IMMUNOLOGICAL STUDIES IN *ETROPLUS SURATENSIS* (BLOCH)

DISSERTATION SUBMITTED BY

Shri. SANTHOSH, N. K.

IN PARTIAL FULFILMENT FOR THE DEGREE OF
MASTER OF SCIENCE (MARICULTURE)
OF THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

भाकृअनुप
ICAR

NOVEMBER - 1993

POST-GRADUATE PROGRAMME IN MARICULTURE

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

COCHIN - 682 014

CERTIFICATE

This is to certify that this Dissertation is a bonafide record of work carried out by Shri. SANTHOSH N.K. under my supervision and that no part thereof has been presented before for any other degree.

DR. P.C. THOMAS,
SENIOR SCIENTIST,
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE,
COCHIN - 14.

Countersigned by :

DR. P.S.B.R. JAMES,
DIRECTOR,
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE,
COCHIN - 14.

C O N T E N T S

PREFACE

PREFACE

Aquaculture is developing into a prime industry to tap the enormous turnover of bioenergy for the benefit of mankind. Since aquaculture depends on renewable natural resources it holds immense potential for food production with infinite future for hundreds of years to come. When man began to enclose water and create ponds for fish culture, he appreciated the problems of over-population, diseases, the influence of temperature and other factors involved in the production of fish.

The importance of disease factor in the fishery has been appreciated by man only lately. Fish diseases are being understood as a limiting factor and various methods are being sought to control the diseases. The prevention of disease is becoming more important than its therapy. The relevance of immunization of fish becomes more clear in this context. Immunization against diseases can and should play an important role in the management of intensively cultured fish.

Vaccination of fish is the main forte of immunology applied to fisheries. This needs an indepth knowledge of the immune system of fish which in turn calls for detailed investigations into the fish immune system.

Study of fish immunology assumes importance even on other counts. Immunological systems of fishes potentially can give clues to the presence of human disease antigens present in the environment (Janssen, 1970).

Also the fish 's immune system may help to monitor pollution levels of toxins. Phylogenetics, teleology and ontogeny are some of the other specialized fields concerned with the development of the immune response. The creation and maintenance of large fishery resources depend upon a delicate ecological web. The antigen-antibody balances in the environment are integral factors of biochemical interactions which build up a fish population. These populations are of importance to the commercial fisheries, the sport fisheries as well as the hobbyists dealing with exotic tropical fishes.

Of late considerable interest has been developed in the study of fish immunology, following the rapid expansion of fish farming. However, even today our knowledge about fish immunology is limited to only a very few species. Hence any generalisation about fish immunology will be premature as fish is the most varied of all vertebrates. There is hardly any study on the immune system of the Banded pearl spot Etroplus suratensis which is an important brackish water fish cultured extensively in ponds and tanks. Hence an attempt was made through the present study to investigate the different aspects of immune response in Etroplus suratensis with the following objectives.

1. To evaluate the humoral immune response to different antigens.

2. To assess the differential response to different doses of antigen.

3. To evaluate the variations if any in immunological response dependent on the route of antigen administration.

4. To study and compare the immunological response in different size groups.

5. To assess the immunosuppressive effect of the heavy metal Copper (Cu) if any.

6. To test the effectiveness of immunization in protecting the fish against the bacterial infection viz., Aeromonas hydrophilla.

7. To evaluate the cellular response along with humoral immune response.

I wish to express my deep sense of gratitude to my supervising teacher Dr. P.C. Thomas M.V.Sc; Ph.D. Senior Scientist, Central Marine Fisheries Research Institute (C.M.F.R.I.), Cochin for his valuable guidance, constant encouragement, whole hearted support throughout the course of this study and the preparation of the manuscript. I am thankful to Dr. P.S.B.R. James Director, C.M.F.R.I., Cochin for the facilities provided during the tenure of this work. I express my thanks to Dr. C. Suseelan Principal Scientist for his encouragements. I am grateful to Dr. I.S.Bright Singh Lecturer, School of Environmental Studies, Cochin University of Science and Technology for providing the bacterial culture. My thanks are also due to Mr.P.M.Aboobacker Technical Officer and Mr.A.Nandakumar. I wish to express my sincere thanks to all my classmates and senior scholars especially Mr. Sini Joys Mathew for their timely help.

I am thankful to the Indian Council of Agricultural Research(I.C.A.R.) for awarding me a Junior Research Fellowship (J.R.F) during the tenure of which this work was carried out.

INTRODUCTION

INTRODUCTION

Aquaculture is becoming an increasingly important source for meeting the ever increasing need of protein rich food. In spite of the all round developments in various aspects of aquaculture, the occurance of disease still remains as a major problem to be solved. Hence development of preventive measures against diseases is of vital importance for the success of aquaculture programmes.

Traditional methods of disease control using chemical treatments may not be always effective. They can be expensive and can cause problems of pollution (Samuelsen, 1989) and of drug resistance in the pathogen (Bjorklund et al., 1990 and Holmer, 1991). Many of the chemotherapeutic agents may have toxic side effects and are often growth retardants. In addition to this, prophylaxis is only for short periods and toxic chemicals may be retained in the animal.

Considering the numerous disadvantages of antibiotics and chemotherapeutic agents, tapping the innate defense system viz., the immune system of the animal itself should form the back bone of the disease control measures.

All animals have the ability to differentiate 'self' from 'nonself', the essence of disease prevention (Newman, 1993). Though mechanisms vary considerably between phyla, they all serve the same basic purpose, to prevent foreign organisms from invading the host and causing diseases.

Fishes are the oldest and most diverse of all vertebrate groups. The immune system of fish are quite varied and appear associated with fish phylogeny (Sharma, 1981). The more premitive groups often possess a very simple immune system. In contrast, the more advanced Teleost fish possess immune structures approaching the complexity and efficiency of higher vertebrates.

The proper application of immunology techniques for diagnosis, prevention and treatment of diseases in the field of human and veterinary medicine have lead to successful eradication of many diseases and increasing the efficiency and quality of life. Yet the application of immunology principles in the fishery field has been slow.

Immunology could be used in fisheries outside the relam of disease prevention to explore racial and genetic differences among marine and fresh water fishes (Anderson, 1974), to monitor pollution levels' of toxins etc. (Goncharov and Mikryakov, 1970).

Most of the available information on the immune responses of Teleosts is based on studies of relatively few species, and these are principally Salmonids and Cyprinids of temperate region. Information available on the immune system of tropical fish species and the tropical fish diseases is limited. In India very few immunological studies have been done in fish, and the available information is in Tilapia and fresh water Carps.

The Banded pearl spot, Etroplus suratensis (Bloch) is an excellent delicious fish extensively cultured. It is very common in the brackishwaters

of the coastal regions of Kerala, Tamil Nadu, Andhra Pradesh and Orissa (Talwar and Jhingran, 1991). Since the immunological aspects of Etroplus suratensis have not yet been investigated, the present study was taken up in this species.

The immune response of fish have been categorized classically as specific or adaptive and nonspecific or nonadaptive (Papermaster et al., 1964). "Specific immunity is induced in response to presence of a foreign antigen, an adaptive acquired defense system with humoral (antibody) and cellular (graft rejection, delayed hypersensitivity) components. Non-specific immunity is a natural or innate nonantibody defense system rendering animals reistant to microbial infection, with humoral (complement, lysozyme, interferon, transferrin, lectins, C-reactive protein, natural agglutinins, lysins and precipitins), cellular (phagocyte) components and structural (skin, gills, mucus, and alimentary canal) components" (Ingram, 1980).

The specific immune response has two arms: humoral immunity mediated through the production of antigen specific antibodies by 'B' lymphocytes and cell mediated immunity which is mainly depending on 'T' lymphocytes. These two mechanisms co-operate each other. Exposure to an antigen results in the stimulation of a small number of virgin lymphocytes which are capable of recognizing the antigen through specific antigen receptors.

The fishes have a variety of lymphopoietic tissues, the pronephros acting as the main stem cell generator in Teleostei, with the thymus and spleen playing roles as primary and secondary lymphoid organs (Fange, 1982). The production of antibodies in response to antigenic stimulation in fish was demonstrated in the beginning of this century itself (Babes and Riegler, 1903 & Noguchi, 1903 cited by Corbel, 1975). However, the progress in the field of fish immunology is comparatively slow.

Antibodies are glycoprotein molecules which exhibit the properties of immunoglobulins (Singleton and Sainsbury, 1978). In fish only IgM class of immunoglobulin has been definitely identified (Hodgins et al., 1967; Clem and Leslie, 1969; Fletcher and Grant, 1969; Shelton and Smith, 1970; Marchalonis, 1971; Singel, 1974; Ellis and Parkhouse, 1975; Litman, 1975; Emmrich et al., 1976; Etlinger et al., 1976; Clem et al., 1977; Warr et al., 1977; Roberts, 1978; Warr and Marchalonis, 1980; Manning et al., 1982 and Ellis, 1985) although other classes have been reported in Elasmobranchs and Dipnoids (Marchalonis, 1969; Chartrand and Finstad, 1971).

With few exceptions (Uhr, et al., 1962; Marchalonis, 1971 op. cit.) immunoglobulin of Teleosts has been recorded as a tetrameric molecule with a molecular weight of approximately 6,50,000 daltons (Shelton and Smith, 1970 op. cit.; Acton et al., 1971; Hall et al., 1973; Cisar and Fryer, 1974).

Immunochemical properties of fish antibodies were studied by various investigators such as Grey, 1963; Ambrosius, 1966; Clem and Small, 1967;

Clem et al., 1967, Marchalonis and Edelman, 1968; Fidler et al., 1969 and Alexander et al., 1970. The general impression gained from the literature is that Teleost immunoglobulins are predominantly of macroglobulin type. Different biological properties such as agglutination, precipitation, complement fixation and virus neutralization had been demonstrated in fish indicating functional heterogenity of antibodies (Lobb and Clem, 1981).

Cellular immune response have been reviewed by Rijkers (1982a) who included under this mixed leucocyte reactions, migration inhibition, delayed hypersensitivity, allograft rejection and tumor immunity. Presence of subpopulations of lymphocytes in fish has been reported by Secombes et al., 1983. There are evidence for the presence of delayed hypersensitivity and allograft rejection in fish (Dreyer and King, 1948; Finstad and Good, 1964; Baldo and Fletcher, 1975; Timur et al., 1977 and Goven et al., 1980).

The nonspecific cellular responses are phagocytosis, activity of natural killer (NK) cells and cytotoxicity of phagocytic cells. The phagocytic system not only ingest and kill microbes but also stimulate antigen-specific immune response enhanced by activation of the complement system (Rijkers, 1982b).

A number of substances are found in the blood of fish such as lectins, lysozyme, interferon, C-reactive protein, transferrin, complement, properdin, other naturally occuring agglutinins, lysins, opsonins and precipitins having protective functions (Sindermann, 1990).

Secretory antibodies have been identified in skin and intestinal mucus of Teleosts. Antibodies were detected in the mucus of the Plaice, Pleuronectes platessa (Fletcher and Grant, 1968; 1969 op. cit.) and the Snapper, Lutjanus grisens (Bardshaw et al., 1971).

The immune responses of fish are influenced by several environmental and physiological factors. These include environmental temperature, water quality, stress, seasonal effects, pollutants, drugs, hormonal changes, pheromones, hierarchial position of the individual in social order, sex ratio of the population, stocking density, intercurrent infections and a variety of other factors (Bisset, 1948; Barrow, 1955; Avtalion 1969; Perlmutter et al., 1973; Pfuderer et al., 1974; Avtalion et al., 1976, 1980; Yamáguchi et al., 1980; Lamers and Pilarczyk, 1982; Stolen et al., 1982; Manning and Mughal, 1985).

Many studies have shown that antibody production is slower at low temperatures within the normal physiological range of a particular species of fish (Cushing, 1942; Bisset, 1949; Kuhns et al., 1969 and Anderson 1974 op. cit.). Temperature - dependent processes included proliferation and differentiation of'B' cells, as well as antibody synthesis and release (Rijkers, 1982b op. cit.). On the other hand antigen recognition and processing is relatively independent of temperature (Horne et al., 1982).

The existence of immunological memory in fish is of interest both for fundamental research (study of the phylogeny of the immune response) and also for applied research (seeking better methods for immunization

of fish against pathogens). The existence of immunological memory had been proved in a number of species (Avtalion, 1969 op. cit.; Trump and Hildeman, 1970; Ambrosius and Frenzel, 1972; Ingram and Alexander, 1980). Only living tissue can evoke immunological memory (Rijkers, 1982 a op. cit.). The most important aspect of the specific immune system exploited by vaccination is the establishment of immune memory which has a long duration. For a vaccine to have commercial application, the duration of protection induced is of vital importance.

Soluble protein antigens such as ovalbumin and Bovine Serum Albumin (BSA) as well as bacterial antigen Aeromonas hydrophila EMU 20 were used as antigens in the present study. Sudha (1990) reported good antigenic response in Tilapia mossambica by ovalbumin. The immunogenecity of antigens varied between fish species (Ellis, 1988a). According to Avtalion et al., (1980), BSA is a good immunogen in Tilapia even without adjuvant. But Hodgins et al., (1967 op. cit) reported that BSA is a poor immunogen in rainbow trout even when injected with adjuvant. Aeromonas hydrophila is a potential vaccine candidate for many fishes of warm water as well as cold water (Newman, 1993 op. cit.)

The immune response to different levels of antigen was evaluated. The level of antibody production is usually correlated with the dose of antigen administered. Very high doses of antigen injected into fish can induce tolerance (Wishkowsky and Avtalion, 1982). But low antigen levels might induce only weak response and poor memory (Stevenson, 1988). Hence administration of an optimum dose of antigen is important to elicit

a positive immunity to provide protection.

The magnitude of the primary and secondary responses have been shown to be route dependent for injected antigens (Rijkers et al., 1980a). The identification of an effective route for administration of the antigen is also important in successful vaccination programmes. Hence the immunological response to different routes of antigen administration was evaluated in the present study.

The specific immune system will not be fully mature for several weeks in the newly hatched fish. Fishes are capable of mounting humoral and cell mediated immune response over a certain age/size thresholds, though these thresholds have not been worked out in most of the species (Johnson et al., 1982). Application of vaccine in young animal has to be done with lot of precautions as the young fish, if vaccinated before its immune system is properly developed may acquire tolerance (Botham and Manning, 1981; Anderson et al., 1982).

As many diseases affect fry, it is very important to determine the earliest age when fish can be successfully vaccinated. The success of vaccination appears to be a function of the weight of the fish rather than the age (Johnson et al., 1982 op. cit.; Tatner and Horne, 1983). There may be strong incentives for commencing immunization early in development both to afford protection as soon as possible and for motives of economy and convenience. The earliest age a fish can be effectively vaccinated will differ between species and between vaccines. The immune response in different size groups was assessed in the present study.

Fishes are frequently exposed environmentally to a wide variety of pollutants as well as potential toxic agents and they are capable of accumulating high levels of many chemical toxicants (Brungs et al., 1977). It is known that the immune system of fish is affected by various environmental factors. The pollutants include heavy metals, pesticides, oil, sewage, heat etc. Among heavy metals copper is a major pollutant in natural waters. Although the literature on effects of heavy metals upon fish is extensive (Eisler, 1973; Eisler and Wapner, 1975), little of this research sought information on effects upon fish immune system. The heavy metals on which any such information is available include cadmium, chromium, copper, iron, lead, mercury and zinc. The effect of copper on the antibody production in Etroplus suratensis was evaluated in the present study..

Copper is one heavy metal apparently capable of increasing fish bacterial disease incidence, but whether or not this action is through direct interference with fish immune system remains to be demonstrated (Zeeman and Brindley, 1981).

The immune response of many animals to bacterial antigen has received much attention since Koch (cited by Post, 1966) proved certain diseases are caused by specific microorganisms. Fishes are affected by a variety of bacterial diseases. The walls or capsular polysaccharides of bacteria contain well defined immunogenic groups.

Aeromonas hydrophila is a Gram negative bacteria frequently associated with diseases in Carps, Eels, Milk fish, Channel catfish, Ayu, Tilapia

and Etroplus (Amin et al., 1985; Miyazaki and Jo, 1985). This includes Acute bacterial haemorrhagic septicemia, Red sore disease and secondary infection in furunculosis lesions (Elliot and Shotts, 1980).

In Epizootic Ulcerative Syndrome (EUS), studies conducted on the affected fishes in different countries recorded a varied bacterial fauna but consistently showed the predominance of Aeromonas hydrophila from the ulcerated areas of fish. Infection with A. hydrophila may be the final insult to fish already affected by parasites, injuries or other pathogens. A. hydrophila is considered a major economic problem, but it is difficult to distinguish direct losses and those from secondary infections (Amin et al., 1985 op. cit.). A. hydrophila has been isolated from the ulcers of Etroplus suratensis which is affected by Epizootic Ulcerative Syndrome.

The ability of rainbow trout, Salmo gairdneri to produce antibody against A. hydrophila has been reported by Post (1966 op. cit.). However, no such report on Etroplus suratensis is available in literature. Again, the simple presence of antibody in the fish may not always ensure protection against microbial infection. Hence challenge experiments are needed to be carried out to ascertain the protective capacity of the microbial antigen inoculation.

There is an intimate integration between fish haematological and immunological systems. Macrophages, granular leucocytes and lymphocytes are important in immune responses of fish (Hawkins and Hawdesley-Thomas, 1972; Anderson, 1974 op. cit.; Corbel, 1975 op. cit and Ellis et al., 1976).

Studies on the cellular basis for anitybody formation in various fish have been limited (Smith et al., 1967; Chiller et al., 1969a, 1969b). Studies on haematological changes in fish subsequent to antigenic stimulation is scarce.

Weinreb (1958) reported that exogenous Adreno Cortico Trophic Hormone (ACTH) in rainbow trout produced an 80% reduction of circulating lymphocytes within 24 hrs. following administration. Increase in the number of leucoytes was reported by Klontz (1972a) in rainbow trout which was inoculated subcutaneously with bacterial or viral antigen.

The importance of haematology in the diagnosis of fish diseases has been widely accepted (Schumacher et al., 1956). Lymphocytes are believed to be the executive cells of the specific immune mechanisms. Lymphocytes of several fish species (Plaice, Skate, Carp and Trout) have been shown to carry immunoglobulin in their surface membranes (Ellis, 1977a.). The rejection of transplanted tissues in fish is associated with the graft being surrounded and infiltered by cells possessing the morphology of small lymphocytes (Hogarth, 1973). Chiller et al.,(1969 a op. cit.) reported that majority of rosette forming cells have morphological similarity to lymphocytes indicating the production of antibodies by these cells.

Monocytes are regarded as partially differentiated end cells (Gottlieb and Waldman, 1972). They have the capacity to produce enzyme systems and contain small amounts of oxidases, peroxidases, alkaline and acid phosphatases (Tompkins, 1955). Ellis (1976) suggested the presence of receptors for antibody-antigen complexes on the surface of a small percentage of monocytes.

Neutrophils are the most important of the leucocytes as they show the greatest sensitivity to change in the environment (Mahajan and Dheer, 1979b). Neutrophils of fish infiltrate injured tissues early in an inflammatory response (Thorpe and Roberts, 1972; Joy and Jones, 1973; Roberts et al., 1973). Thrope and Roberts (1972 op. cit.) reported the migration of monocytes and neutrophils from blood vessels into the affected tissue of brown trout which was infected by Aeromonas bacteria. The phenomena of neutrophilia has been reported in several fish species by many workers (Finn and Neilson, 1971; Hines and Spira, 1973).

Macrophages are important in the cellular immune response. Antigen containing macrophages are commonly found in the antibody-producing organs of fish (Klontz, 1972 a op. cit). Ellis, 1974 reported that macrophages are capable of taking up soluble protein antigens like Bovine Serum Albumin (BSA) particularly in the pronephros. He also reported the presence of surface antibody on the cell membrances of about 10% of the macrophages in the spleen and kidney of Plaice, Pleuronectes platessa. Since various blood cells are involved in the immune response, an investigation into their differentiation and characterization is needed to be carried out.

Brief Definitions

Adjuvant: Any substance which when administered with (or before) an antigen heightens, or affects qualitatively, the immune response to that antigen.

Agarose: A component of agar

Antigen: Any agent which initiates antibody formation and/or induce a state of active immunological hypersensitivity.

Antibody: A glycoprotein molecule produced in the body in direct response to the introduction of an antigen.

Gel diffusion: A procedure in which antibodies and antigens diffuse independently through a gel medium forming a precipitate within the gel where homologous antigens and antibodies meet in optimal proportions.

Titre: A measure of the concentration of antibodies (or antigen) in a given sample. It is the highest, dilution of the sample which gives a positive serological reaction, (eg. agglutination, precipitation) with antigen (or antibody) under the conditions of the titration.

MATERIAL AND METHODS

MATERIAL AND METHODS

Experimental animals

The Pearl spot Etroplus suratensis was collected by cast net from brackish water ponds of Matsyafed, Narakkal. In order to minimise population differences, fishes were collected from same ponds throughout the study. The salinity, temperature and pH of the water at the collection site were determined and water of the same quality was used for keeping the experimental animals initially in laboratory to minimise the stress.

Acclimation

The animals were disinfected by immersing them in 0.5 ppm $KMnO_4$ solution for 5 minutes on arrival at laboratory. Acclimation of the animals to the laboratory condition was done by maintaining them in fibre glass tanks of 1 ton capacity containing water with salinity 6 ± 1‰, pH 6.2 ± 0.5 and temperature 22 ± 2°C. The animals were fed two times a day with dry pelletised feed and wet feed (clam meat). The pelletised feed was prepared using Fish meal (25%), Soya Bean meal (25%), Tapioca powder (25%), Ground nut oil cake (20%), Gelatin (2%), Soya oil (1.5%), Mineral premix (1%) and Vitamin premix (0.5%). The faecal matter and other waste materials were daily siphoned off, and to reduce ammonia content in water, the biological filter was set up. The water in the tank was changed in every second day. Electrically operated aerators were used for aeration continuously. The animals were acclimatized for 2 weeks prior to the experiments.

Test containers

Fibreglass tanks of 100 litre capacity were used as test containers. Each tank was covered with velon screen netting to prevent the jumping out of the animals.

Antigens

Soluble protein antigens such as ovalbumin and Bovine Serum Albumin (BSA) and bacterial antigen Aeromonas hydrophila EMU 20 were used as antigens in the present study.

A. Bacterial antigen

Bacterial culture of Aeromonas hydrophila EMU 20 was obtained from the Microbiology Laboratory of School of Environmental Studies, Cochin University of Science and Technology. The bacteria was cultured by lown culture method on plates of Zobell's agar (Peptone 0.5%, Yeast extract 0.1%, Ferric phosphate 0.01%, Beaf extract 0.5%, Agar 2% and Seawater 1 ml with pH 7.5).

After 18 hrs. of incubation, the culture was harvested into sterile 0.85% NaCl solution in double distilled water. The cells were killed by adding 0.5 ml of 1% formalin to the culture and maintaining at 37°C for 12 hrs. The cells were centrifuged and washed repeatedly in saline. The cell suspension was adjusted to contain approximately 1×10^8 cells in 0.2 ml.

B. Soluble protein antigens

Ovalbumin and Bovine Serum Albumin (BSA) were prepared by dissolving 0.5 g ovalbumin flakes (Loba-Chemie Indo Austranal Co.) and BSA powder (SISCO, India) in 100 ml of physiological saline (0.85% NaCl). It was filtered through Whatman No. 1 filter paper.

Adjuvant

The antigens were inoculated after homogenizing with the adjuvant. The adjuvant was prepared by homogenizing 1% Tween 80 (Hi-media) in paraffin oil (s.d. Fine - Chem. Ltd.) phase at 30:70 ratio (Ward et al., 1985).

Anesthetization

Fish was anesthetized in 0.4 ml phenoxyethanol per litre of water or by a mild eletric shock.

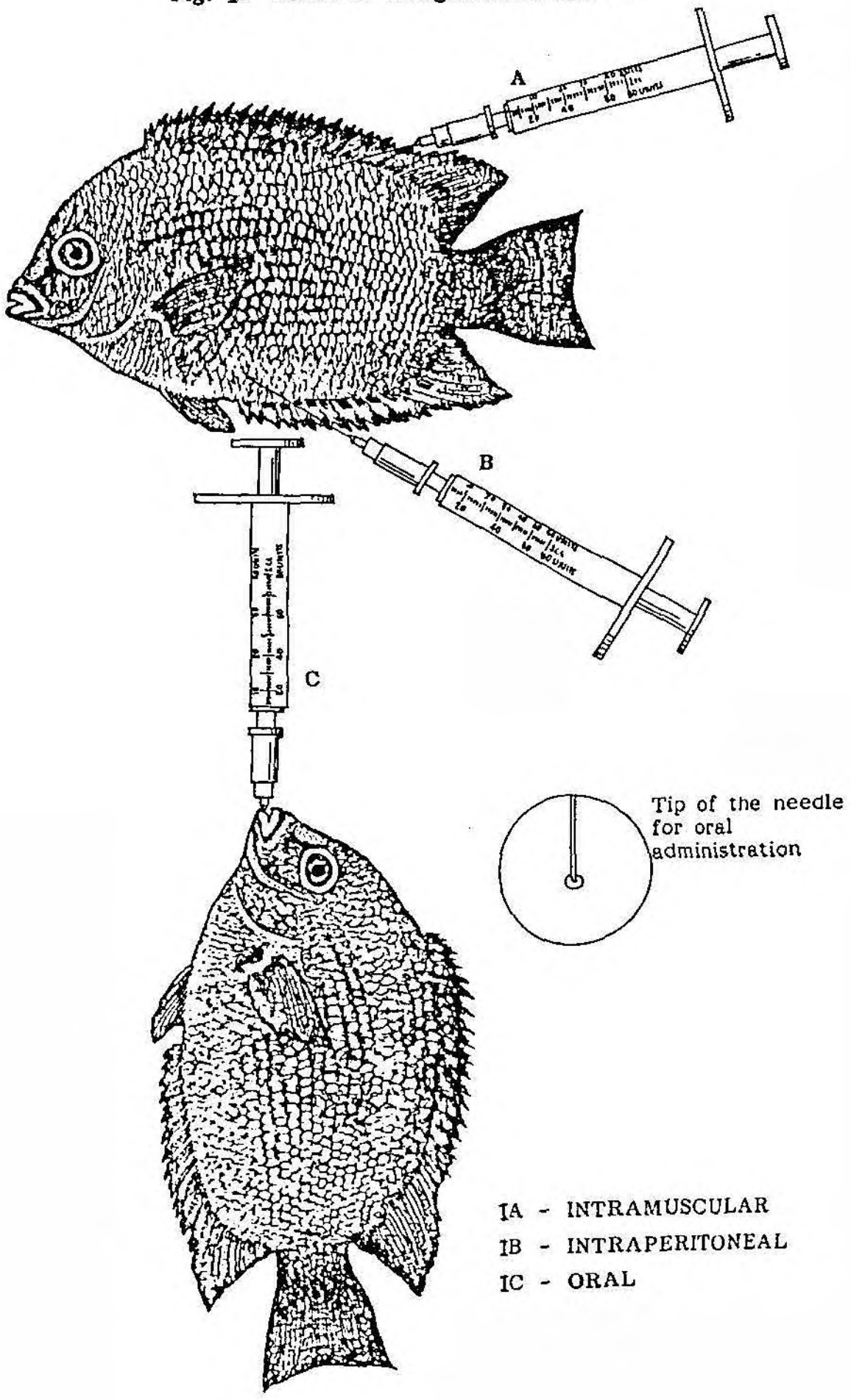
Inoculation

The antigens were administered through either intramuscular or intraperitoneal or oral routes.

a. Intramuscular Inoculation

The anesthetized fish was gently, yet firmly held in the left hand, facing away from the handler. The inoculation needle was inserted at a site lateral and middle to the dorsal fin. The needle was pointed down into the soft musculature and slowly deposited the antigen (Fig. I A).

Fig. I. Routes of Antigen administration



Then the needle was slowly removed to avoid tearing of the tissues and leakage of the inoculum from the site of deposit. The site was gently massaged with cotton soaked in rectified spirit to prevent the infection at the site of inoculation.

b. Intraperitoneal inoculation

The inoculation needle was inserted at a site laterally and immediately anterior to the anus (Fig. 1B). Care was taken not to penetrate the internal organs in the area.

c. Oral inoculation

Oral administration of the antigen was carried out following the method adopted by Mughal and Manning (1985). In this method the mouth of the fish was opened and the needle was introduced through the mouth opening and passed back into the buccal cavity towards the opening of the oesophagus where the antigen was delivered cautiously. In order to avoid any injury from the pointed end of the needle, a plastic sphere of 2 mm diameter having a central hole was inserted at the tip of the needle as shown in Fig. 1C. The fish was held vertically in water for 3 minutes and released.

Blood collection

Blood collection was done aseptically from anesthetized fish by heart puncture using sterile hypodermic 2.5 ml syringe. The point of insertion of the needle in Etroplus is lateral, midway between the base

Plate I. Photograph showing a part of the experimental set up.

Plate II. Collection of blood from anesthetized fish by heart puncture.

of pectoral and pelvic fin at an angle of about 40° with the body surface of the fish (Plate II). A slight negative pressure was kept on the plunger to facilitate ready flow of blood into the syringe as soon as the lumen of the heart is entered. The needle was then held at that position until the desired amount of blood was drawn. Care was taken not to damage the internal organs in that area. The advantages of heart puncture are that the collection of blood is without sacrificing the animal and sequential blood collection is also possible.

The blood samples from different fishes were kept separately in small, sterile glass bottles and allowed to clot and retract in room temperature for 1 hour. Then the blood samples were kept in sterile conditions for 24 hours at 4°C. The serum was separated asceptically and centrifuged at 4000 r.p.m. for 10 minutes. The supernatent serum was collected and heated for 15 minutes at 45°C, in a water bath. Then the serum was used for antibody detection tests.

Antibody detection

Antibody was detected and quantified by precipitation and agglutination tests (Davis et al., 1968).

A. Precipitation test

When an antigen is added to the antiserum, the complexes that form become insoluble and precipitate from solution. From the time of its discovery in 1847, the precipitin reaction was used extensively as

a qualitative or semiquantitative assay for estimating antibody titres in sera. Precipitation reaction can be carried out in liquid media as well as in gel.

a) Precipitin reaction in liquid media

The antiserum from each fish was taken in clean sterile test tubes. The serum was diluted to different dilutions of 0, 10, 20, 50, 80, 100, 120, 150, 180, 200, 250, 280, 300, 330, 350, 380, 400, 430, 450, 480, 500, 530, 550, 580, 600 with physiological saline (0.85% NaCl). To each test tube of antiserum 0.2 ml of antigen was added through the sides of the test tube without shaking and kept in room temperature overnight and examined for preipitation ring or any cloudiness at the junction of the two phases (Davis et al., 1968 op. cit.; Anderson, 1974 and Cruichshank et al., 1975).

b) Precipitin reaction in agarose gel

When antibodies and antigen are inroduced into different regions of an agarose gel, they diffuse freely towards each other. If their reaction can lead to precipitation readily visible opaque bands of precipitate appear at the junction of their diffusion fronts. Gel diffusion can be conducted by the following methods.

i) Single Diffusion in One Dimension

This was carried out as per the method described by Oudin in France. 0.65% agarose was dissolved in phosphate buffer pH 7.2 containing

0.1% sodium azide. The agarose was completely dissolved at 100°C in a water bath. Then it was cooled to 50°C and mixed with the antiserum (0.1 ml antiserum in 0.5 ml agarose solution) in a sterile test tube and cooled to solidify. Antigen solution was layered over the solidified agarose. The preparation was kept at room temperature overnight and examined for the development of precipitation band.

ii) Double Diffusion in One Dimension

This was carried out as per the method described by Oakley and Fulthrope, 1953. In this method the antiserum in agarose is overlaid by a column of 0.65% agarose which in turn is overlaid by antigen, added either as liquid or incorporated into agarose gel in a clean, sterile test tube.

iii) Double Diffusion in Two Dimensions

This was performed by the simple and elegant procedure developed mainly by Ouchterlony in Sweden (Ouchterlony, 1949). This was done using 0.6% agarose in 4% saline containing 0.1% soidum azide. The agarose gel was poured into small, flat bottomed petri dishes or microslides to give a perfectly even surface. When this was cooled and solidified, wells were cut in the agarose. The wells were sealed with one drop of melted agarose. Then separate wells were filled with antigen and antiserm. The agarose plates were covered and placed in a damp cool chamber. Diffusion is allowed to occur at room temperature. The plate was examined by means of incident light after 12-18 hours.

B. Agglutination test

Agglutination test was used for the detection of antibodies against the bacterial antigen, Aeromonas hydrophila. Slide agglutination test was carried out on ordinary glass slide. A drop of saline (0.15 M NaCl) was placed on the slide and a small amount of bacterial culture from a solid medium emulsified in it by means of an inoculating loop. It was then examined under the microscope to ascertain that the suspension of the bacteria was well separated and not in visible clumps. A drop of antiserum was placed on the slide just beside the bacterial suspension with the help of a platinum loop. They were mixed and incubated for 15-20 minutes. This was made to a smear and the bacteria was stained using Gram's sufranine solution (Merck) for 30 seconds and washed with double distilled water. The preparation was examined under the microscope. Serum was diluted to different dilutions and performed the agglutination test and determined the agglutination titre values.

EXPERIMENTAL DESIGNS

Humoral Immune Response

Preliminary trials were carried out to ascertain the ability of Etroplus suratensis to respond immunologically to soluble protein antigens such as ovalbumin and Bovine Serum Albumin (BSA) as well as bacterial antigen viz., Aeromonas hydrophila. These trials indicated humoral immune response to all these antigens. However, considering thicker precipitation band formation with ovalbumin, it was selected as the antigen of choice for detailed investigations on differential response to various doses of

antigen and different routes of antigen administration as well as the effect of fish body size on immunological response.

The kinetics of primary response and secondary response were also investigated using ovalbumin. An experiment to determine the immunosuppressive effect of the heavy metal copper (Cu) was also carried out as a part of this study using ovalbumin. The magnitude of primary and secondary immune response to ovalbumin, Bovine Serum Albumin (BSA) and Aeromonas hydrophila inoulation in Etroplus suratensis was also compared.

Experimental fishes in the weight group of 80 - 150 g were used in all these studies except the one to determine the effect of fish size on immune response in which case animals weighing 5 g and above were also included. The details of the various investigations are presented hereunder.

EXPERIMENTS USING OVALBUMIN

1. Dose determination

A trial was carried out to compare the primary and secondary immune response to different levels of ovalbumin inoculation and to determine the suitable dose for further studies. Ovalbumin doses in geometric scale such as 12.5, 25 and 50 ug/g body weight were tested. For this study 40 fishes in the 80 - 130 g size group were divided into four groups at random. The first group served as the control while the second, third

and fourth group received ovalbumin inoculation at the rate of 12.5, 25 and 50 /ug/g body weight respectively. Control group was maintained without antigen administration.

Blood was collected on 9th day and serum from each fish tested for precipitation titre values individually. The animals were given a booster inoculation on 10th day at the same dose as that of the primary inoculation but without adjuvant. The animals were bled five days after the administration of booster dose and antibody titre in the sera was again determined.

2. Comparison of the routes of antigen administration

Antibody production can vary with the route of antigen administration. Hence a study was carried out to compare the immune response to ovalbumin inoculation through two different routes viz., intramuscular and intraperitoneal routes. Ovalbumin at the rate of 25 /ug/g body weight was administered intramuscularly in one group of 12 fishes while another group of 12 fishes received the same dose intraperitoneally. The control group was maintained without antigen administration. Booster dose was administered to both groups on 10th day.

Blood samples were collected from first day onwards till the antibody was detected in the sera. Again blood samples were collected on 9th and 15th day. Antibody precipitation titre values were determined to evaluate the primary and secondary immune response using sera from 9th and 15th day blood samples respectively.

3. Study of immune response in different size groups

It is important to know at what age the fish immune system becomes reactive to a foreign material. So humoral immune response in different size groups were evaluated. The size groups selected for the study were given below.

Group	Mean length with S.D. in cm.	Mean weight with S.D. *in gms.*
I	6.4 ± 0.9	5.074 ± 3.5
II	8.6 ± 1.2	15.133 ± 5.4
III	11.2 ± 1.4	32.49 ± 6.9
IV	16.2 ± 1.7	100.21 ± 20.5

Ovalbumin was administered intramusularly at the rate of 25 /ug/g body weight. Blood samples were collected on 9th day from all the groups and again on 15th day which was after the booster dose on 10th day. The sera were tested for precipitation titre values.

4. Kinetics of antibody production

Concurrently with the earlier described trials a group of 12 fish were inoculated with ovalbumin intramuscularly at the rate of 25 /ug/g body weight. Pooled blood samples were collected from 3 fishes daily from first day onwards till the 19th day. The sera from the daily blood samples were tested for the presence of antibody to determine the day of antibody appearance. Once the antibody was detected, the samples were diluted serially and percipitation titre value determined to monitor

the daily change in antibody titre on account of primary response.

Blood from a second group which had received a booster dose also of 25 µg/g body weight on 10th day was collected on 12th, 15th, 24th and 126th day to monitor the secondary response through antibody precipitation titre values.

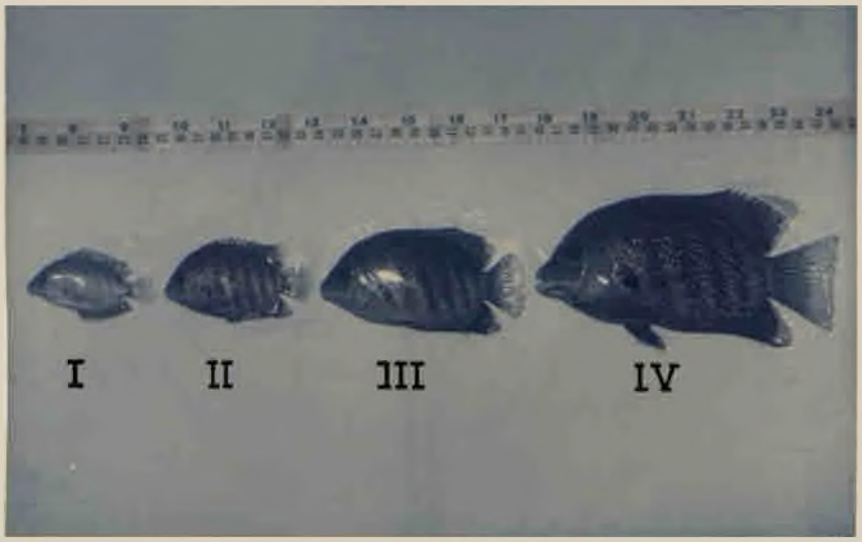
5. Immunotoxicity of heavy metal Copper (Cu)

To study the immunotoxic effect of short term exposure of fish to heavy metal copper an experiment was carried out using two groups of fishes. The group (i) was used as control. The group (ii) was exposed to the heavy metal Cu at 5 ppm level in water for 3 hours and then transferred to water free of copper. The total hardness of the water was 838 mg $CaCo_3$/l and was determined by the standard method of APHA, 1955.

After two days, ovalbumin was administered intramusularly to both groups of fish at the rate of 25 µg/g body weight. Booster dose was given on 10th day.

Blood samples were collected from both groups of fish on 4th, 5th, 7th and 9th days to determine primary response. Again blood was collected on 15th and 31st days to assess secondary response. All the sera were tested by precipitation test and determined the titre values.

Plate III. Different size groups of animals (I, II, III & IV) used to compare immunological response.


I
II
III
IV

EXPERIMENT USING AEROMONAS HYOROPHILA EMU 20

Formalin killed Aeromonas hydrophila EMU 20 cells were administered through intramuscular, intraperitoneal and oral routes at the dose of 1×10^8 cells. A group of control fish also was maintained. Blood samples were collected on 12th day after the primary dose. Booster dose was given on 19th day and blood was collected again on 24th day from all the groups. Sera were tested for antibodies by agglutination and precipitation tests and determined the titre values.

All the above groups of fish (immunized and non-immunized) were challenged with live Aeromonas hydrophila intraperitoneally at a dose of 1×10^4 cells.

Cellular response to soluble protein inoculation

Attempts were made to study the cellular changes in response to the inoculation of soluble protein antigen. For this purpose blood samples were collected from the fish inoculated with ovalbumin at the rate of 25 μ/g body weight as well as the control group both before the inoculation and 15 days after the inoculation. Booster dose was administered on 10th day of primary dose administration. Various haematological parameters were studied using standard techniques.

1. Blood cell counts

Improved Neubauer ruling haemocytometer was used for blood cell count.

A. Erythrocyte count

The pipette with red glass bead was used for charging the counting chamber. The RBC pipette gives a dilution of 1:200 when blood is drawn to the 0.5 mark and diluted upto 10 ml mark. Hayem's solution with the composition of Mercuric chloride 0.5 g. Sodium sulphate 5 g. Sodium chloride 1 g and double distilled water 100 ml was used as the diluent. All counting were done in triplicates.

Calculation

The number of red cells per cu.mm. of blood

$$= \frac{\text{Number of smaller squares counted}}{\text{No. of red cells counted x dilution}} \times 400$$

When dilution is 200 times the formula is

$$= \frac{\text{No. of red cells counted x 200 x 400}}{80}$$

B. Leucocyte count

The pipette with white glass bead was used for charging the counting chamber. The WBC pipette gives a dilution of 1:20 when blood is drawn to 0.5 mark and diluted upto 11 mark. The diluting fluid used was Turk's solution which is 8% acetic acid solution coloured by 1 ml. of Gension - Violet stain and made upto 100 ml. with double distilled water. Counting was done under high power of microscope with light adjusted to the minimum.

Calculation

The number of white cells per cu.mm. of blood

$$= \frac{\text{Number of white blood cells counted x 10 x 20}}{4}$$

2. Typing of leucocytes

An attempt was made to identify the different types of leucocytes of Etroplus suratensis on the basis of morphology and staining characteristics. Stained blood smears on microslide were prepared for this purpose.

Preparation of blood smears

Smears of the circulating blood sample obtained by cardiac puncture were prepared (after making sure all cells have been resuspended) by placing a drop of the sample on one edge of a clean and sterile slide and distributing it the length of the slide by another slide held at 45° to the first. The smear was allowed to air dry, immersed in absolute methanol for 5 minutes for fixation and again air dried before staining.

Staining

Different Romanowsky stains were tried i.e., Weight's, Leishman's and Giemsa's. Of these Giemsa's stain (Merck) was found to give the best results. Blood smears were stained with Giemsa (1 ml. Giemsa : 9 ml. distilled water) for about 18 minutes. The slides were gently washed in distilled water with pH adjusted to 6.5 and dried. The slides were examined under light microscope and different leucocytes were identified.

3. Morphological and morphometric studies of RBC

Morphometric measurements of red cells were made with an ocular micrometer standardized with stage micrometer as parallel magnification. The diameter of the cell or nucleus was calculated by adding the mean

length and width and then dividing the total by two. The values represented the mean for a total for seventy cells. The nucleus to cell diameter ratio (n-c ratio) was calculated by dividing the whole cell diameter by that of the diameter of the nucleus (Mahajan and Dheer 1979a).

Coloured photomicrographs were made in NIKON OPTIPHOT No.1 (Japan) Microscope using 35 mm 100 X G Konica colour negative.

Statistical Analysis

All antibody titre values were converted into log 2 values and these values only were used for the estimation of mean titre values, standard deviation (S.D.) as well as for all other statistical analysis. One way Anova (F test) followed by critical difference analysis was carried out to assess the significance of difference in antibody production in response to different doses of antigen. Student's 't' test was carried out to ascertain the significance of difference in antibody production resulted from different routes of antigen administration. The significance of difference in the antibody production in response to ovalbumin and Bovine Serum Albumin in the same dose and route of administration was also evaluated by Student's 't' test. Chi-square test was applied to evaluate the data on the effectiveness of immunization in protecting the fish against Aeromonas hydrophila infection.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

The humoral immune response elicited in Etroplus suratensis monitored by precipitation test and/or agglutination test as well as the associated cellular response observed in the different experiments are presented below.

Humoral Immune Response

Preliminary trials

Preliminary trials with ovalbumin, Bovine Serum Albumin (BSA) and Aeromonas hydrophila resulted in the production of antibodies. Presence of these antibodies in blood serum was demonstrated by precipitation and agglutination tests. Thus the preliminary trials indicated that Etroplus suratensis is capable of mounting immunological response to these antigens. However, ovalbumin produced thicker preciptiation bands in the precipitation reaction in liquid media suggesting its strong immunogenic capacity in Etroplus suratensis.

Precipitation was observed in liquid media (Plate IV) as well as in agarose gel by single diffusion in one dimension and double diffusion in one dimension (Plate V). Double diffusion in two dimensions did not result in consistent precipitation lines.

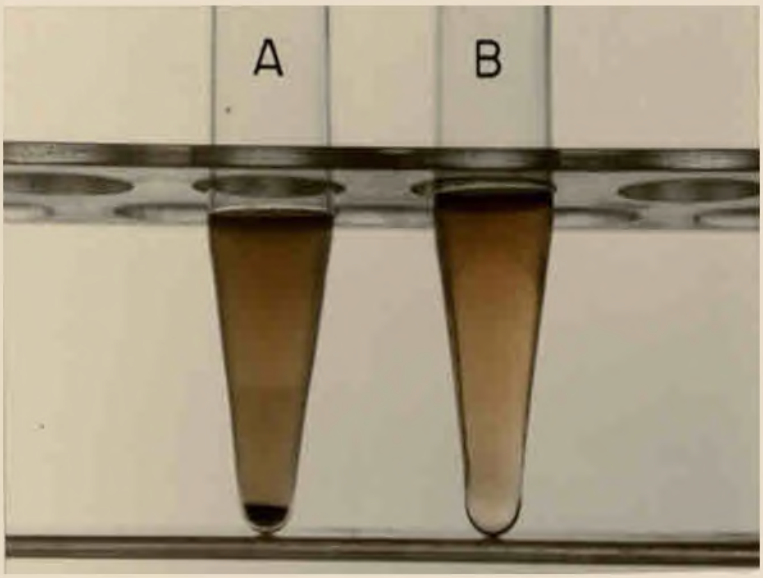
EXPERIMENTS USING OVALBUMIN

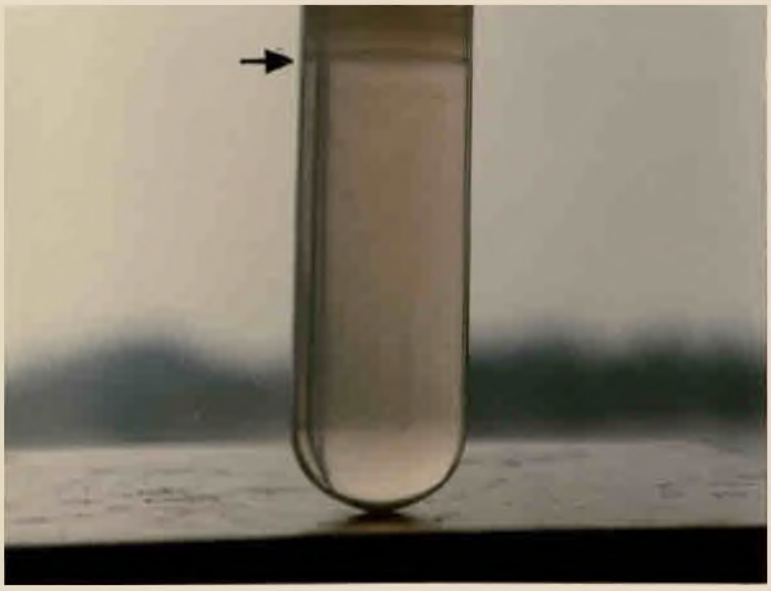
1. **Immune Response to different doses**

The primary immune response on 9th day and secondary response on

Plate IV. Photograph depicting the precipitation reaction in liquid medium. The centrifuging tube A contains serum from the treated fish. Tube B contains serum from control animals. Both the tubes were finally layered with a solution of ovalbumin. The preparations were centrifuged and precipitate had settled to the bottom.

Plate V. Photograph showing precipitation by double diffusion in one dimension in agarose gel column. The arrow mark points to the precipitation band in the gel column.


A
B



15th day in three groups of experimental fishes that received intramuscular inoculation of ovalbumin at the rate of 12.5, 25 and 50 µg/g body weight on first day (with adjuvant) and again on 10th day (without adjuvant) were determined along with the control group which did not receive any antigen. The responses in different groups measured as precipitation titre values using serially diluted blood serum collected on 9th and 15th day were converted to log-2 values for better comparison. While all the fishes that received ovalbumin inoculation produced precipitation band both on 9th and 15th day, the same could not be detected in control group. Comparison of the magnitude of response are presented below.

Primary Response

The mean precipitation titre values are presented in Table 1 and Fig.II. It can be seen from the above table that the highest primary response was from the inoculation of 25 µg ovalbumin/g body weight. The mean precipitation titre in this group was 8.058 ± 0.22 compared to 7.618 ± 0.28 and 6.388 ± 0.59 that received the lower dose (12.5 µg) and higher dose (50 µg) respectively.

One way Anova (F test) carried out to ascertain the significance of difference in the primary response indicated a highly significant difference ($P < 0.01$) among the primary response to different doses of ovalbumin inoculation.

The critical difference of the mean antibody titre values of primary responses were estimated to find out means which differed significantly from one another.

The critical difference estimates indicated that though the mean titre value obtained from the dose of 25 /ug/g body wt. was higher than that of 12.5 /ug dose, the difference was not statistically significant. However, the response from both 12.5 /ug and 25 /ug inoculations were higher than that of 50 /ug inoculation and this differences were highly significant statistically ($P < 0.01$).

The weak immune response with high dose (50 /ug/g body wt.) may be due to the tolerance. In fish, phenomena suggestive of tolerance have been described by Lopez et al., (1974); Avtalion et al., (1980 op. cit.); Manning et al., (1982 op. cit); Wishkovsky and Avtalion (1982 op.cit.). They have observed immunosuppression or tolerance with high doses of antigen. Serero and Avtalion (1978) demonstrated tolerance in Carp using high doses of BSA. The present study is in agreement with the above reports.

Secondary Response

As can be seen from the Table 1, the secondary response was higher than the primary response ($P < 0.01$) in all cases. The mean precipitation titre values were 8.506 ± 0.15, 8.574 ± 0.16 and 8.814 ± 0.09 (expressed in log 2) in the low, medium and high doses respectively.

Amnestic response after the booster dose was reported by Fujii et al., (1979) in Lamprey and Rijkers (1982 b op. cit.) in Carp using Sheep Red Blood Cells (SRBC), Mughal and Manning (1985 op. cit) in Grey mullet using Human Chorionic Gonadotrophin (HCG) and Sudha (1990 op. cit) in Tilapia mossambica using ovalbumin as antigen.

One way Anova showed that the antibody titre values on 15th day also differed significantly. Analysis of critical difference showed highly significant difference in the mean antibody titre values between low and high doses (12.5 and 50 /ug/g body wt.) as well as medium and high doses (25 and 50 /ug/g body wt.), ($P < 0.01$). As in the case of primary response, though the antibody titre values of medium dose (25 /ug/g body wt.) was higher than that of lower dose (12.5 /ug/g body wt.), this was not significant statistically.

The fishes which received 50 /ug ovalbumin/g body wt. developed necrotic lesion and growth of wool like fungus at the incoulation site. Such cases have been reported by other workers also. Considering the high primary response and absence of tissue damage, 25 /ug/g body weight was selected as an appropriate dose for inoculation of ovalbumin for further studies.

2. Comparison of the routes of antigen administration

The appearance of antibodies in the sera of the two groups of fishes which were inoculated with ovalbumin at the rate of 25 /ug/g body weight intramuscularly or intraperitoneally were monitored from the next day onwards along with the control group which received no antigen. These fishes were maintained at 23°C.

Antibody was detected on 3rd day in the sera of fish which received i.m. inoculation whereas in i.p. inoculation it was on 6th day. Sailendri and Muthukkaruppan (1975) reported that anti-BSA and anti-Sheep Red Blood Cell (SRBC) antibodies appeared within two days in the serum of

Tilapia mosambica after intravenous immunization at a dose of 25mg/ animal at 30°C. It is a well known fact that the antibody production is influenced by a variety of factors including the nature of the antigen, temperature, dose and route of entry. In the present study intramuscular route of ovalbumin administration has induced an early production of antibody.

Primary and secondary responses were worked out using the sera collected on 9th day after primary inoculation and 5th day after booster dose respectively. The mean precipitation titre values of both i.m. and i.p. routes are presented in Table 2 and Fig. III. The mean titre values for 9th day sera (expressed in log 2) were 8.058 ± 0.22 for i.m. inoculation and 6.531 ± 0.48 for i.p. inoculation.

The sera collected on the 5th day after booster dose gave mean precipitation titre value of 8.574 ± 0.16 (serum dilution 1:381) and 7.588 ± 0.18 (serum dilution 1:192) in the i.m. and i.p. routes respectively.

Student's 't' test carried out to assess the equality of mean titre values of i.m. and i.p. routes of antigen administration indicated highly significant difference between the two ($P < 0.01$). This result indicate that the i.m. inoculation of ovalbumin produce significantly higher secondary response as compared to i.p. inocuation.

Rijkers et al., (1980 a op. cit.) reported that the magnitude of the primary and the secondary response is route dependent. He has reported

Table 1. **Antibody production in response to different doses of ovalbumin.**

Dose /ug/g body wt.	Route	Mean Precipitation Titre value with S.D.		
		9th day (Primary response)	Booster dose	15th day (Secondary response)
12.5	i.m.	7.618±0.28a	10th day	8.506±0.15a
25	i.m.	8.058±0.22b	10th day	8.574±0.16b
50	i.m.	6.388±0.59a,b	10th day	8.811±0.09a,b

i.m. -intramuscular.
aa, aa: Highly significant ($P<0.01$)
bb, bb: Highly significant "

Table 2. **Antibody production in response to different routes of ovalbumin administration**

Dose /ug/g body wt.	Route	Anti-body Dete-ction	Mean Precipitation Titre value with S.D.		
			9th day (Primary response)	Booster dose	15th day (Secondary response)
25	i.m.	3rd day	8.058±0.22**	10th day	8.574±0.16**
25	i.p.	6th day	6.531±0.48	10th day	7.588±0.18

i.p. - intraperitoneal

** Highly significant ($P<0.01$)

Fig. II **Comparison of antibody production to different doses of ovalbumin**

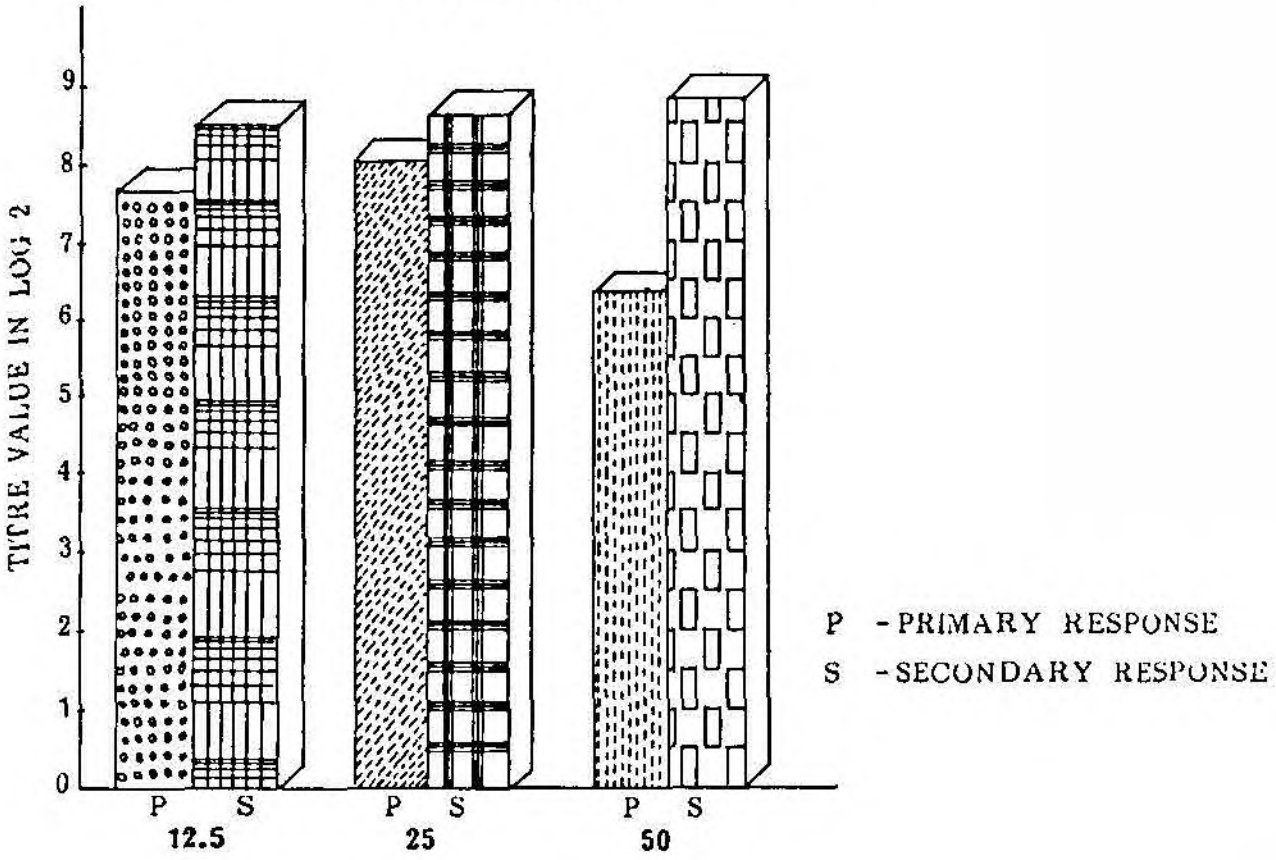
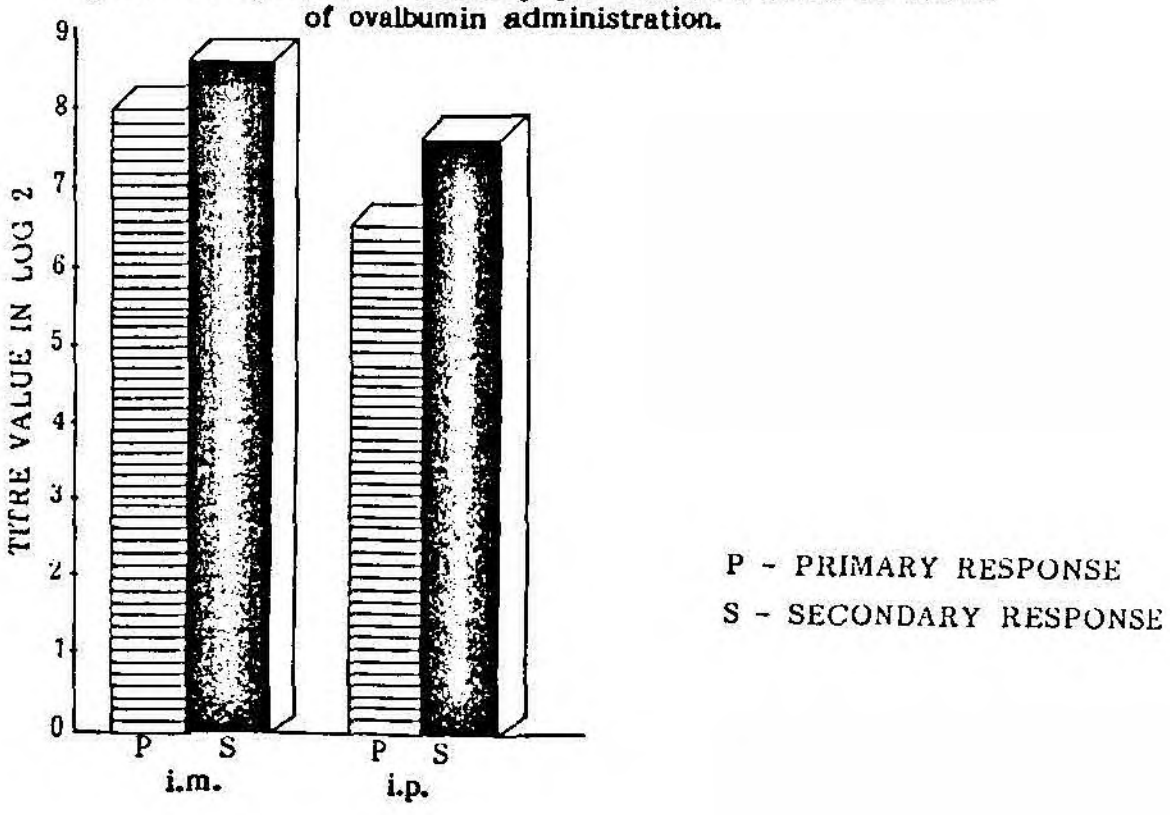


Fig.III. **Comparison of antibody production in different routes of ovalbumin administration.**



that Carp primed with sheep erythrocytes intramuscularly developed better response as compared to the intravenous route. Several authors have reported that intramuscular injection of antigen elicits higher antibody titres than intraperitioneal injection. Mughal and Manning (1985 op.cit.) have reported that in Carp, Cyprinus carpio i.m. inoculation of Human Chorionic Gonadotrophin (HCG) produced antibody titre of 9 (expressed in log 2) whereas i.p. inoculation produced antibody titre of only 8 (expressed in log 2) at 18-22°C. The observations in the present study also compares well with the above reports.

3. Immune response in different size groups

Antibody titres were determined on 9th day in all the four size groups viz, I, II, III and IV which were inoculated with ovalbumin intramuscularly at a dose of 25 /ug/g body weight. Control fishes received no antigen. All fishes were given a booster dose on 10th day. The secondary response was evaluated on 15th day.

The mean precipitation titre values were 8.22, 8.13, 8.20 and 8.07 on 9th day and 8.78, 8.81, 8.72 and 8.80 on 15th day in the groups I, II, III and IV respectively (Table 3). One way Anova showed no significant difference between these groups in the antibody production neither on 9th day nor on 15th day. It can be concluded that Etroplus suratensis is capable of mounting humoral antibody titres even at the size of 6.4cm in length and 5g in weight like an adult one. Even at this size also, the immune system is well developed as in the adult fish to produce specific antibodies against an antigen.

Table 3. **Antibody production in different size groups (Antigen: ovalbumin)**

Group	Dose µg/g body wt.	Route	Mean length in cm. with S.D.	Mean weight in g. with S.D.	Mean Precipitation Titre value		
					9th day *(Primary response)*	Booster dose	15th day *(Secondary response)*
I	25	i.m.	6.4±0.9	5.07±3.5	8.22	10th day	8.78
II	25	i.m.	8.6±1.2	15.13±5.4	8.13	10th day	8.81
III	25	i.m.	11.2±1.4	32.49±6.9	8.20	10th day	8.72
IV	25	i.m.	16.2±1.7	100.21±20.5	8.07	10th day	8.80

i.m. - intramuscular

Similar results have been reported by many authors in other fishes. According to Amend and Johnson (1981) and Tebbit et al., (1981), the minimum size of Salmonids for successful vaccination against Vibriosis and Enteric red mouth disease is about 1g. though the treatment is most effective on fish over 4.5g. Sailendri and Muthukkaruppan (1975 op. cit.) reported that young adults of Tilapia mossambica (2.5-3 months old) can mount both humoral and cellular responses. In this species immunological maturation occurs at 1 to 2 months of hatching (Sailendri 1973). Ward et al., (1985 op. cit.), reported that Salmo gairdneri of 0.15g are capable of mounting a low level of immune response to Vibrio anguillarum vaccine and this protective response increased as the fish grows, to the threshold level of 0.5g and the vaccination becomes more efficient. Paterson and Fryer (1974) reported that Coho Salmon, Oncorhynchus kisutch even at the size 1.2g can be vaccinated successfully against Aeromonas salmocida and their immune state persists for at least 1 year after injection.

The result in the present study also suggests the possibility of immunization of Etroplus suratensis in the very young stage of 5g itself against infectious diseases and expect a degree of protection. It was difficult to extent this study to still smaller animals because of the difficulty in the collection of sufficient volume of blood. However, further studies with still smaller animals are needed to determine the lower threshold size/age of immune response in this species.

4. Kinetics of antibody production

The first appearance of precipitating antibody in the serum and

the daily change in the precipitation titre values were monitored using pooled blood from the animals which were inoculated with ovalbumin at the rate of 25 µg/g body weight. The daily changes in the primary response were estimated for 19 days in the animals that received only the primary dose. The change in secondary response was estimated from the blood collected on 12th, 15th, 24th and 126th day from the animals which received the booster dose on 10th day. The kinetics of the antibody response are presented below. The graphic representation of the same is given in Fig. V.

Primary response

The precipitating antibody was detected in the serum on 3rd day where the mean titre value was 1 (expressed in log 2) with corresponding serum dilution of 1:2. On 4th day the mean titre value increased suddenly to 4.9 where the serum dilution was 1:30. Thereafter the precipitation titre increased only gradually and reached the peak value on 9th day. At the peak, the mean precipitation titre was 8.18 (log 2) with the serum dilution of 1:290. Thereafter a gradual decrease in the titre values were observed over the days. Mean titre values were 7.32, 6.64, 6.12 and 5.9 on 12th, 14th, 16th and 18th day respectively. On 19th day the mean titre value declined to 5.70 where the serum dilution was 1:60.

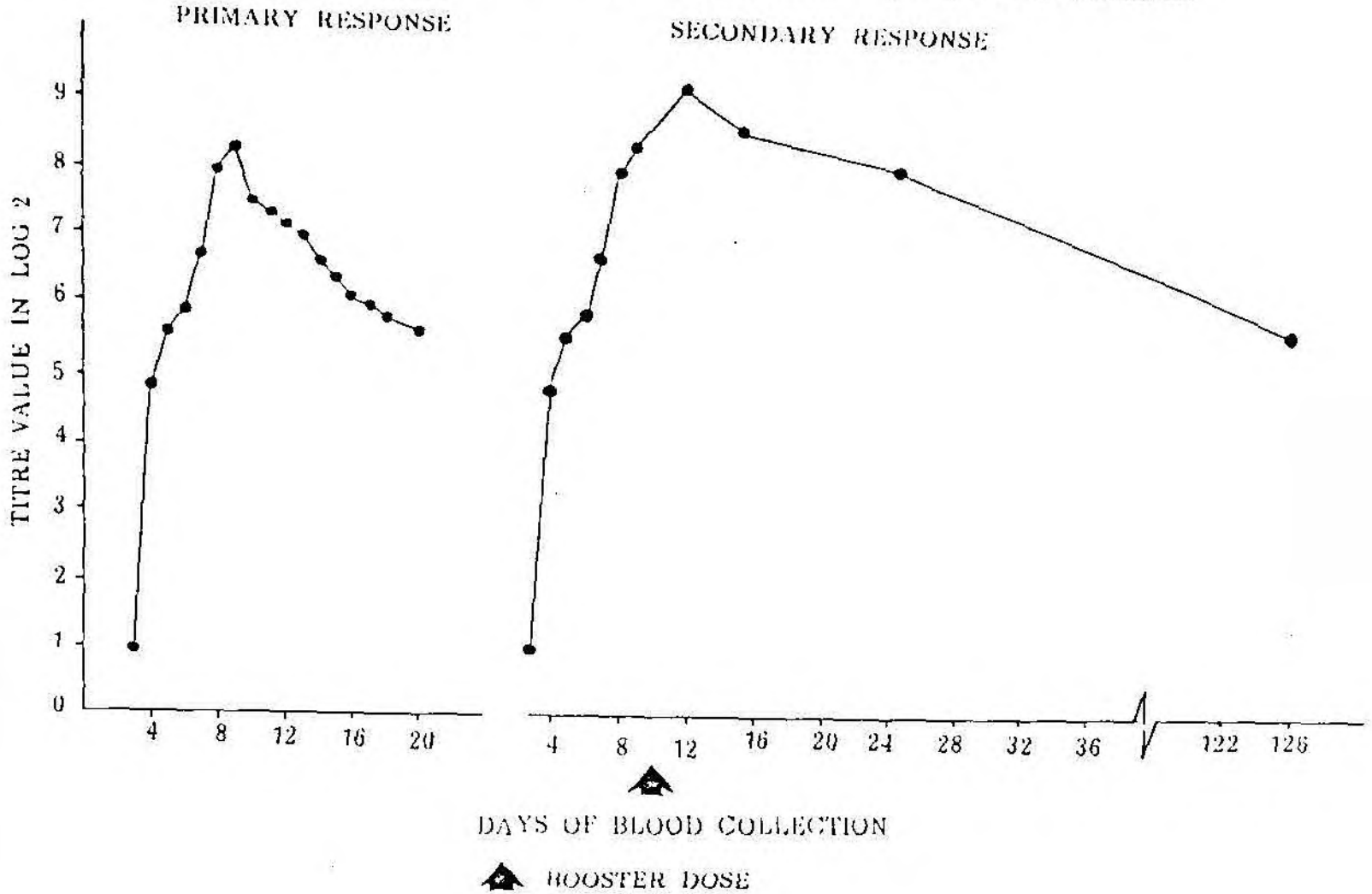
Secondary response

The fishes received the booster dose on 10th day showed a sudden increase in the titre. The mean titre value reached 9.1 (serum dilution 1:550) on 12th day as against 8.18 (serum dilution 1:290) on 9th day. Titre value showed a decreasing trend thereafter with 8.64 on 15th day

Table 8. Comparison of Precipitation Titre values in fish with and without booster dose

Day	Fish without booster dose		Fish with booster dose on 10th day	
	Serum dilution	Titre value in log 2	Serum dilution	Titre value in log 2
9	290	8.18	290	8.18
12	160	7.32	550	9.1
15	85	6.41	400	8.64
19	60	5.70	-	-
24	-	-	260	8

Fig. V. Kinetics of primary and secondary response to ovalbumin



and 8 on 24th day. The sera collected from these fishes on 126th day gave a mean precipitation titre value of 5.64 (serum dilution 1:50). A comparison of titre values of fish without booster dose and with booster dose is presented in the Table 8.

The result shows that the secondary response was higher and prolonged than the primary response. In the fish which were given booster dose, the presence of specific antibody could be demonstrated even on 126th day. Prolonged existance of antibody in serum may be pointing out towards a correspondingly long period of immune coverage and is a favourable point.

5. Immunotoxicity of Copper

To investigate the immunotoxic effect of copper in Etroplus suratensis, ovalbumin was administered intramuscularly to two groups of fishes of which one group had a prior exposure to the heavy metal copper (Cu) at 5 ppm. level in water. The primary response in both the groups were monitored on 4th, 5th, 7th and 9th days. Booster dose was given to both groups on 10th day and secondary response was tested on 15th and 31st day. The precipitation titre values in both the groups were compared. The result is shown in the Table 5 and Fig. VI.

On 4th day the mean precipitation titre values were equal in both the groups (4.90 expressed in log 2). Though the titre values went on increasing in both the groups thereafter, the rate of increase in copper exposed animals was much lower than that in the unexposed controls. There were wide differences in the mean titre values of *Cu treated and*

control fishes on the 7th and 9th day when sera were tested. On 7th day control fish gave precipitation upto a serum dilution of 1:110 (6.78 in log 2) whereas in the Cu treated group it was 1:60 (5.9 in log 2) and on 9th day it was 1:290 and 1:65 respectively (8.18&6.02 in log 2).

After the administration of booster dose, there was a sudden increase in the titre value in the control group whereas in the Cu treated group the increase was marginal. On 15th day the mean precipitation titre value in the control fishes was 8.64 (serum dilution 1:400) whereas in the Cu treated group it was only 6.49 (serum dilution 1:90). On the 31st day also the control group maintained an appreciable level of specific antibody in the serum giving rise to a mean precipitation titre of 7.96 (in log 2) with serum dilution 1:250 while in the Cu treated group the mean precipitation titre value was 3.32 (in log 2) with serum dilution 1:10.

This result indicate a clear cut immunosuppressive effect of copper in Etroplus suratensis. Copper may be exerting its immunosuppressiveness through its toxic effect on the antibody producing centres of fish such as anterior kidney, spleen and thymus causing damage to such centres partially or fully or else reducing the specific antibody production capacity of the fish.

Reports on the immunosuppressive effect of copper and other heavy metals on fish are available in literature. Kolomitseva et al., (1969) reported the immunosuppressive effect of copper. Stevens (1977) immunized

Table 4. Antibody production in response to different antigens.

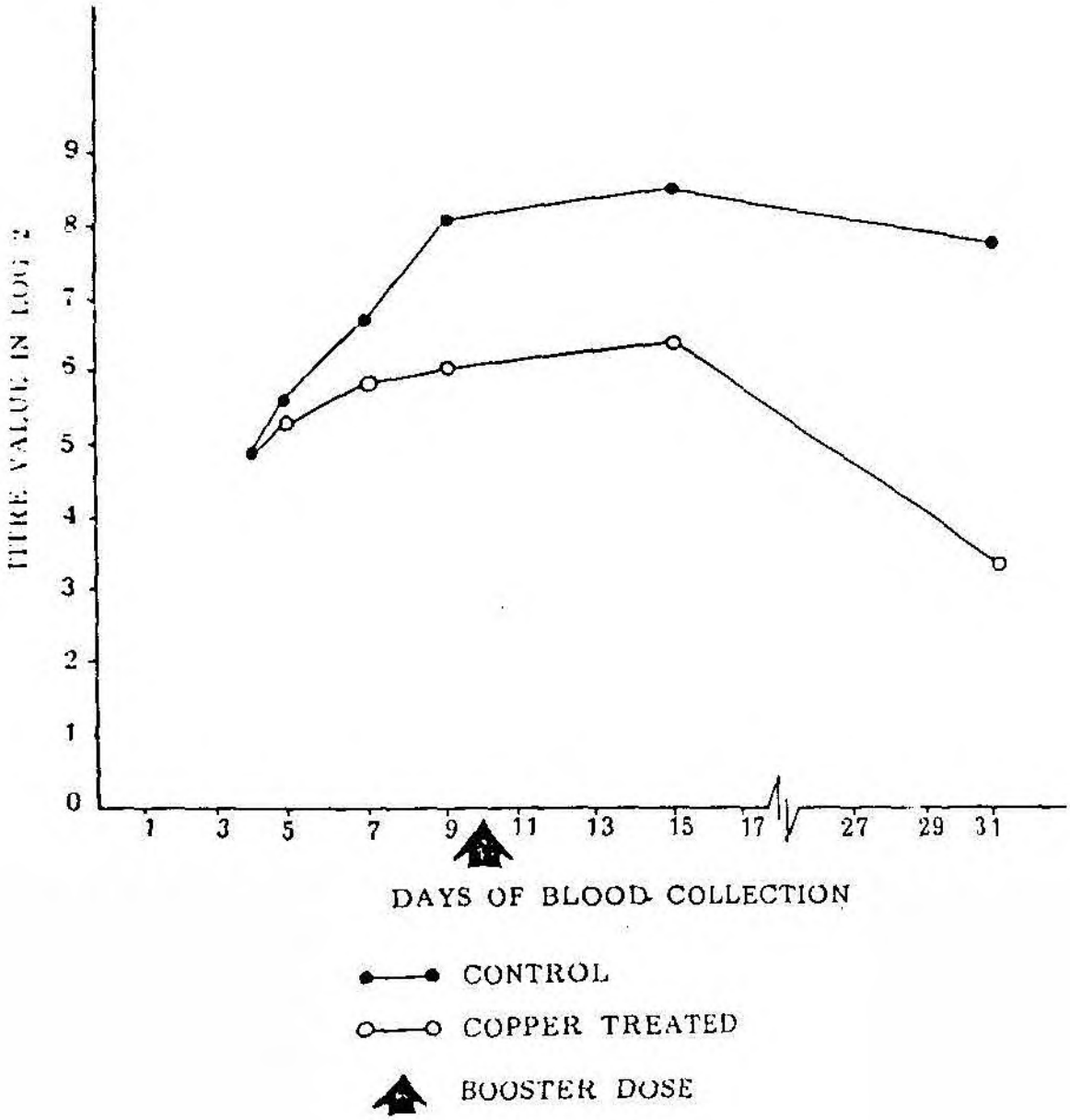
Antigen	Dose /ug/g body wt.	Route	Mean Precipitation Titre value with S.D.		
			9th day (Primary response)	Booster dose	15th day (Secondary response)
B.S.A.	25	i.m.	3.17 ± 0.46	10th day	5.04 ± 0.25
Ovalbumin	25	i.m.	8.058 ± 0.22**	10th day	8.574 ± 0.16**

** Highly significant ($P<0.01$)

Table 5. Antibody production in Cu treated and control fishes.

Antigen	Dose /ug/g body wt.	Route	Days of Blood collection	Precipitation Titre value	
				Cu. treated	Control
			4	4.90	4.90
			5	5.34	5.64
			7	5.90	6.78
Ovalbumin	25	i.m.	9	6.04	8.18
				Booster dose on 10th day	
			15	6.49	8.64
			31	3.32	7.96

Fig. VI. Comparison of antibody production in Cu treated and control fishes



juvenile Coho Salmon, Onchorhynchus kisutch intraperitoneally with formalin-killed Vibrio anguillarum. Immunized Salmon were exposed to 10.1-32.9 ppb. levels of Cu in water for 31 days. The survivers were then acclimated and naturally challenged by V. anguillarum for upto 34 days. He observed strong decrease in the bacterial agglutination titres after Cu exposure.

Roales (1974) treated Blue gourami, Trichogaster trichopterus with Cu at 9 ppb.levels for 4-5 weeks and immunized with viral or bacterial antigens. He reported that viral neutralization titres were drastically reduced while bacterial agglutination titres were not even detectable. There is hardly any report on the immunosuppressive effect of Cu on any of the Indian fish.

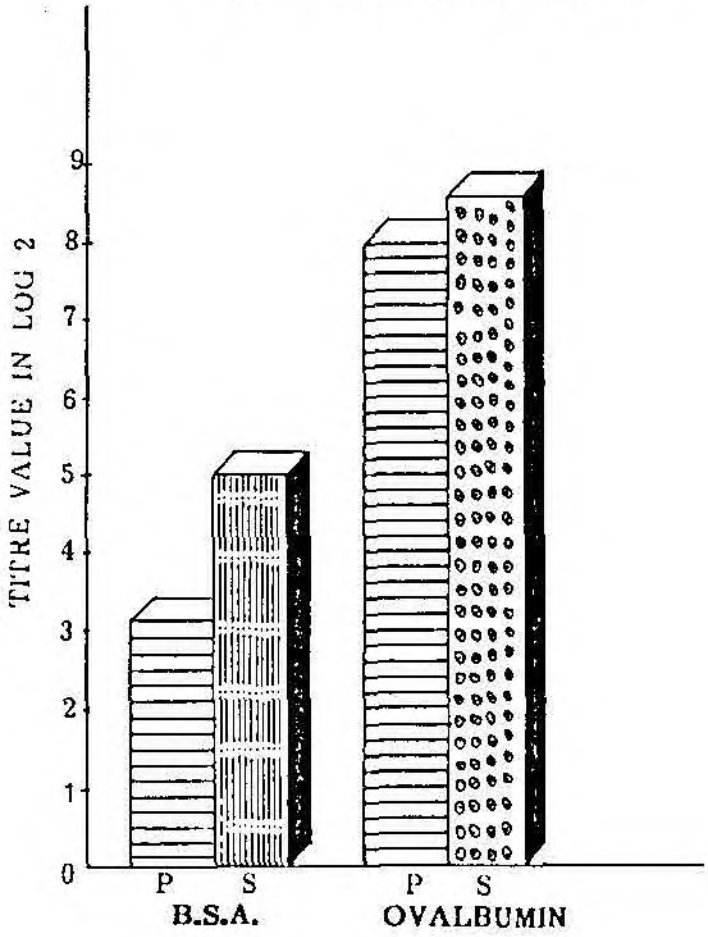
Specific antibody response is susceptable to suppression by many heavy metals. Among them copper is apparently capable of increasing fish bacterial disease incidence (Zeeman and Brindley 1981 op. cit.).

The present study suggests the ability of copper to weaken the immune system of Etroplus suratensis. This may render the host more susceptible to infectious diseases.

Immune Response to Bovine Serum Albumin (BSA)

The immune response to BSA in Etroplus suratensis was also evaluated as a part of the present study. The experimental animals received BSA at the rate of25 /ug/g body weight along with the adjuvant. Primary response was determined on 9th day using precipitation test. The mean titre value expressed in log 2 was 3.17 ± 0.46 (serum dilution 1:9)

Fig IV. Comparison of antibody production in response to different antigens.



P - PRIMARY RESPONSE

S - SECONDARY RESPONSE

The secondary response observed on 5th day of the injection of booster dose of same amount of BSA without adjuvant was higher than the primary response. The mean titre value expressed in log 2 was 5.04 ± 0.25 (serum dilution 1:33) as against 3.17 ± 0.46 in the primary response. Higher secondary response is as per the logical expectation only.

The primary and secondary responses to BSA and ovalbumin are presented in Table 4 to provide a comparison. The two groups of fishes received a similar dose of 25 µg/g body weight of either BSA or ovalbumin in the first day along with adjuvant and a similar dose on 10th day without adjuvant. The antigen was administered intramuscularly.

It can be seen from the table that compared to BSA, the response was of high magnitude with ovalbumin. This is true for both primary response and secondary response. The mean titre values for ovalbumin and BSA were 8.058 ± 0.22 and 3.17 ± 0.46 respectively on 9th day and 8.574 ± 0.16 and 5.04 ± 0.25 on 15th day. The student's 't' test revealed these differences in the response to be highly significant ($P < 0.01$). The present observation is similar to the report of Sudha (1990 op. cit.) in Tilapia mossambica. She has reported that compared to ovalbumin, BSA produced a very low antibody precipitation titre on primary injection and a moderate value on secondary injection.

IMMUNE RESPONSE TO AEROMONAS HYDROPHILA EMU 20

Three groups of 10 fishes each were given formalin killed Aeromonas hydrophila cells at a dose of $1x10^8$ cells through intramuscular,

intraperitoneal and oral routes. Control group was maintained without antigen injection. The primary response was evaluated on 12th day and secondary response on 24th day with booster dose administered on 19th day. The results of agglutination and precipitation tests are shown in the Table 6 & 7 and Fig. VII & VIII.

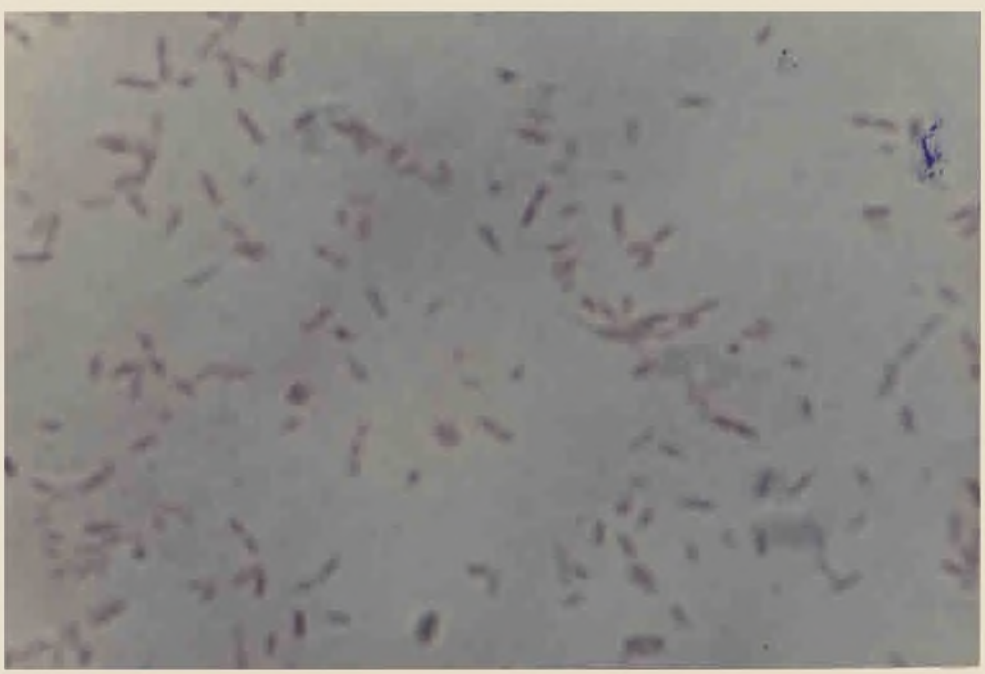
Agglutination test using 12th day sera from i.m. inoculation showed clumping of bacterial cells. The mean agglutination titre value in log 2 was 1 (serum dilution 1:2). The clumping of bacterial cells on microslide is shown in the Plate VI. The sera from control fishes did not show any clumping, suggesting the absence of antibody against Aeromonas hydrophila in the blood (Plate VII).

Serum from i.p. inoculation gave mean agglutination titre value of 2 (serum dilution 1:4). But oral route did not produce any agglutinating antibody in the circulating blood. The booster dose administration through i.m. and i.p. routes resulted in the increase of mean agglutination titre to 3.00 and 4.32 respectively. But in the case of oral inoculation, agglutinating antibody could not be detected in the serum even after the booster dose.

Sera from all the above groups were tested for precipitins also. On 12th day, the mean precipitation titre value of i.m. and i.p. inoculations were 2 and 4.32 respectively (serum dilutions 1:4 and 1:20). Contrary to the agglutinating antibodies, precipitating antibody was detected in the serum of fish which received oral inoculation of the bacteria. The titre value was 1 (Serum dilution 1:2).

Plate VI. Aeromonas hydrophila suspension in normal saline mixed with sera of immunized fish. Mark the clumping of bacterial cells (stained with Gram's sufranine solution, Merck x 1000).

Plate VII. Aeromonas hydrophila suspension in normal saline mixed with control sera. Note the evenly distributed bacterial cells (stained with Gram's sufranine solution, Merck x 1000).



As in the case of agglutination titre, the precipitation titre also increased after the booster dose administration. However the magnitude of increase was more with the i.p. inoculation. The mean precipitation titres on 24th day were 4 (serum dilution 1:16) and 6.64 (serum dilution 1:160) in i.m. and i.p. inoculations respectively. In the case of oral administration, the mean precipitation titre value increased to 3.17. Agglutinating and/or precipitating antibody was not detected in the serum of control fishes.

The result shows that i.p. inoculation is the route of choice for Aeromonas hydrophila. Oral inoculation resulted in poor immunological response. In the case of i.p. and i.m. administrations, the antigen directly comes in contact with the vascular system. This may help better antibody production whereas in the case of oral administration, antigen has to cross the barriers of the intestinal tract to enter into the blood system. Moreover, in oral inoculation there is the major problem of the destruction of antigen in the stomach and foregut before they reach the immune sensitive areas of the lower gut (Rombout et al., 1985).

The primary response observed in the present study with a dose of $1x10^8$ cells could be considered as low. Lamers et al., (1985) used Aeromonas hydrophila as a model antigen in Carp and reported that with 10^5 cells injected per 100g body weight, the primary antibody titres were low. In the present study also the primary response was supporting the above cited report. However, the secondary response was high especially in the i.p. inoculation.

Table 6. Agglutinating Antibody production in response to different routes of Aeromonas hydrophila administration.

Dose	Route	Mean Agglutination Titre value		
		12th day (Primary response)	Booster dose	24th day (Secondary response)
$1x10^8$ cells	i.m.	1	19th day	3
$1x10^8$ cells	i.p.	2	19th day	4.32
$1x10^8$ cells	oral	Non detectable	19th day	Non detectable

Table 7. Precipitating Antibody production in response to different routes of Aeromonas hydrophila administration.

Dose	Route	Mean Precipitation Titre value		
		12th day (Primary response)	Booster dose	24th day (Secondary response)
$1x10^8$ cells	i.m.	2	19th day	4
$1x10^8$ cells	i.p.	4.32	19th day	6.64
$1x10^8$ cells	oral	1	19th day	3.17

Fig. VII. Comparison of agglutinating antibody production in different routes of A. hydrophila administration.

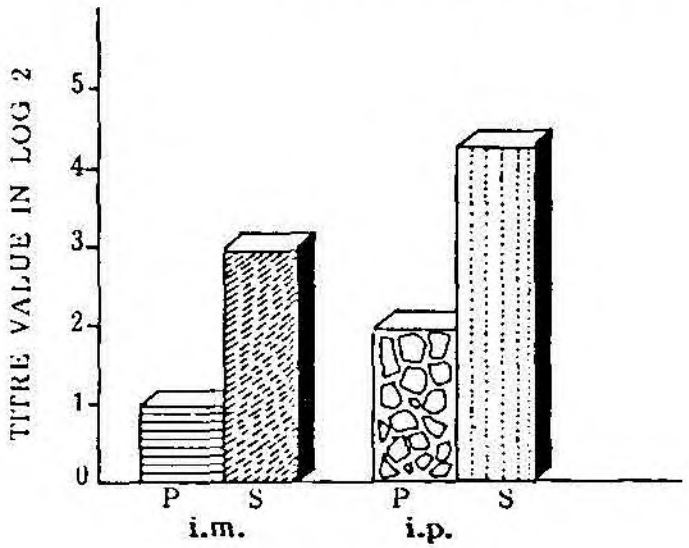
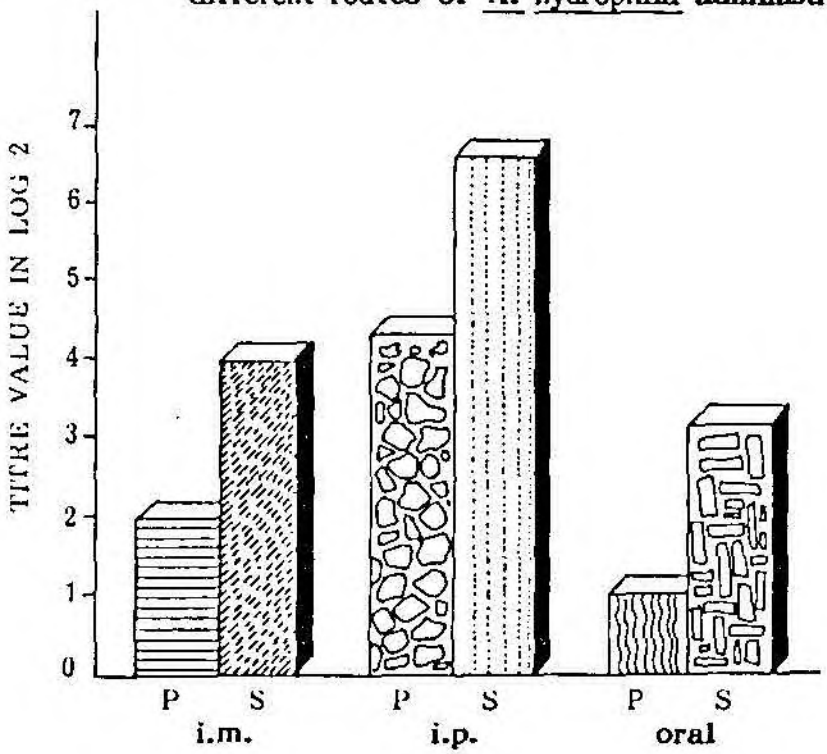


Fig. VIII. Comparison of precipitating antibody production in different routes of A. hydrophila administration.



Post (1966 op. cit.) reported that i.p. route of delivery of A. hydrophila was the most rapid method of producing protective anitbody titres in rainbow trout, Salmo gairdneri. He also reported very slow and weak response in the orally inoculated group of fish. The result of the present study is in agreement with the above cited observations.

Challenging experiment

All the above four groups of fishes (10 No. in each group) were challenged with live Aeromonas hydrophila at a dose at $1x10^4$ cells intramuscularly. The mortalities of fish in each group were recorded.

In the control group, out of the 10 fishes challenged, all but one died. In the group which received oral immunization the mortality was 70% and in the intramuscular immunization 60%. In the case of intraperitioneal inoculation the mortality was only 20%. The dead fishes showed rotted fins and reddish lesions on the body surface. The gills were pale, peripheral blood vessels congested, heart appeared constricted and chambers were empty. Peritoneal and pericardial cavities showed accumulation of clear fluid.

Chi-square test was carried out to assess the effectiveness of immunization in the above groups of fishes, and the result is shown in the Table 9. The Chi-square test showed that the i.p. immunization was the most effective where the mortality was only 20% compared to the 90% mortality in the control group. The Chi-square value was significant at 1% level. It is to be recalled here that it was in i.p. inoculation of A. hydrophila that has given rise to the highest agglutination titre

Table 9. Challenge of immunized and non immunized fish with live Aeromonas hydrophila cells

Number of fishes	Route of immunization	Precipitation titre value (24th day)	Rout of challenge inoculation	% Mortality	Chi-square Value
10	i.p.	6.64	i.p.	20%	7.27**
10	i.m.	4	i.p.	60%	1.89
10	Oral	3.17	i.p.	70%	0.92
10	Control	Not detectable	i.p.	90%	

** Highly significant ($P < 0.01$)

value of 4.32 (log 2) with serum dilution 1:20 and precipitation titre value of 6.64 (log 2) with serum dilution 1:100. The high titre value and lowest mortality in this group points towards the presence of appreciable quantity of specific antibodies against the bacteria A. hydrophila in the blood and its effectiveness in protecting the fish against the A. hydrophila challenge.

It is significant to mention that the mortality in the i.m. inoculation group was 60% in which the mean preciptiation titre value was 4 (serum dilution 1:16). But in the group which received oral immunization, the mortality was still high i.e., 70% in which the mean precipitation titre value was only 3.17 (serum dilution 1:9). In control fish no antibody was detected and the mortality was 90% on challenge. This shows that when the antibody titre is high, the mortality is less and vice versa. The 10% survival in the control group may be due to the non specific immunity innatly present in the individuals of a population. However, the highest degrees of protection was from the i.p. inoculation followed by i.m. and oral inoculations.

Schäperclaus (1954 &1970 cited by Lamers, 1985) first demonstrated protection of Carp against A. hydrophila measured as increased survival of laboratory fish. Post (1963) inoculated heat killed A. hydrophila in rainbow trout, Salmo gairdneri at the rate of 1 mg/fish in intramuscular, intraperitoneal and oral routes, and reported that the highest degree of protection was in the intraperitoneal inoculation followed by intramuscular and the least by oral inoculation.

Post (1966 op. cit.) provided comprehensive information about protection in the rainbow trout, Salmo gairdneri. He reported that i.p. route of delivery of A. hydrophila was the most rapid method of producing protective antibody titres in Salmo gairdneri. He reported a slow response in the orally inoculated group of fish. The result in the present study is in agreement with the above reports.

In inducing positive immunity, administration by injection is more efficient than oral immunization or direct immersion techniques, although the latter methods produce reasonable results and may be preferable for other reasons. Ward et al., (1985 op. cit.) compared the antibody response in Salmo gairdneri vaccinated against Vibrio anguillarum through different routes. He noticed that i.p. injection produced good antibody response but the oral and immersion (non-parenteral) routes produced little or no circulating antibody. Oral immunization has the problem of the destruction of antigen in the stomach and foregest before they reach the immune sensitive areas of the lower gut (Rombout et al., 1985 op. cit.) The low titre value in the orally inoculated group of fish may be due to the above reason.

Ruangapan et al., (1986) vaccinated Nile tilapia (Tilapia nilotica) with fomalin killed Aeromonas hydrophila by i.p. injection. At 1 week post-vaccination, some degree of protection against an injected challenge was observed. Between 2 and 5 weeks, there were no mortalities in vaccinated groups, compared with 73-80% in the controls.

The present study suggests the possibility of immunization of Etroplus suratensis through intraperitoneal inoculation against Aeromonas hydrophila infection.

CELLULAR IMMUNE RESPONSE

The cellular responses observed in Etroplus suratensis on the 15th day following the i.m. inoculation of ovalbumin at the rate of 25 /ug/g body weight on first day as primary dose and a similar booster dose on 10th day are presented below.

Erythrocyte count

The erythrocyte count, carried out using the improved Neubauer ruling haemocytometer ranged from $2.14 \times 10^6 mm^{-3}$ to $3.2 \times 10^6 mm^{-3}$ in control fish. The mean value was $2.67 \times 10^{6} mm^{-3}$. In Teleosts high variation in erythrocyte count have been reported. Following are erythrocyte counts in different fishes reported by different authors.

Author	Fish	Mean	Range
Haws and Goodnight (1962)	Channel catfish Ictalurus punctatus	$2.16 \times 10^6 mm^{-3}$	-
"	Brown bull head Ictalurus nebulosus	$1.27 \times 10^6 mm^{-3}$	-
McKnight (1966)	Mountain whitefish Prosopium williamsoni	$1.57 \times 10^6 mm^{-3}$	1.01-2.34
Conroy (1972)	Atlantic salmon Salmo salar	$1.165 \times 10^6 mm^{-3}$	0.94-1.39
Denyes and Joseph (1956)	Large mounth bass Micropterus salmoides	$2.6 \times 10^4 mm^{-3}$	1.72-3.48
Clark et al., (1979)	"	$1.81 \times 10^4 mm^{-3}$	0.98-2.76

Author	Fish	Mean	Range
Siddiqui and Naseem (1979)	Rohu Labeo rohita	$2.29 \times 10^6 mm^{-3}$	1.65-2.93
Pellitero and Pinto (1987)	Sea bass Dicentrarchus labrax	$3.16 \times 10^6 mm^{-3}$	-
Shyni (1993)	Anabas testeudineus	$4.67 \times 10^6 mm^{-3}$	4.41-4.93

In the present study there was no significant difference in the erythrocyte count before inoculation and on 15th day after the inoculation. The erythrocyte count recorded in Etroplus suratensis is within the range reported for other species mentioned above and is comparable to the values reported for Carassius auratus (Anthony 1961), Merluccius merluccius (Conroy and Rodrigues 1965) and Anabas testudineus (Banerjee 1966).

Leucocyte count

The leucocyte count ranged from 5,200 mm^{-3} to 11,500 mm^{-3} before inoculation. The mean value was 8,350 mm^{-3}. There was a marked increase in the number of leucocytes after the antigen administration. On the 15th day of inoculation of ovalbumin, the mean leucocyte count reached 40,310 mm^{-3} which ranged from 35,640 mm^{-3} to 44,980 mm^{-3}.

Siddiqui and Naseem (1979 op. cit.) reported that the mean leucocyte count of Labeo rohita was 6,250 mm^{-3}. Siwicki and Studnicka (1987) reported marked increase in the leucocyte numbers on 7th and 14th days in Carp, Cyprinus carpio L. which were experimentally inoculated with Pseudomonas alcaligenes and Aeromonas punctata.

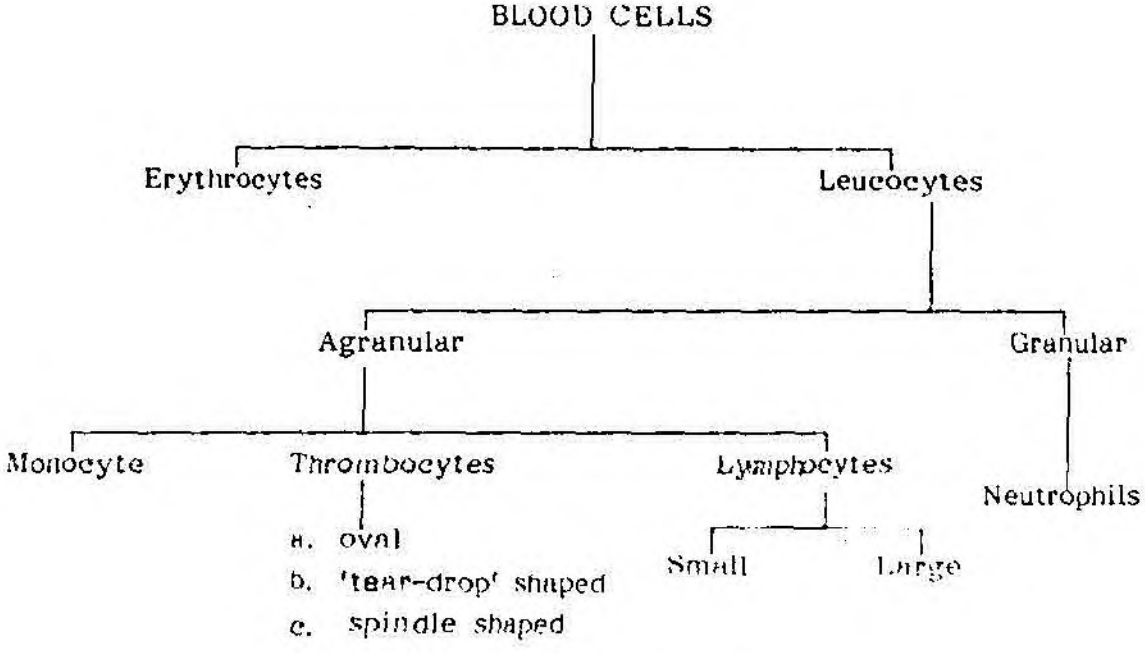
The differential count of leucocytes showed that the lymphocytes constituted 27% of the leucocytes before the antigen inoculation. Increase in the lymphocyte count was observed following i.m. injection of ovalbumin. On 15th day of primary dose (booster dose on 10th day) lymphocyte constitued 55% of the total leucocytes. Klontz (1972 op. cit) has reported marked increase in the numbers of lymphocytes and macrophages on 5th day in rainbow trout, Salmo gairdneri which were subcutaneously inoculated by a bacterial or viral antigen. It is now established in most vertebrate phyla that lymphocytes are highly differentiated cells which respond to immunological stimuli in a variety of ways (Ellis 1977 a op. cit.).

Lymphocyte are believed to be responsible for the production of specific antibodies (Ellis 1977b). The exposure to an antigen results in the stimulation of a small number of virgin lymphocytes and this in turn leads to its proliferation with the differentiation of daughter lymphocytes (Ellis 1988 b). Therefore on the administration of the antigen, an increase in the count of lymphocytes and other leucocytes which play a role in the immune response and a resultant enhancement of specific antibodies in the serum are logically to be expected.

Morphological characteristics of blood cells

The intimate relationship between immunology and haematology is well established. Though lot of literature on fish haematology is available, the nomenclature of blood cells still presents a problem. The terminology applied to fish blood cells has been borrowed from mammalian

haematology. But the functional, ontogenetic and morphological similarities between fish and mammalian blood cells are not standardized. Haematological studies in Etroplus suratensis is very limited. Therefore, an effort was made in the present work to study the morphology and to identify and characterize the various blood cells of Etroplus suratensis. Blood from control animals were utilized for this study. The blood cells observed in the present study could be identified into the following cells types.



Leucocytes

Lymphocytes, thrombocytes, monocytes and neutrophils were the major types of leucocytes identified in the blood smears of Etroplus suratensis. Characteristic morphological features of different types of leucocytes are presented below.

Lymphocytes

Small and large lymphocytes were observed. The small lymphocyte is round and contains a large round nucleus which occupies nearly the entire cell leaving only a small portion of clear blue cytoplasm. The nucleus stains intensely blue (Plate VIII a). The large lymphocyte is an ovoid cell with an eccentric round or irregular nucleus. The cytoplasm stains pale blue with Giemsa's stain.

Monocyte

Monocytes are larger cells often with cytoplasmic projections and pseudopodia. The cytoplasm takes little stain when exposed to Giemsa's stain. Vacuoles are present in the cytoplasm. Plate VIII b shows a developing stage of the monocyte. The nucleus is usually situated eccentrically. Many workers have faced difficulty in identifying monocytes in fish blood (Mahajan and Dheer 1979 a op. cit.).

Thrombocytes

Thrombocytes were observed in groups. Three types of thrombocytes such as oval shaped, 'tear-drop' shaped or fusiform and spindle shaped were identified. Oval shaped thrombocytes are small than the other two forms. Its nucleus is oval. The cell shape is mainly provided by the nucleus. The cytoplasm surrounding the nucleus is small in quantity and stains faintly (Plate IX d). Oval shaped thrombocyte with kidney shaped nucleus also was identified (Plate IX e). In 'tear-drop' shaped thrombocyte one end is pointed and the other end rounded (Plate X f).

The spindle shaped thrombocyteis elongated in shape and its nucleus is also elongated. The nucleus which is seen in the middle is basophilic and cytoplasm pale (Plate XI g).

Neutrophil

They are round cells. The cytoplasm contains numerous neutrophilic granules which give the cytoplasm an almost translucent pink appearance. Small vacuoles are found in the cytoplasm. The nucleus is placed eccentrically (Plate VIII c).

Macrophage

Macrophages are large cells, variable in shape, and frequently possessing numerous small vacuoles within the cytoplasm. Nucleus is small in proportion to the total cell volume. The cytoplasm stains blue with Giemsa's stain (Plate XII h).

Of the four types of thrombocytes reported in fishes (round, oval, fusiform and 'tear-drop' shaped) all types except the round type were observed in Etroplus suratensis. The function of thrombocytes is the clotting of blood. Basophils and eosinophils are rare in fish blood and not reported in many species studied. They were not observed in the blood smears of Etroplus suratensis also.

Red Blood Cells (RBC)

The RBC of Etroplus suratensis was oval and the nucleus clearly visible. The cytoplasm was lightly stained and developed violet colour

Plate VIII. a. Small lymphocyte, Giemsa x 1000

b. Developing stage of monocyte, Giemsa x 1000

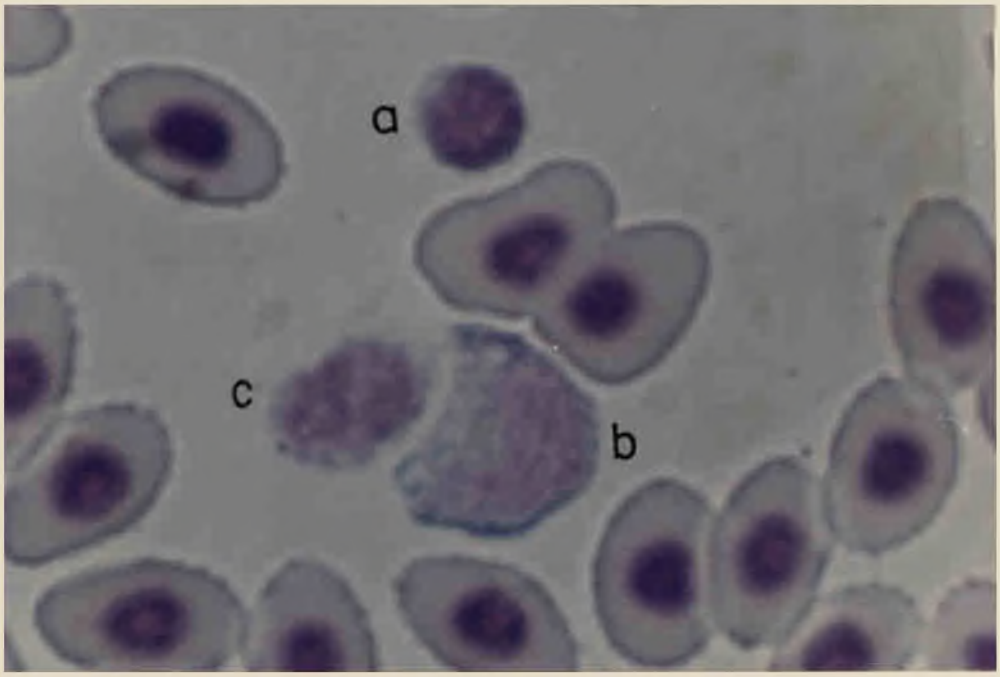
c. *Neutrophil*, Giemsa x 1000

Plate IX. d. Oval shaped thrombocyte, Giemsa x 1000

e. Oval shaped thrombocyte with kidney shaped nucleus, Giemsa x 1000

j. Round RBC, Giemsa x 1000

k. Triangular RBC, Giemsa x 1000


a
c
b

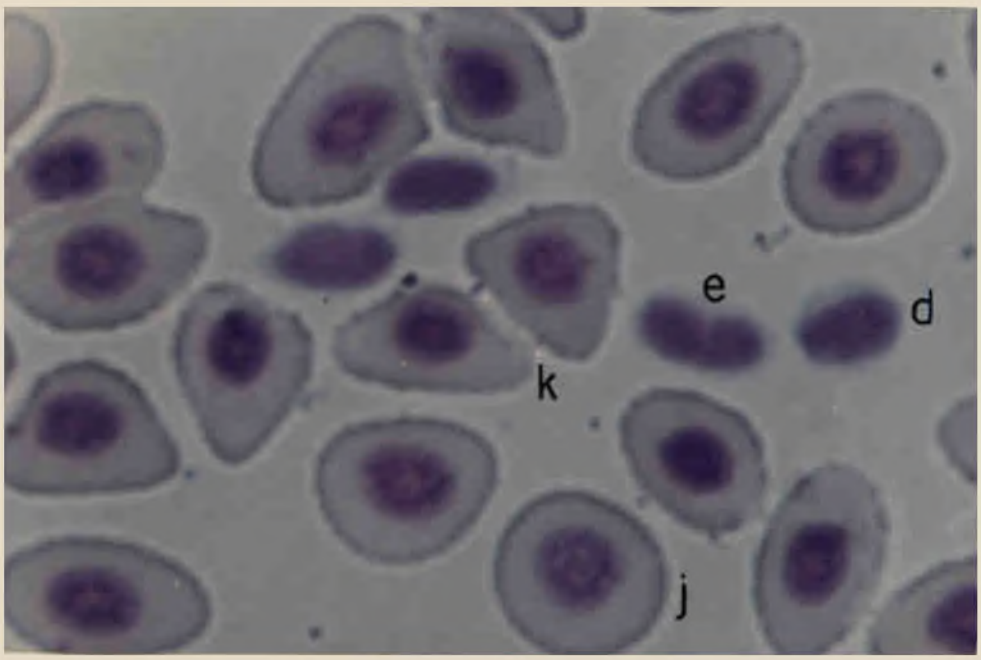
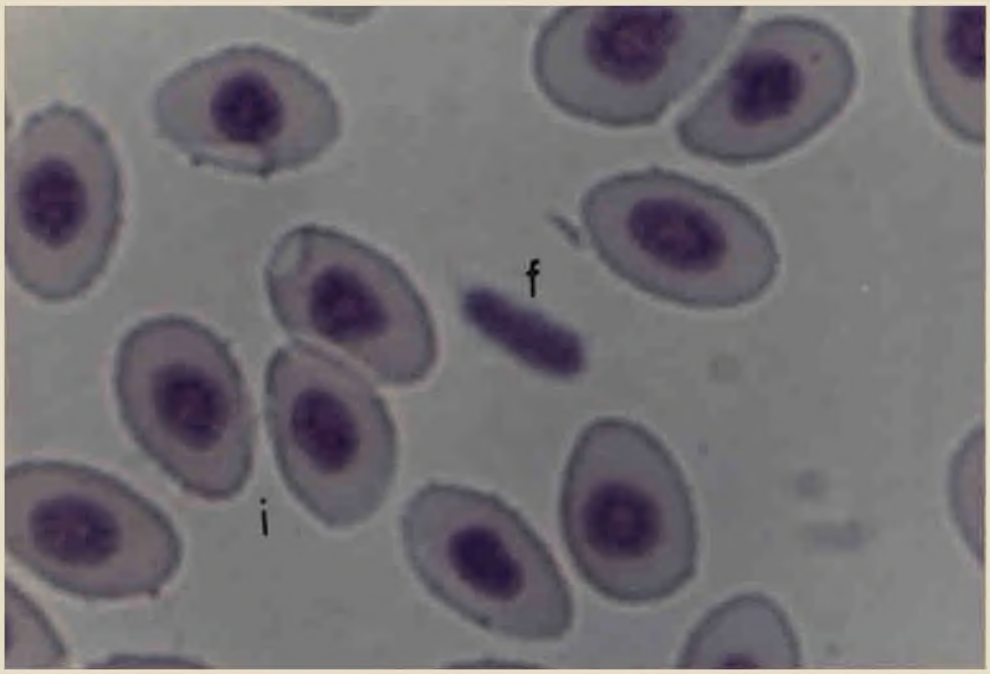

e
d
k
j

Plate X. i. Oval shaped RBC, Giemsa x 1000

f. 'Tear-drop' shaped thrombocyte, Giemsa x 1000

Plate XI. g. Spindle shaped thrombocyte, Giemsa x 1000


f
i

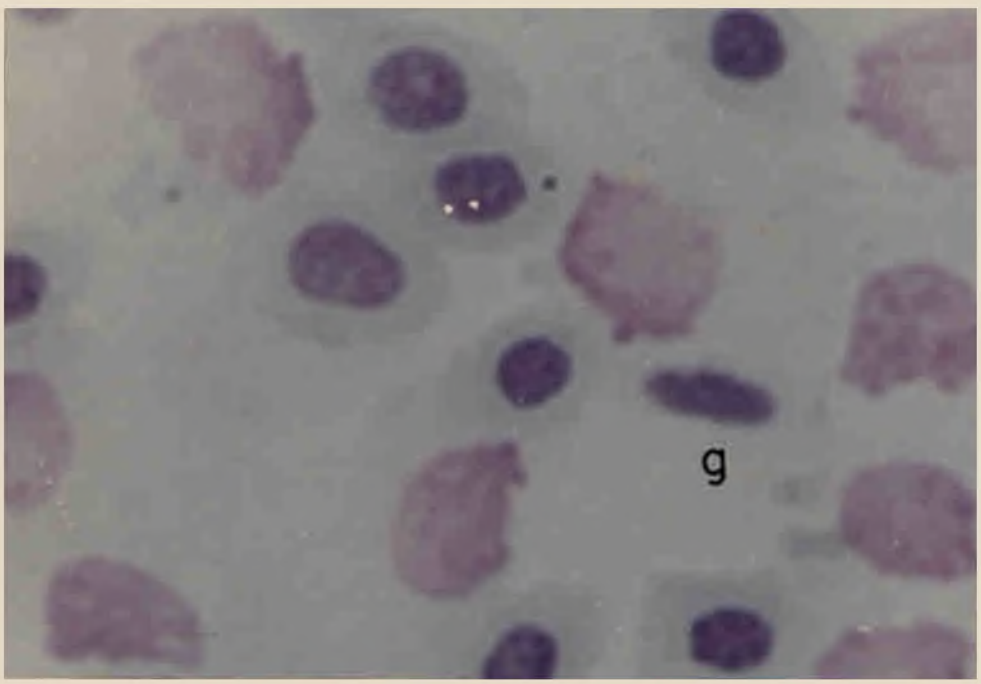
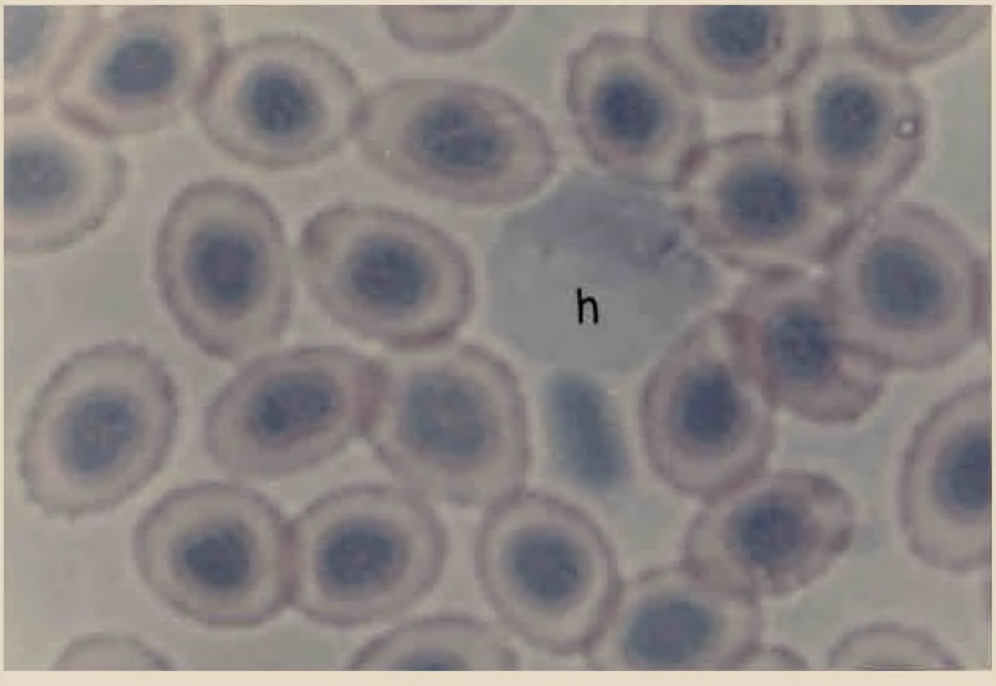

g

Plate XII. h. Macrophage, Giemsa x 1000



with Giemsa's stain. The nucleus stained deep violet. No vacuoles were observed in the cytoplasm of the cell (Plate X i). In addition to oval cells, round and triangular red blood cells also were observed (Plate IX j,k). Conroy (1972 op. cit.) observed large number of rounded RBC in Atlantic salmon, Salmo salar and attributed into active erythropoiesis. The physiological efficiency of the cells is related to its shape and volume (Hartman and Lessler 1964).

The morphometric measurements of Red Blood Cells are shown in the Table 10 below.

Cell			Nucleus			Ratio of mean cell diameter to mean nuclear diameter /um
Mean Length /um	Mean Width /um	Mean diameter /um	Mean Length /um	Mean Width /um	Mean diameter /um	
10.828 ± 1.079	7.508 ± 0.949	9.168 ± 1.014	5.315 ± 1.326	3.502 ± 0.536	4.40 ± 0.931	2.083

The mature RBCs varied in length from 8.3 to 13.28 /um and in width from 5.87 to 8.3 /um. The mean diameter of the cell was 9.168 /um and of the nucleus 4.40 /um. According to Mahajan and Dheer (1979a op. cit.), the mean RBC diameter of Channa punctatus was 9.5 ± 0.14/um and that of nucleus 3.5 ± 0.31/um. Boomker (1981) reported that the mean cell diameter and nucleus diameter of Channa gariepinus was 7.65 ± 1.35 /um and 2.93 ± 0.225 /um respectively. Shyni (1993 op. cit).

reported that mature RBCs varied in length from 9.80 to 12.74 /um and in width from 4.90 to 9.80 /um in Anabas testeudineus. The present study indicate that the morphometric parameters of the Red Blood Cells of Etroplus suratensis is within the range reported for other species.

SUMMARY

SUMMARY

The present study was carried out to evaluate the humoral and cellular immune response in Etroplus suratensis to soluble protein antigens such as ovalbumin and Bovine Serum Albumin as well as bacterial antigen Aeromonas hydrophila.

The specific antibodies produced were detected and quantified by precipitation and agglutination tests. Antibody response to different doses of ovalbumin was compared to determine the appropriate dose. The immunological response to intramuscular and intraperitoneal routes of ovalbumin inoculations were compared. Comparison of the immunological response to ovalbumin by different size groups were also carried out. The immunosuppressive effect of copper was assessed. Immunological response to different antigens were also compared.

Fishes were immunized against Aeromonas hydrophila through i.p., i.m. and oral routes and their efficiency in protecting the fish against live bacterial challenge was assessed.

Cellular changes to ovalbumin administration was also investigated. Different cell types in the blood also were identified. The salient findings of the study are summerised below.

1. Etroplus suratensis immunologically responded to all the antigens tested viz., ovalbumin, Bovine Serum Albumin and Aeromonas hydrophila. The specific antibodies produced against the above antigens could be detected by precipitation and/or aggultination tests.

2. Clear precipitation bands were produced in liquid medium as well as in agarose gel by single diffusion in one dimension and double diffusion in one dimension. Agglutination test, characterized by the clumping of bacterial cells was demonstrated on glass slides.

3. Ovalbumin at the rate of 25 ug/g weight was found to be the appropriate dose for evoking good primary and secondary responses. A high dose of 50 ug/g ovalbumin produced immunological tolerance with low primary antibody titres.

4. Intramuscular inoculation of ovalbumin produced higher antibody titres than the intraperitoneal route both in the primary and the secondary responses. Precipitating antibodies appeared in the serum on 3rd day in i.m. inoculation whereas in i.p. inoculation it was on 6th day.

5. There was no significant difference in the antibody titre values among different size groups (5-120 g) of Etroplus suratensis with ovalbumin as the antigen. This suggests the possibility of immunization of this species even at the size of 5g body weight.

6. In the primary response phase, the antibody titre reached its peak on 9th day. A booster dose stimulated antibody production resulting in the sudden increase of titre values. The secondary response phase was found to be prolonged also.

7. Copper was shown to have immunosuppressive effect in Etroplus suratensis. There was considerable reduction in the antibody titre values in the fish which were exposed to copper in the water.

8. Bovine Serum Albumin had a weaker immunological response when compared to that of ovalbumin.

9. Aeromonas hydrophila produced precipitating and agglutinating antibodies in Etroplus suratensis. Intraperitoneal route produced the highest antibody titres compared to intramuscular and oral inoculations.

10. On challenge experiment with live bacteria, mortality was least in intraperitoneally immunized fish followed by intramuscular and oral immunizations. Intraperitoneal immunization gave 80% protection. The result pointed to the possibility of immunization of Etroplus suratensis against A. hydrophila injection through i.p. route.

11. Leucocyte count had increased significantly following ovalbumin inoculation. There was a marked increase in the number of lymphocytes. RBC count did not show any change.

12. Morphological characteristics of blood cells were studied. Oval, round and triangular Red Blood Cells were observed in the blood smears. Monocytes, lymphocyte (small and large), neutrophils, macrophages and three types of thrombocytes viz., oval, 'tear-drop' shaped and spindle shaped could be identified in the blood smears.

REFERENCES

REFERENCES

ACTON, R.T., WEINHEIMER, P.F., HALL, S.J., NEIDERMEIER W., SHELTON, E. and BENNETT, J.C. 1971. Tetrameric immune macroglobulins in three orders of bony fishes. Proc. natn. Acad. Sci. U.S.A. 68: 107-111.

ALEXANDER, J.B., WILSON, J.G.M. and KERSHAW, W.E. 1970. Immune response in Salmon. Abstract of paper presented at meeting of Fisheries Society of British Isles, 24th April 1970. J. Fish Biol. 2: 384

AMBROSIUS, H. 1966. Comparative investigation of immune globulins of various vertebrate classes. Nature, London, 209: 524.

* _____. and FRENZEL E.V. 1972. Anti-DNP antibodies in Carps and Tortoises. Imm. Chem., 9: 65-71.

AMEND, D.F. and JOHNSON, K.A. 1981. Current status and future needs of Vibrio anguillarum bacterins. In: Fish Biologies: Serodiagnostics and Vaccines. Dev. Biol. Standard. 49: 403-417.

AMIN, N.E., ABDALLAH, I.S., ELALLAWY, T. and AHMED, S.M. 1985. Motile Aeromonas septicaemia among Tilapia nilotica (Sarotherodon niloticus) in Upper Egypt. Fish Pathol., 20: 93-97.

ANDERSON, D.P., 1974. Fish Immunology, T.F.H. Publications, Inc-Ltd. 10-160.

_____, MERCHANT ., B., DIXON, O.W. and LIZZIO, E.F. 1982. Investigations of immunological memory in rainbow trout, Salmo gairdneri to DNP conjugates. Develop. Comp. Immunol. Suppl. 2: 115-122.

* Not refered in original.

ANTHONY, E.H. 1961. The oxygen capacity of goldfish, Carassius auratus in relation to the thermal environment. J. exp. Biol. 38: 93-107.

APHA, 1955. Standard methods for the examination of water, sewage and industrial waters. Am. Publ. Health Org. (10th edn.), New York: 522.

AVTALION, R.R. 1969. Temperature effect on antibody production and immunological memory in Carp, Cyprinus carpio immunized against Bovine Serum Albumin (BSA). Immunol., 17: 927-931.

_____. 1981. Environmental control of the immune response in fish. Crit. Rev. Environ. Control, 11: 163-188.

_____, WEISS, E. and MOALEM, T. 1976. Regulatory effects of temperature upon immunity in ectothermic vertebrates. In "Comparative Immunology" Ed. MARCHALONIS, J.J., Blackwell Scientific Publications, Oxford: 227-238.

_____, WISHKOWSKY, A. and KATZ, D. 1980. Regulatory effect of temperature on specific suppression and enhancement of the humoral immune response in fish. In: Phylogeny of Immunological Memory, Ed. MANNING, M.J., Amsterdam: Elsevier/North Holland: 113-121.

BALDO, B.A. and FLETCHER, T.C. 1975. Phylogenetic aspects of hypersensitivity reactions in flat fish. In: Immunologic phylogeny, Eds. HILDEMANN, W.H. and BENEDICT, A.A., Plenum Press, London: 365-375.

*BANERJEE, V. 1966. A note on haematological studies of Anabas testeudineus (Bloch). Sci. Cult. 32: 326.

BARDSHAW, C.M., RICHARD, A.S. and SIGEL, M.M. 1971. IgM antibodies in fish mucus. Proc. Soc. Exp. Biol. Med. 136: 1122-1124.

BARROW, T.H. 1955. Social behaviour in fresh water fish and its effect on resistance to trypnosomes. Proc.Natu. Acad. Sci. U.S.A., 41: 676-679.

*BISSET, K.A. 1948. The effect of temperature on antibody production in cold blood vertebrates. J. Path. Bact., 60: 87-92.

_____. 1949. The influence of adrenal cortical hormones upon immunity in cold-blooded vertebrates. J. Endocr. 6: 99-103.

BJORKLUND, H., BONDESTAM, J. and BYLUND, G. 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture, 86: 359-367.

*BOOMKER, J. 1981. The haemocytology and histology of the haemopoietic organs of South African freshwater fish. III. The leucocytes, plasma cells and macrophages of Clarias gariepinus and Sarotherodon mossambicus. Onderstepoort J. Vet. Res., 48.

BOTHAM, J.W. and MANNING, M.J. 1981. The histogenesis of the lymphoid organs in the Carp, Cyprinus carpio L. and the ontogenetic development of allograft reactivity. J. Fish Biol. 19: 403-414.

BRUNGS, W.A., Mc CORMICK, J.H., NEIHEISEL, T.W. SPEHAR, R.L., STEPHAN, C.E. and STOKES, G. N. 1977. Effects of pollution on fresh water fish. J. Water Pollut. Control. Fed., 49: 1425.

CHARTRAND, S.L. and FINSTAD, J. 1971. A structurally unique immunoglobulin of the African lungfish. Fedn. Proc. Fedn. Am. Socs exp. Biol. 30: Abstr.886.

CHILLER, J.M., HODGINS, H.O., CHAMBERS, V.C. and WEISER, R.S. 1969a. Antibody response in rainbow trout, Salmo gairdneri. I. Immunocompetent cells in the spleen and anterior kidney. J. Immunol., 102: 1193-1201.

_____. and WEISSER, R.S. 1969b. Antibody response in rainbow trout, Salmo gairdneri. II. Studies on the kinetics of development of antibody-producing cells and on complement and natural haemolysin, J. Immunol. 102: 1202-1207.

*CISAR, J.O. and FRYER, J.L. 1974. Characteristics of anti-Aeromonas salmonicida antibodies from Coho Salmon. Infect. Immun. 9: 236-243

CLARK, S., WHITMORE, Jr. D.H. and Mc MOHAN, R.F. 1979. Considerations of blood parameters of large mouth bass, Micropterus salmoides. J. Fish Biol. 14(2): 147-158.

CLEM, L.W., De BOUTAUD, F. and SIGEL, M.M. 1967. Phylogeny of immunoglobulin structure and function. II. Immunoglobulins of the nurse shark. J. Immun. 99: 1226-1235.

_____. and LESLIE, G.A. 1969. Phylogeny of immunoglobulin structure and function, In: Immunology and Development, Ed. ADINOLFI, M., Leverham Press, London: 62.

_____, Mc LEAN, W.E., SHANKEY, V.T. and CUCHENS, M.L. 1977. Phylogeny of lymphocyte heterogeneity. I. Membrane immunoglobulins on Teleost lymphocytes. Devel. Compar. Immunol., 1: 105-118.

_____. and SMALL, P.A. Jr. 1967. Phylogeny of immunoglobulin structure and function. I. Immunoglobulin of the lemon shark. J. exp. Med. 125: 843-920.

CONROY, D.A. 1972. Studies on the haematology of the Atlantic Salmon, Salmo salar L. Symp. Zool. Soc. Lond. 30: 101-127.

_____. and RODRIGUES, J.L. 1965. Haematological study of the hake, Merluccius merluccius from the South West Atlantic. J. Fish. Res. Bd. Can. 22: 869-971.

CORBEL, M.J. 1975. The immune response in fish: a review, J. Fish Biol., 7: 539-563.

CRUICHSHANK, R., DUGUID J.P., MARMION B.P. and SWAIN B.H.A. 1979. In: Medical Microbiology. Vol. 1 and 2. Longman Group Ltd.

CUSHING, J.E. 1942. An effect of temperature upon antibody production in fish. J. Immunol. 45: 123-126.

DAVIS B.D., DULBECCO R., EISEN H.N., GINSBERG H.S. and WOOD W.B. 1968. Principles of Microbiology and Immunology: 360-507.

*DENYES, H. and JOSEPH, J.M. 1956. Relationship between temperature and blood oxygen in largemouth bass. J. Wildl. Mgmt. 20: 56-64.

DREYER, N.B. and KING J.W. 1948. Anaphylaxis in fish. J. Immunol., 60: 277-282.

EISLER, R. 1973. Annotated Bibliography on Biological Effects of Metals in Aquatic Environments. EPA, Environmental Research Ser., Report No. R3-73-007, U.S. Environmental Protection Agency, Washington, D.C.

_____. and WAPNER, M. 1975. Second Annotated Bibliography on Biological Effects of Metals in Aquatic Environments. EPA, Environmental Research Ser., Report No. 600/3-75-008, U.S. Environmental Protection Agency, Washington, D.C.

*ELLIS, A.E., 1974. Aspects of lymphoid and reticuloendothelial system in the Plaice, Pleuronectes platessa L. Ph.D. thesis: Aberdeen University.

_____. 1976. Leucocytes and related cells in the Plaice, Pleuronectes platessa. J. Fish Biol., 8: 143-156.

_____. 1977 a. The leucocytes of fish: A review. J. Fish Biol. 11: 453-491.

_____. 1977b. Ontogeny of the immune response in Salmo salar, Histogenesis of the lymphoid organs and appearance of membrane immunologobulin and mixed leucocyte activity. In: Developmental immunobiology, Eds. SOLOMON, J.B. and HORTON J.D., Elsevier/North-Holland Bimedical Press, Amsterdam: 225-231.

_____. 1985. Fish serum precipitins to Aeromonas salmonicida ecotoxins and Protease - lipoprotein reactions, A critical appraisal, In: Fish Immunology, Eds, MANNING, M.J. and TATNER, M.F., Academic Press. Inc. (London) Ltd., 107-122.

_____. 1988a. Optimizing factors for fish vaccination, In: Fish vaccination Ed. ELLIS, A.E., Academic Press, 32-46.

_____. 1988b. General principles of fish vaccination. In: Fish vaccination Ed. ELLIS, A.E., Academic Press, 1-19.

_____, MUNROE, A.L.S. and ROBERTS, R.J. 1976. Defence mechanisms in fish. I. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice, Pleuronectes platessa, J. Fish. Biol., 8: 67.

_____ and PARKHOUSE, R.M.E. 1975. Surface immunologlobulin on the lymphocytes of the skate, Rama naevus, Europ. J. Immunol., 5: 726-728.

ELLIOT D. and SHOTTS, E. Jr. 1980. Aetiology of an ulcerative disease in gold fish, Carassius auratus: Experimental induction of the disease. J. Fish Dis., 3: 687-693.

EMMRICH, F., RICHTER, R.F. and AMBROSIUS , H. 1976. Immunoglobulin determinants on the surface of lymphoid cells in carp. Europ. J. Immunol., 5: 76-78.

ETLINGER, H.M., HODGINS, H.O. and CHILLER, J.M. 1976. Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity in rainbow trout revealed by the organ distribution of Mitogenic responses J. Immunol., 116: 1547-1553.

FANGE, R. 1982. A comparative study of lymphomyeloid tissue in fish. Devel. comp. Immunol. Suppl. 2: 23-33.

*FIDLER, J.E., CLEM, L.W. and SMALL, P.A. Jr. 1969. Immunoglobulin synthesis in neonatal nurse sharks, Ginglymostoma cirratus, Comp. Biochem. Physiol. 31: 365-371.

FINN, J.P. and NEILSON, N.O. 1971. Inflammatory response in rainbow trout. J. Fish Biol. 3: 463-478.

FINSTAD, J. and GOOD, R.A. 1964. The evolution of the immune response. III. Immunological responses in the lamprey. J. exp. Med. 120: 1151-1167.

FLETCHER, T.C. and GRANT, P.T. 1968. Glycoproteins in the external mucus secretions of the Plaice, Pleuronectes platessa, and other fishes. Biochem. J. 106: 12.

_____. 1969. Immunoglobulins in the serum & mucus of the Plaice, Pleuronectes platessa. Biochem. J. 115: 65.

FUJII, T., NAKAGAWA, H. and MURAKAWA, S. 1979. Immunity in lamprey II. Antigen binding responses to sheep erythrocytes in the ammocoete Develop. Comp. Immunol. 3: 609-620.

*GONCHAROV, G.D. and MIKRYAKOV, V.R. 1970. The effect of low concentrations of phenol on antibody formation in Carp, Cyprinus carpio L. Problems of Aquatic Toxicology, Published by "Nauka" Moscow. Translated by: Bur. of Sport Fisheries and Wildlife, Div. of Fish. Research. 1971:7.

GOTTLIEB, A.A. and WALDMAN, S.R. 1972. The multiple functions of macrophages in immunity. In: Macrophages and Cellular Immunity. Eds. LASKIN, A. and LECHEVALIER, H., London: Butterworths, 13-44.

GOVEN, B.A., DAWE, D.L. and GRATZER, J.B. 1980. In. vivo and in. vitro anaphylactic type reactions in fish. Dev. Compar. Immunol., 4: 55-64.

GREY, H.M. 1963. Phylogeny of the immune response. Studies on some physical, chemical and serological characteristics of antibody produced in the turtle. J. Immun. 91: 819-825.

HALL, S.J., EVANS, E.E., DUPREE, H.K., ACTON , R.T., WEINHEIMER, P.F. and BENNET, J.C. 1973. Characterisation of a teleost immunoglobulin: the immune macroglobulin from the channel catfish, Ictalurus punctatus. Comp. Biochem. Physiol. 46: 187-197.

HARTMAN, F.A. and LESSLER, M.A. 1964. Erythrocyte measurements in fishes, amphibia and reptiles. Biol. Bull. mar. biol. Lab., Woods Hole 126: 83-88.

HAWKINS, R.I. and MAWDESLEY - THOMAS, L.E. 1972. Fish haematology - a bibliography, J. Fish Biol., 4: 193.

HAWS, T.G. and GOODNIGHT, C.J. 1962. Some aspects of the haematology of two species of catfish in relation to their habitats. Physiol. Zool. 35: 8-17.

HINES, R. and SPIRA, D.T. 1973. Ichthyophthiriasis in the mirror carp. III. Leucocyte response. J. Fish Biol. 5: 527-534.

HODGINS, H.O., WEISER, R.S. and RIDGEWAY, G.J. 1967. The nature of antibodies and the immune response in rainbow trout, Salmo gairdneri. J. Immunol., 99: 534-544.

HOGARTH, P.J. 1973. Immune relations between mother and foetus in the viviparous poecilid fish, Xiphophorus hellerei Haecket. III. Survival of embryos after ectopic transplantation. J. Fish Biol. 5: 109-113.

HOLMER, M. 1991. Impacts of aquaculture on surrounding sediments: generation of organic-rich sediments. Eur. Aquacult. Soc. Spec. Publ., 16.

HORNE, M.T., TATNER, M.F., Mc DERMENT, S., AGIUS, C. and WARD, P.D. 1982. Vaccination of rainbow trout, Salmo gairdneri Richardson at low temperature and long-term persistence of protection. J. Fish Dis. 5: 343-345.

INGRAM, G.A. 1980. Substances involved in the natural resistance of fish to infection - A review. J. Fish Biol. 16: 23-60.

_____ and ALEXANDER 1980. The immune response of brown trout, Salmo trutta to lipopolysaccharide. J. Fish Biol., 16: 181-197.

JANSSEN, W.A. 1970. Fish as potential vectors of human bacterial diseases. In: A symposium on Diseases of Fishes and Shellfishes. Am. Fish. Soc., Sp. Publi. 5. Washington, D.C. 284-290.

JIRGENSONS, B., SPRINGER, G.F. and DESAI, P.R. 1970. Comparative circular dichroism studies of individual eel and human antibodies. Comp. Biochem. Physiol. 34: 721-725.

JOHNSON, K.A., FLYNN, J.K. and AMEND, D.F. 1982. Duration of immunity in Salmonids vaccinated by direct immersion with Yersinia ruckeri and Vibrio anguillarum bacterins. J. Fish Dis. 5: 207-213.

JOY, J.E. and JONES, L.P. 1973. Observations on the inflammatory response within the dermis of a white bass, Morone chrysops, infected with Lernea cruciata. J. Fish Biol. 5: 21-23.

KLONTZ, G.W. 1972a. Haematological techniques and the immune response in rainbow trout. In: Diseases of Fish, Ed. MAWDESLEY, L.E. - THOMAS, Academic Press, London, 30: 89-99.

*KOLOMITSEVA, M.G., VOZNESENSKAIA, F.M. and ISAEVA, E.A. 1969. Immunobiologic reactivity of the animal organism with regard to various concentration of copper in its rations, Zh. Mikrobiol. Epidemiol. Immunobiol. 46:99.

KUHNS,W.J., NIGRELLI, R.F. and CHUBA, J.V. 1969. Effects of temperature upon "Naturally occuring" blood group agglutinins in fresh water catfish. J. Immunol., 103: 62-65.

LAMERS, C.H.J. 1985. The reaction of the immune system of fish to vaccination. Thesis, Agricultural University of Wageningen, Netherlands.

_____, De HASS, M.J.H. and VAN MUISWINKEL, W.B. 1985. The reaction of the immune system of fish to vaccination: development of immunological memory in Carp, Cyprinus carpio following direct immersion in Aeromonas hydropnila bacterin. J. Fish Dis., 8: 253-262.

_____ and PILARCZYK, A. 1982. Immune response and antigen localization in Carp, Cyprinus carpio after administration of Yersinia ruckeri O-antigen. Develop. comp. Immunol. Suppl. 2: 107-114.

LITMAN, G.W. 1975. Physical properties of immunoglobulins of lower species: A comparison with immunoglobulins of mammals. In: "Compar. Immunol," Ed. MARCHALONIS, J.J., Blackwell, Oxford 239-275.

LOBB, C.J. and CLEM, L.W. 1981. The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. J. Immunol. 127: 1525-1529.

LOPEZ, O.M., SIGEL, M.M. and LEE, J.C. 1974. Phylogenetic studies on T-cells. Lymphocytes of the shark with differential response to phytohaemagglutinin and concanavation A. Cell. Immunol. 10: 287-293.

MAHAJAN, C.L. and DHEER, J.S. 1979a. Cell types in the peripheral blood of an air-breathing fish, Channa punctaus. J. Fish Biol. 14(5): 481-487.

_____ 1979b. Seasonal variations in the blood constituents of an air-breathing fish, Channa punctatus. J. Fish Biol. 14: 413-417.

McKNIGHT, I.M. 1966. A haematological study on the Mountan whitefish, Prosopium williamson. J. Fish. Res. Bd. Can. 23: 45-64.

MANNING, M.J., GRACE, M.F. and SECOMBES, C.J. 1982. Developmental aspects of immunity and tolerance in fish. In: Microbial Diseases of Fish, Ed. ROBERTS R.J., Academic Press, New York, London, 31-46.

_____ and MUGHAL, M.S. 1985. Factors affecting the immune responses of immature fish. In: Fish and Shellfish Pathology, Ed. ELLIS, A.E. Academic Press, London, 27-39.

MARCHALONIS, J.J. 1969. Isolation and characterisation of immunoglobulin like proteins of the Australian lungfish, Neoceratodus forsteri. Aust. J. exp. Biol. med. Sci. 47: 405-419.

_____. 1971. Isolation and partial characterisation of immunoglobulins of gold fish, Carassius auratus and Carp, Cyprinus carpio. Immunology 20: 161-173.

_____ and EDELMAN, G.M. 1968. Phylogenetic origins of antibody structure, III. Antibodies in the primary immune response of the sea lamprey, Petromyzon marinus. J. exp. Med. 127: 891-914.

MIYAZAKI, T and JO, Y. 1985. A histopathological study of motile aeromonad disease in ayu, Plecoglossus altivelis. Fish Pathol., 20: 55-60.

MUGHAL, M.S. and MANNING M.J. 1985. Antibody response of young Carp, Cyprinus carpio, and Grey mullet, Chelon labrosus immunized with soluble antigen by various routes. In: Fish Immunology, Eds. MANNING M.J. and TATNER M.F. Academic Press, London 313-326.

NEWMAN, S.G. 1993: Immunization of fish and crustaceans. Infofish International 3: 39-44.

*OAKLEY, C.L. and FULTHROPE, A.J. 1953. Antigenic analysis by diffusion. J. Path. Bact., 65:49.

*OUCHTERLONY, Ö. 1949. Antigen - antibody reactions in gels. Ark. Kemi. Miner. Geol. 1-9.

PAPERMASTER, B.W., CONDIE, R.M., FINSTAD, J. and GOOD, R.A. 1964. Evolution of the immune response. I. The phylogenetic development of adaptive immunologic responsiveness in vertebrates. J. Exp. Med. 119: 105-130.

PATERSON, W.D. and FRYER J.L. 1974. Immune response of juvenile Coho Salmon, Oncorhynchus kisuch to Aeromonas salmonicida cells administered intraperitoneally in Freund's complete adjuvant. J. Fish. Res. Bd. Can., 31 (11): 1751-1755.

PELLITERO, P.A. and PINTO, R.M. 1987. Some blood parameters in sea bass, Dicentrarchus labrax infected by bacteria, virus and parasites.

PERLMUTTER, A., SAROT, D.A. YU, M., FILAZZOLA,R.J. and SEELEY,R.J. 1973. The effect of crowding on the immune response of the blue gourami, Trichogaster trichopterus to the infectious pancreatic necrosis (IPN) virus, Life Sci. 13: 363-375.

PFUDERER, P., WILLIAMS, P. and FRANCIS, A.A. 1974. Partial purification of the crowding factor from Carassius auratus and Cyprinus Carpio. J. Exp. Zool., 187: 375-382.

PLUMB, J.A.1984. Immunization of warm water fish against five important pathogens, In: symposium on Fish Vaccination, Ed.P.de. KINKELIN Paris: Office International desp Epizooties, 199-222.

* POST,G. 1963. The immune response of rainbow trout, Salmo gairdneri to Aeromonas hydrophila. Dept. Inform. Bull. Utah State Dept. of Fish and Game. 63:7.

_____. 1966. Response of rainbow trout, Salmo gairdneri to antigens of Aeromonas hydrophila. J. Fish Res. Bd. Can. 23: 1487-1494.

RIJKERS, G.T. 1982a. Kinetics of cellular and humoral immune reaction in fish. Develop. Comp. Immunol. Suppl. 2: 93-100.

_____. 1982b. Non-lymphoid defensive mechanisms in fish. Dev. Comp. Immunol. 6: 1-12.

_____, FREDERIX - WALTERS, L.M.H. and VAN MUISWINKEL,W.B. 1980a. The immune system of cyprinid fish: The effect of antigen dose and route of administration on the development of immunological memory in Carp, Cyprinus carpio. In: "Phylogeny of Immunological Memory". Ed. MANNING, M.J., Elsevier, North Holland, Amsterdam, 93-102.

* ROALES, R.R. 1974. The effects of sub-lethal doses of methyl-mercury and copper, applied separately and jointly, on the immune response of the blue gourami, Trichogaster trichopterus to viral and bacterial antigens, Arch. Environ. Contam. Toxicol., 5: 325.

ROBERTS, R.J. (Ed). 1978. Fish Pathology, Bailliere tindall, London.

_____., Mc QUEEN A., SHEARER, W.M. AND YOUNG,H.1973. The histopathology of Salmon tagging. I. The tagging lesion of newly tagged parr. J. Fish Biol. 5: 497-504.

ROMBOUT, J.H. W.M., LAMERS, C.M.J. HEFFRICH, M.H., DEKKER, A. and TAVERNE-THIELE, J.J. 1985. Uptake and transport of intact macromolecules in the intestinal epithelium of Carp, Cyprinus carpio and the possible immunological implications. Cell and Tissue Research, 239: 519-530.

RUANGAPAN, L. KITAO, T. and YOSHIDA, T. 1986. Protective efficacy of Aeromonas hydrophila vaccines in Nile tilapia. Veter. Immunol. Immunopathol., 12: 345-350.

SAILENDRI, K. 1973. Studies on the development of lymphoid organs and immune responses in the teleost, Tilapia mossambica. Ph.D. Thesis, Madurai University, Madurai.

_____ and MUTHUKKARUPPAN, V.R. 1975. The immune response of the teleost, Tilapia mossambica to soluble and cellular antigens. J. Exp. Zool., 191: 371-382.

SAMUELSEN, O.B. 1989. Degradation of oxytetracycline in seawater at two different temperatures and light intensities and the persistence of oxytetracycline in the sediment of a fish farm. Aquaculture 83: 7-16.

SCHUMACHER, R.E., HAMILTON, C.H. and LONGTIN, E.J. 1956. Blood sedimentation rates of brook trout as affected by furunculosis. Progve. Fish. Cult. 18: 47-148.

SECOMBES, C.J., VAN GRONINGEN, J.J.M. and EGBERTS, E. 1983. Separation of lymphocyte subpopulations in Carp, Cyprinus carpio L. by monoclonal antibodies: immunohistochemical studies. Immunology 48: 165-175.

SERERO, M. and AVTALION, R.R. 1978. Regulatory effect of temperature upon immunity in ectothermic vertebrates. III. Establishment of immunological suppression in fish. Dev. Comp. Immunol., 2: 87-94.

SHARMA, R.P. 1981. Immunity and the immune system,In: Immunologic considerations in toxicology, Vol. 1. CRC Press, Inc. Florida: 9-18.

SHELTON, E. and SMITH, M. 1970. The ultrastructure of Carp, Cyprinus carpio immunoglobulin: at tetrameric macroglobulin. J. Molec. Biol. 53: 615-617.

SHYNI, R. 1993. Studies on some haematological aspects of the air breathing fish. *Anabas testeudineus* (Bloch). M.Sc. Project work submitted to Mahatma Gandhi University, 27-40.

SIDDIQUI, A.Q. and NASEEM, S.M. 1979. The haematology of Ronu, Labeo rohita. J. Fish Biol. 14(1): 67-72.

*SIGEL, M.M. 1974. Primitive immunoglobulins and other proteins with binding functions in the shark. Ann. N.Y. Acad. Sci. 234: 198-215.

SINDERMANN, C.J. 1990. Principal Diseases of Marine Fish and Shellfish Vol. 1. Diseases of Marine Fish. Academic Press, Inc., California, 230-255.

SINGLETON, P. and SAINSUBURY, D. 1978. Dictionary of Microbiology, Wiley-Interscience Publication, 20-21.

SIWICKI, A. and STUDNICKA, M. 1987. The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected Carp, Cyprinus carpio L. J. Fish Biol. 31: 57-60.

SMITH, A.M., POTTER, M. and MERCHANT E.B. 1967. Antibody-forming cells in the pronephros of the teleost, Lepomis macrochirus. J. Immunol. 99: 876-882.

STEVENS, D.G. 1977. Survival and immune response of Coho Salmon exposed to copper. EPA, *Ecological Research* Ser., Report No. 600/3-77-031, U.S. Environmental Protection Agency, Washington, D.C.

STEVENSON, R.M.W. 1988. Vaccination against Aeromonas hydrophila. In: Fish Vaccination, Ed. ELLIS, A.E., Academic Press, 112-123.

STOLEN, J.S., GAHN, T. NAGLE, J. 1982. The humoral antibody formation to erythrocyte antigens in three species of flat fish. Develop. Comp. Immunol. Suppl. 2: 101-106.

SUDHA, P.V. 1990. Evaluation of Immune Response in Finfish. M.Sc. Dissertation submitted to Cochin University of Science and Technology.

TALWAR, P.K. and JHINGRAN, A.G. 1991. Inland Fishes, Vol.2 Oxford and IBH Publishing Co. Pvt. Ltd., 885-887.

TATNER, M.F. and HORNE, M.T. 1983. Susceptibility and immunity to Vibrio anguillarum in post hatching rainbow trout fry, Salmo gairdneri Richardson. Dev. Comp. Immunol. 7: 465-472.

* TEBBIT, G.L., ERICKSON, T.D. and VANDE WATER, R.B. 1981. Development and use of Yersinia ruckeri bacterins to control enteric red mouth disease. In: Fish Biologies: Serodiagnostics and vaccines. Dev. Biol. Standard. 49: 395-401.

TIMUR, M.R.J., ROBERTS, R.J. and Mc QUEEN, A. 1977. Carrageenin granuloma in the plaice, Pleuronectes platessa, a histological study of chronic inflammation in the teleost fish. J. Comp. Path. 87: 89-96.

TOMPKINS, E.N. 1955. The Monocyte. Ann. N.Y. Acad. Sci. 59: 732-745.

TRUMP, G.N. and HILDEMAN W.H. 1970. Antibody responses of goldfish to Bovine Serum Albumin: Primary and secondary responses. Immunology, 19: 621-627.

THORPE, J.E. and ROBERTS, R.J. 1972. An aeromonad epidemic in the brown trout, Salmo trutta L. J. Fish Biol. 4: 441-451.

UHR, J.W., FINKELSTEIN, M.S. and FRANKLIN, E.C. 1962. Antibody response to bacteriophage X174 in non-mammalian vertebrates. Proc. Soc. Exp. Biol. Med., 111: 13-15.

WARD P.D., TATNER,M.F. and HORNE M.T. 1985. Factors influencing the efficiency of vaccines against vibriosis caused by Vibrio anguillarum. In: Fish Immunology, Eds. MANNING M.J. and TATNER M.F. Academic Press, London, 22-229.

WARR, G.W.A.D., De LUCA, DECKER, J.M., MARCHALONIS, J.J. and RUBEN, L.N. 1977. Lymphoid heterogenesity in teleost fish: Studies on the genus Carassius. In: Developmental Immunology, Eds. SOLOMEN, J.B. and HORTON, J.D. Elsevier/North-Holland Biomedical press, Amsterdam, 241-248.

_____ and MARCHALONIS, J.J. 1980. Membrane immunoglobulins of teleost fish lymphocytes. In: "Aspects of Developmental and comparative immunology I". Ed. SOLOMON, J.B., Pergamon Press, Oxford, 33-37.

*WEINREB, E.L. 1958. Studies on the histology and histopathology of the rainbow trout, Salmo gairdneri irideus. I. Haematology under normal and experimental conditions of inflammation. Zoologica, N. Y. 43 (4): 145-155.

WISHKOWSKY, A. and AVTALION, R.R. 1982. Induction of helper and suppressor functions in Carp, Cyprinus carpio and their possible implications in seasonal disease in fish. Dev. Comp. Immunol., suppl. 2: 83-91.

YAMAGUCHI, N., TESHIMA, C.,KURASHIGE,S., SAITO,T. and MITSUHASHI,S. 1980. Seasonal modulation of antibody formation in rainbow trout, Salmo gairdneri In: "Aspects of Developmental and comparative immunology I". Ed. SOLOMON, J.B., Pergamon Press, Oxford, 483-484.

ZEEMAN, M.G. and BRINDLEY W.A. 1981. Effects of toxic agents upon fish immune systems: a review. In: Immunological considerations in Toxicology, Vol 2, Ed. SHARMA R.P. CRC Press, Inc. Florida, 1-60.