary lamellae; this stage is accompanied by a move to exogenous feeding. However, as the developmental aspects of gill morphology in Oreochromis species have not been investigated, this appears to be an interesting avenue of research.

The present investigation indicated that there is a critical level of heterotrophic bacteria (including fish pathogens) in the hatchery water beyond which the hatchability of O.mossambicus eggs rapidly decreases. This could be of importance to the pond-based Oreochromis hatchery operations where the broodstock is kept in relatively nutrient rich water. The reported fry production under such pond conditions is low, 257-467 per female per month (Melard and Philippart 1980), however, the present incubation system with UV water sterilisation produced over 1000-1300 fry per female per month (unpublished 1985). Reported low fry production is likely to be associated with egg and fry mortality, none of which has been quantified. Therefore, it may be desirable to monitor the microbial flora of the pond water both quantitatively and qualitatively and measures such as reduced manuring etc. could be important in order to overcome undesirable levels of microbial flora.

The inability to demonstrate antimicrobial activity in the oral mucus of O.mossambicus in the present investigation still does not rule out the possibility of some protection of the eggs from microbial pathogens during brooding. Though some structural changes in the buccal cavity take place prior to spawning which facilitates brooding, nevertheless fry mortality due to mechanical injuries within the buccal cavity has been

clearly demonstrated (Rana 1986b). Considering this evidence, good artificial incubation might be more efficient, in terms of percentage survival, than oral incubation under crowded hatchery conditions.

Besides the fry loss due to mechanical injuries and pathogenic microorganisms, it was already noted that tilapia fry are very susceptible to parasitic infections such as I. multifiliis. The observed very low lethal levels of tomites for yolk sac fry emphasise the severity of an outbreak of Ichthyophthiriasis in a hatchery. The parasite's life cycle is such that propagation of large numbers of infective stages within a short period of time is possible and the slightest contamination of an early fry rearing system with this parasite could be disastrous.

Adult O. mossambicus were found to mount an immune response to I. multifiliis earlier than that reported for all the other species of fish examined to date. This could be the result of a long term evolutionary process and further investigations into the immune system of the species could be important in explaining this interesting phenomenon.

The ability of the fish to retain immunity over the observed nine month period when maintained in an infectious environment could be of some importance to aquaculturists. This suggests that when fish

are kept in relatively poor husbandry conditions where recurrent host parasite encounters are likely, they are able to remain refractory to I. *multifiliis* provided that the initial exposure was sublethal. Though there is no effective vaccine developed yet, the protection of fish, especially the valuable broodstock, could be achieved by careful manipulation of well controlled infection regimes in environments where the parasite is endemic.

This deliberate exposure of naive fish and fingerlings to infection by I. *multifiliis* would however be rather an extreme measure. Though it would be effective in stimulating immunity, the stress would inevitably have an adverse effect on growth performance. The production of a vaccine which could be administered by bath, injection or orally is expensive and unlikely to be cost effective for most low technology culture operations. At this stage, a control method for the parasite during natural outbreaks which prevents lethal infections, and the enhancing of the fishes ability to mount a natural immunity would seem to be the only realistic procedures.

The aspects of passive immunity from mother to fry via eggs in certain fish species has already been discussed. However, the evidence generated from the present investigation did not reveal any passive immunity related protection of mouth-brooding fry against I. *multifiliis* infections by immunized females. It appeared

that the protection offered by the females might be exogenous through provision of a hostile environment to the parasite by secretion of parasite immobilisation factors through the oral mucus thus bathing the offspring in a protective mucous coat. However, carefully designed further investigations into the developmental and passive immunity of Oreochromis species should be encouraged to elucidate the actual mechanism.

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APPENDIX 1

Water quality parameters measured in the broodstock system during the investigation.

Parameter	Range
DO mg/l.	6.3 - 7.4
pH	6.0 - 7.9
Temperature 'C	26.4 - 27.5
Total NH3-N mg/l.	0.24 - 1.43
Total NO2-N	0.00 - 0.39
Total Viable Heterotrophic bacteria cells/ml.	1.24×10^{3} - 2.65×10^{3}
Total Viable Fungal Spores per l.	9.28×10^{4} - 1.56×10^{5}
Total Viable Saprolegniaceae Spores per l.	4.3×10^{3} - 8.7×10^{4}

APPENDIX 2.

The formula and the preparation procedure of dilution water used for the tolerance trials.

Stock solution 1

Dissolve 400g $CaCl_2.6H_2O$, 36g NaCl and 11g $NaNO_3$ in de-ionised distilled water and make up to 1 litre.

Stock solution 2

Dissolve 189g $MgSO_4.7H_2O$ and 99g Na_2SO_4 in de-ionised distilled water and make up to 1 litre.

Stock solution 3

Dissolve 34g $NaHCO_3$ in de-ionised distilled water and make up to 1 litre.

For standard water of 50mg/l hardness add 20ml each of solution 1 and 2 and 200ml of solution 3 to 100l of de-ionised water.

(After Anon 1969, <u>Fish Toxicity Tests</u>, Ministry of Housing and Local Government).

APPENDIX 3.

Chemical characteristics of the dilution water used in the experiments.

All values are in mg/l unless otherwise noted.

Parameter	Mean	Range
Total oxidised nitrogen	0.30	0.30 - 0.40
Total alkalinity as CaCO3	42.50	40.0 - 45.0
Total hardness as CaCO3	58.50	57.0 - 60.0
Total ammonia - nitrogen	0.02	0.01 - 0.02
Conductivity	220.0	194 - 245 US/CM
Suspended solids	1.0	1.0 -2.0
Orthophosphateas P	0.01	<0.01 -0.02
Chloride	31.5	31.0 - 32.0
Potassium	0.7	0.5 - 1.0
Sodium	27.4	26.5 - 28.3
Copper	0.01	0.01 -0.02
Iron	0.01	<0.01 - 0.02
Lead	<0.005	-
Manganese	<0.005	-
Magnesium	3.65	3.20 - 4.10
Calcium	7.05	6.90 - 7.20
Zinc	0.006	<0.005 - 0.017

APPENDIX 4

Histological methods

Processing schedule

50% Methylated spirit	1 hour
80% " "	2 "
8% Phenol in methylated spirit	3 "
8% " " "	2 "
8% " " "	2 "
Absolute alcohol	2 "
" "	1 "
Chloroform	1 "
"	1 "
Wax	2 "
"	2 "
"	1 "

Haematoxylin and Eosin (H&E) staining schedule

1	Xylene	5 minutes
2	Absolute alcohol	2 "
3	Methylated spirit	2 "
	Wash in tap water	
4	Mayer's Haematoxylin	10 "
	Wash in tap water	
5	1% Acid alcohol	1 - 3 dips
6	Scott's tap water substitute *	3 minutes
	Wash in tap water	
7	Eosin	5 "
	Wash in tap water	
8	Methylated spirit	30 seconds
9	Absolute alcohol I	2 minutes
10	Absolute alcohol II	1 "
11	Xylene	5 "

* After Scott's tap water substitute, sections are examined microscopically and
if too dark, differentiated again in 5
if too light, process is repeated from 4

APPANDIX 5

Fixation and dehydration procedure used for SEM.

Fixation

Primary fixative - 2.5% Glutaraldehyde in cacodylate buffer

Secondary fixative - Cacodylate buffered 1% osmium tetroxide

Dehydration

50% ethanol in water 45 min

70% ethanol in water 45 min

95% ethanol in water 45 min

100% ethanol 1 hr

100% ethanol 1 hr

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