THE HISTOGENESIS OF LYMPHOID ORGANS IN *TILAPIA MOSSAMBICA* AND ANTIBODY RESPONSES IN ADULTS AND FRY

by

HEKMAT MOHAMED ALI

A thesis presented for the degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF ASTON IN BIRMINGHAM

April 1987

UNIVERSITY OF ASTON IN BIRMINGHAM

THE HISTOGENESIS OF LYMPHOID ORGANS IN *TILAPIA MOSSAMBICA* AND ANTIBODY RESPONSES IN ADULTS AND FRY

by

Hekmat Mohamed Ali

Submitted for the degree of Doctor of Philosophy, 1987 in the University of Aston in Birmingham.

SUMMARY

The thymic anlagen appears in *Tilapia mossambica* at 2 days post hatching and becomes lymphoid at 5 days. Lymphoid cells were first seen in the pronephros at 14 days and in the spleen at approximately five weeks of age. Differentiation into red and white pulp regions was seen by 10 weeks of age.

Light and electron microscopic studies of adult lymphoid organs revealed increases in size and lymphoid cell numbers. Adult thymus develops a clearer corticomedullary differentiation of thymic corpuscles in the medulla and the splenic red and white pulp became more distinct. Melanomacrophage centres were seen in spleen and pronephros.

Adult fish gave primary and secondary antibody responses following challenge with sheep red blood cells (SRBC), *Escherichia coli (E. coli)* and human gamma globulin (HGG).

Plaque forming cell and immunocytoadherence assays revealed that head kidney and spleen were major sites for antibody production and development of antigen reactive cells. Proliferative activity in these organs was revealed using autoradiography and scintillation counting. Increased levels of pyroninophilia were also seen following antigenic challenge. Pilot studies on adults revealed that they were capable of rejecting first and second set allografts and leucocytes from spleen and head kidney proliferated in mixed leucocyte cultures.

Antibody responses to SRBC, *E. coli* and HGG develop at about 10 - 12 weeks of age. Fry given either a single injection of SRBC at 10 weeks or two injections of the same antigen at 10 weeks and 12 days later, failed to respond to a further challenge with SRBC 56 days after the first injection (A time when animals would normally respond positively to this antigen). Injection of *E. coli* at the same times resulted in a prolonged antibody response.

Key Words: Immunity in *Tilapia mossambica*
Histogenesis of lymphoid organs
Ontogeny of humoral immunity
Tolerance

DEDICATION

To my husband, Samy Sabah El Kheir, my two daughters, Nancy and Nehal and my parents who stood by me during my research period.

To the memory of my brother whose interest and encouragement was vital throughout the years of my education.

ACKNOWLEDGEMENTS

My special gratitude goes to my supervisor, Dr. Jim Rimmer who has always and continously given the help, advice and guidance without which this could not have been accomplished satisfactorily.

The continuous interest and encouragement given by my advisory supervisor, Dr. Nial Bromage are gratefully acknowledged.

My thanks are due to Professor W.R. McWhinnie and Professor J.A. Blair, of the department of Molecular Sciences who kindly allowed access to the departmental facilities.

I am also grateful to the Institute of Aquaculture, Stirling University in Scotland for supplying the *Tilapia mossambica* especially the young stages.

I would also like to extend my gratitude to my colleagues, staff, secretary and technicians in the Molecular Sciences department for their practical help, advice and encouragement especially Dr. Thornburn for his guidance, Mrs E.R. Ince for the bacterial preparation and Mr K. Hughes and Mr S.G. Howitt for their valuable and technical assistance. I would also like to thank Miss Rebecca Holt of the Pharmacy department for her help in E.M. techniques.

I have also enjoyed the co-operation of all my colleagues in the Biology department.

I have a special word of thanks for Miss Iloria Whittier who has shown patience in typing this thesis.

Of course, these acknowledgements would not be complete without mention of the encouragement and good will of my husband, two daughters and my family at home in Egypt.

Finally, I am indebted to the Ministry of Higher Education and Scientific Research of Egypt and our Egyptian Education Bureau in London for financial support throughout the whole time spent in this work.

DECLARATION

Some of the work presented in Chapter 4 of this thesis was performed in collaboration with Dr. J.J. Rimmer.

LIST OF CONTENTS

	Page No.
Title Page	1
Thesis Summary	2
Dedication	3
Acknowledgements	4
List of Contents	6
List of Tables	8
List of Figures	9
List of Appendices	12
Chapter 1	
GENERAL INTRODUCTION	14
Chapter 2	
ONTOGENY OF LYMPHOID ORGANS IN *TILAPIA MOSSAMBICA*	30
2.1 Introduction	30
2.2 Materials and Methods	32
2.3 Results	33
2.4 Discussion	35
Chapter 3	
LIGHT AND ELECTRON MICROSCOPIC STUDIES OF LYMPHOID TISSUES IN 10 WEEK OLD FRY AND ADULT *TILAPIA MOSSAMBICA*	54
3.1 Introduction	54
3.2 Materials and Methods	54
3.2.1 Fish Immunisation	55
3.2.2 Light Microscopic Studies	55
3.2.3 Preparation of Tissues for E.M.	55
3.2.4 Preparation of thick sections (0.5 - 1 mm) for viewing under the light microscope.	57
3.3 Results	57
3.3.1 Thymus	57
3.3.2 Head Kidney (The pronephros)	59
3.3.3 Kidney (The mesonephros)	60
3.3.4 Spleen	60

Page No.

3.4 Discussion 61

Chapter 4

THE IMMUNE RESPONSE OF *TILAPIA MOSSAMBICA* TO DIFFERENT ANTIGENS 78

4.1 Introduction 78

4.2 Materials and Methods 79

4.2.1 Animals 79
4.2.2 Antigens and Immunisation
4.2.3 Ontogenetic Studies on Humoral Immunity in Fry 81
4.2.4 Antigen Injection 82
4.2.5 Collection of Serum 84
4.2.6 Complement 84
4.2.7 Plaque Forming Cell Assay (PFC) 84
4.2.8 Haemagglutination Assay 85
4.2.9 Assessment of the Immune Response of *Tilapia mossambica* to Human Gamma Globulin (HGG) using the Passive Haemagglutination Test 85
4.2.10 Immunocytoadherence (Rosette Forming Cell) Assay 86
4.2.11 Slide Agglutination Test for Bacterial Antigen 86
4.2.12 Histology 87
4.2.13 Scintillation Counting 87
4.2.14 Scale Transplantation 88
4.2.15 Mixed Leucocyte Culture 89

4.3 Results 90

4.3.1 Serum Antibody Responses in Adult Fish 90
4.3.2 Plaque Forming Cells 90
4.3.3. Production of Antigen Reactive Cells in Lymphoid Organs 92
4.3.4 Antibody Response to *Escherichia coli* 95
4.3.5 Hyperosmotic Administration of *Escherichia coli* 95
4.3.6 Antibody Response to Human Gamma Globulin (HGG) 97
4.3.7 Ontogenesis of Humoral Immunity in Fry 97
4.3.8 Histological and Proliferative Changes following Exposure to Antigen 107
4.3.9 Proliferation in Spleen of Adult *Tilapia* 7 days after Injection of SRBC 116
4.3.10 Mixed Leucocyte Culture 117
4.3.11 Scale Transplantation 117

4.4 Discussion 117

Chapter 5

GENERAL DISCUSSION 129

Appendices 137

References 153

LIST OF TABLES

		Page No.
2.1	Summary of the Histogenesis of the Major Lymphoid Organs in *Tilapia mossambica.*	41
2.2	Histogenesis of Lymphoid Organs in Fish	44
4.1	Antibody Titres of *Tilapia mossambica* Immunised with *Escherichia coli* by Hyperosmotic Infiltration.	95
4.2	Ontogenetic Emergence of the Antibody Response of Fry to SRBC.	99
4.3	Plaque Forming Cell Response in Spleen and Head Kidney of Fry 14 Days after Immunisation with SRBC.	101
4.4	Rosette Forming Cell Response in Spleen and Head Kidney of Fry (14 days) after Immunisation with SRBC.	103
4.5	Ontogenetic Emergence of the Antibody Response of Fry to *Escherichia coli.*	104
4.6	Ontogenetic Emergence of the Antibody Response of Fry to Human Gamma Globulin.	106
4.7	Tolerogenic Effects of Early Exposure to SRBC.	110
4.8	Long Term Effects of Early Exposure to *Escherichia coli*	112
4.9	Proliferation in Spleen of Adult *Tilapia.*	116
4.10	Mixed Leucocyte Reactivity in Spleen and Pronephros of *Tilapia.*	117

In Appendices

4.11	Scale Transplantation Allograft Rejection Studies on Adult *Tilapia.*	149
4.12	The Immune Response of Adult *Tilapia mossambica* to SRBC.	150
4.13	Histology of Lymphoid Organs in Immunised Fish.	151

LIST OF FIGURES

		Page No.
1.1	Evolution of fish groups in relation to the Animal Kingdom	15
2.1	General view of a 5-day old *Tilapia* (Sarotherodon) *mossambica*, showing the caudal and head regions with yolk sac attached.	40
2.2	Longitudinal section through head region of Tilapia fry (2 - 5 days post hatching).	45
2.3	L.S. through developing thymus at (2 - 5) days post hatching.	45
2.4	Lymphoblast like cells in the thymus (4 days post hatching).	46
2.5	T.S. of head region at 4 days post hatch to show the position of the thymus.	46
2.6	The thymus at 8 days post hatching.	47
2.7	Thymus at day 9 showing the appearance of thymic cysts.	47
2.8	L.S. through trunk region of fry 2 - 5 days post hatch showing kidney tubules in the mesonephros surrounded by haemopoietic tissue.	48
2.9	L.S. of *Tilapia mossambica* at 5 days post hatching showing the tubules of the pronephros and haemopoietic tissue.	48
2.10	L.S. through head kidney (pronephros) of *Tilapia mossambica* at 6 days post hatching to show undifferentiated stem cells.	49
2.11	L.S. of pronephric region at 7 days post-hatching showing increase in the amount of intertubular haemopoietic tissue.	49
2.12	Section through head kidney at 30 days post hatch.	50
2.13	Head kidney at 5 weeks showing erythrocytes and lymphoid cells.	50
2.14	L.S. through 2 week old fry showing the relative positions of the thymus and pronephros.	51
2.15	L.S. of spleen at 5 - 6 days post hatch showing the presence of large haemopoietic stem cells.	51
2.16	Section through 6 day old spleen showing large stem cells scattered through the reticular framework.	52
2.17	Section through spleen in 10 day old fry showing the presence of erythrocytes with other lightly staining cells.	52
3.1	Cross section of a 10 week old fish showing the dorsal position of the thymus in relation to the gills.	67
3.2	Section showing part of the thymic medulla in adult *Tilapia mossambica*.	68

		Page No.
3.3	Detail of medulla in thymus of *Tilapia mossambica* showing thymic corpuscle.	68
3.4	Autoradiograph showing extensive proliferative activity in the thymus of 10 week old fry.	69
3.5	E.M. of thymus showing fenestrated endothelial cell cytoplasm.	69
3.6	E.M. of granulocyte (?) in thymus of 10 week old fry.	70
3.7	Thymocyte in 10 week old fry thymus.	70
3.8	Histological section of head kidney of *Tilapia mossambica*, 5 days after immunisation with sheep red blood cells.	71
3.9	Enlarged view of lymphoid area in the head kidney of adult *Tilapia* showing highly concentrated lymphocytes.	71
3.10	Thick embedded section of head kidney of fry showing cellular proliferation.	72
3.11	Electron micrograph of pronephros of 10 week old fry.	72
3.12	The haemopoietic tissue in 10 week old pronephros.	73
3.13	Transverse section in the mesonephros showing kidney tubules and lack of lymphoid tissue.	73
3.14	Low power view of adult *Tilapia* spleen.	74
3.15	Section of spleen of *Tilapia mossambica* showing white pulp (WP) and red pulp (RP) of control fish.	75
3.16	Section of spleen from immunised fish 7 days after injection with 20% SRBC.	75
3.17	Electron micrograph of spleen from 10 week old *Tilapia* fry.	76
3.18	Cell with electron dense cytoplasmic inclusions in spleen of adult fish injected with bacteria (*Escherichia coli)* 7 days earlier.	76
4.1	Primary and secondary humoral immune response of adult *Tilapia mossambica* to SRBC.	91
4.2	Kinetics of plaque forming cells (PFC) in the spleen and head kidney of adult *Tilapia mossambica* following primary and secondary challenge with SRBC.	93
4.3	Kinetics of rosette forming cells (RFC) in the spleen and head kidney of adult *Tilapia mossambica* following primary and secondary challenge with SRBC.	94
4.4	Primary and secondary humoral immune response of adult *Tilapia mossambica* to *Escherichia coli* .	96
4.5	Primary and secondary humoral immune response of adult *Tilapia mossambica* to HGG.	98

Page No.

4.6 Primary immune response of *Tilapia mossambica* fry (10 weeks old) to sheep red blood cells. 100

4.7 Effect of adjuvant on immune response of *Tilapia mossambica* fry to *Escherichia coli.* 105

4.8 Antibody response of fry injected at 10 weeks of age and 12 days later with HGG in FCA. 108

4.9 Antibody response of fry injected at 10 weeks of age and 12 days later with HGG in saline. 109

4.10 Antibody response of *Tilapia mossambica* fry immunised at 126 days of age to SRBC. 111

4.11 The effect of adjuvant on the immune response of fry to *E. coli.* 113

4.12 Autoradiograph of kidney imprints from fish immunised with 20% SRBC and killed 3 days after $3^{+}H$ thymidine injection. 115

4.13 Autoradiograph of spleen section from fish immunised with 20% SRBC and killed 4 days after $3^{+}H$ thymidine injection. 115

LIST OF APPENDICES

		Page No.
Appendix 1	Preparation of *Escherichia coli* suspension	137
Appendix 2	Preparation of Hank's solution and different buffers	138
Appendix 3	Preparation of fixatives for histology	141
Appendix 4	Slide agglutination test using latex particles	142
Appendix 5	Tissue processing for histological techniques.	143
Appendix 6	Methyl green pyronin.	144
Appendix 7	Autoradiographic techniques	145
Appendix 8	Methods to stain autoradiographs using methyl green pyronin	147
Appendix 9	Preparation of L-15 [Liebovitz] liquid medium	148
Appendix 10	Scale Transplantation	149
Appendix 11	Immune response of adult *Tilapia mossambica* to SRBC at (22 - 27^{o}C).	150
Appendix 12	Histology of lymphoid organs in immunised fish.	151

CHAPTER 1

CHAPTER 1

GENERAL INTRODUCTION

The specific immune system is one component of the protective system of all vertebrates which enables the individual to survive and maintain its homeostasis in a hostile environment. In other words the immune system of vertebrates protects them from cancer cells and disease causing pathogenic micro-organisms, such as bacteria and viruses (Hood, Weissman and Wood, 1978).

Immunity is an important physiological mechanism in all animals and in vertebrates it can be divided into two types: innate immunity and adaptive immunity. The former depends on innate defence mechanisms which are non-specifically operative against a wide range of offending organisms which may enter the body. These defences include the action of phagocytes, interferons and various naturally occurring substances such as lysozyme, C-reactive protein, agglutinins and lysins. Adaptive immunity is an acquired state and depends on the body's ability to respond adaptively and specifically against particular offending organisms by producing specific reactive lymphocytes (cell-mediated immunity) or circulating antibodies (humoral immunity) against them; the latter are protein molecules also termed immunoglobulins. Both cell-mediated and humoral immunity are present in the teleosts. The bony fishes with over 20,000 species are the largest and most diverse vertebrate group and the Teleosti are the most numerous and highly-developed. As masters of the aquatic environment, they are as diverse and highly specialised in their own right as the higher tetrapods are on land (Young, 1981). In keeping with their position as the most highly advanced fishes (see Fig 1.1) teleosts exhibit a wide range of immune responses.

The study of fish immunity began in the eighteenth century with the work of William Hewson who was the first to describe "Lymphatic vessels" in a number of fish (Gulliver, 1846). He found that fish had no lymph nodes although he did not describe any other lymphoid organs either. Detailed descriptions of fish immunity did not appear in any number until after 1945, flowering in the 1960's with the growth of mammalian

Fig. 1.1 Evolution of fish groups in relation to the Animal Kingdom (from various sources, after Young, Y.Z., 1981).

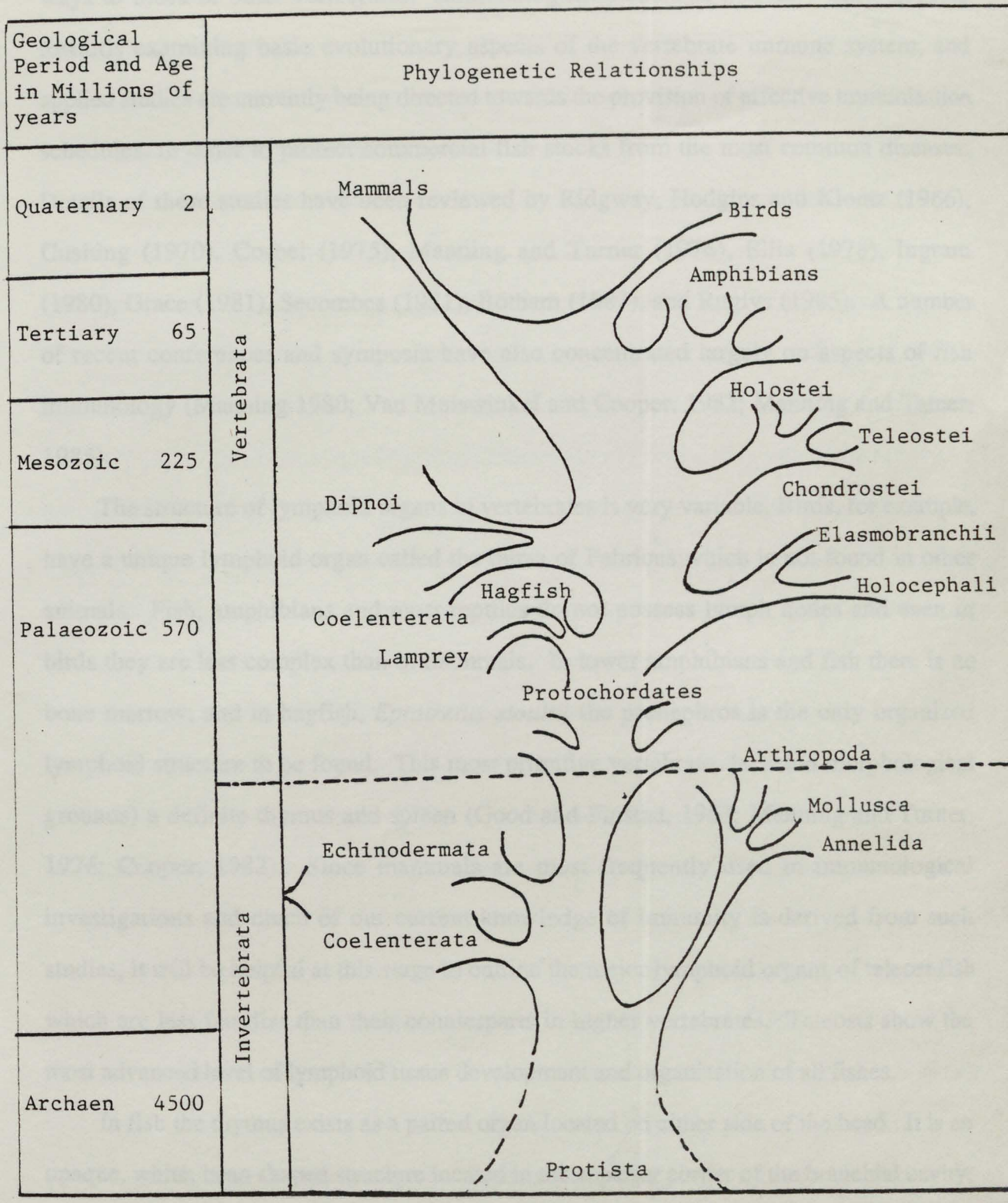
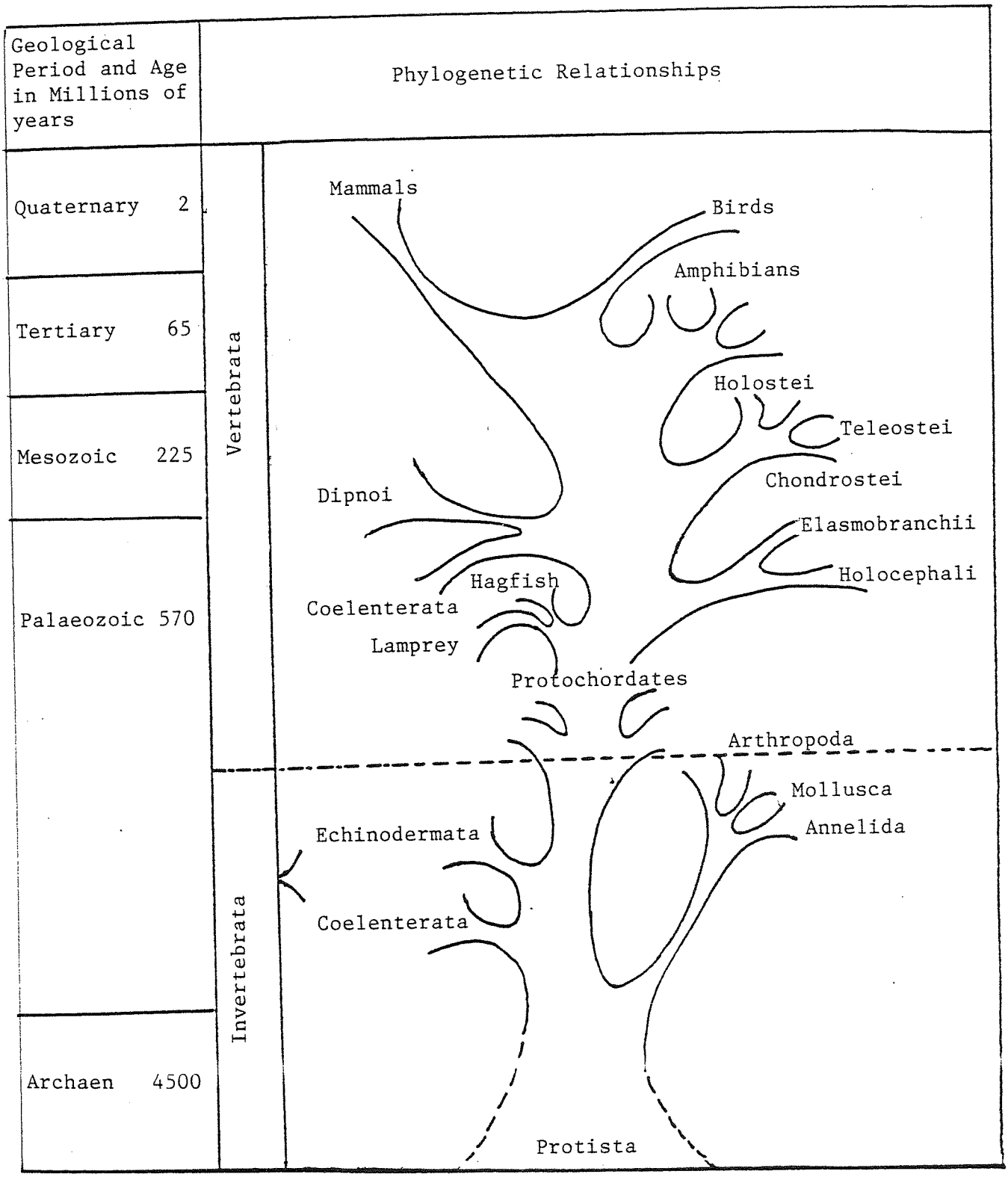


Fig. 1.1 Evolution of fish groups in relation to the Animal Kingdom (from various sources, after Young, Y.Z., 1981).



immunology (Cushing, 1970). More recently the immune responses of the class Pisces or Fishes have received additional attention. Knowledge of fish immunity is still expanding and it appears that in many respects the immune system of fish may function in similar ways to those of other vertebrates. Immunological studies of fish have been directed towards examining basic evolutionary aspects of the vertebrate immune system, and applied studies are currently being directed towards the provision of effective immunisation schedules, in order to protect commercial fish stocks from the most common diseases. Details of these studies have been reviewed by Ridgway, Hodgins and Klontz (1966), Cushing (1970), Corbel (1975), Manning and Turner (1976), Ellis (1978), Ingram (1980), Grace (1981), Secombes (1981), Botham (1982), and Ruglys (1985). A number of recent conferences and symposia have also concentrated largely on aspects of fish immunology (Manning 1980; Van Muiswinkel and Cooper, 1982; Manning and Tatner, 1985).

The structure of lymphoid organs in vertebrates is very variable. Birds, for example, have a unique lymphoid organ called the bursa of Fabricus which is not found in other animals. Fish, amphibians and most reptiles do not possess lymph nodes and even in birds they are less complex than in mammals. In lower amphibians and fish there is no bone marrow; and in hagfish, *Eptatretus stoutii* the pronephros is the only organized lymphoid structure to be found. This most primitive vertebrate lacks(on morphological grounds) a definite thymus and spleen (Good and Finstad, 1967; Manning and Turner, 1976; Cooper, 1982). Since mammals are most frequently used in immunological investigations and much of our current knowledge of immunity is derived from such studies, it will be helpful at this stage to outline the major lymphoid organs of teleost fish which are less familiar than their counterparts in higher vertebrates. Teleosts show the most advanced level of lymphoid tissue development and organization of all fishes.

In fish the thymus exists as a paired organ located on either side of the head. It is an opaque, white, bean shaped structure located in the superior corner of the branchial cavity, under the operculum. The development of the thymus in fish has been described by several authors (Deanesley, 1927 ~~to 1972~~; Hafter 1952), and the sequence of histogenesis in relation to the other lymphoid organs has also been investigated (Sailendri 1973; Grace

and Manning 1980). It has been suggested that the fish thymus is a central lymphoid organ (Ellis, 1978). Using the mammalian technique of '*in situ*' thymic labelling, the thymocytes of the rainbow trout, *Salmo gairdneri* Richardson, were successfully labelled with $3^{+}H$ thymidine. The migration of the cells to the peripheral lymphoid organs was followed using autoradiography and scintillation counting and it was shown that twice as many thymocytes migrated to the spleen, as to the kidney (Tatner., 1985).

Thymocytes, are similar throughout the fishes although thymic organization varies. In *Astyanax mexicanus* there are cortical and medullary areas (Hafter, 1952) but in many fish the morphology is undifferentiated as, for instance in the rainbow trout, *Salmo gairdneri* (Grace, 1981). Young carp (*Cyprinus carpio*) have an undifferentiated thymus but division into medulla and cortex has been observed in older animals (Botham, 1982). Apart from thymocytes, other cells are present in the thymus including granulocytes (Zapata, 1981; Chilmonczyk, 1983) and Botham (1981) found that plasma cells may be associated with blood vessels. The outer membrane of the rainbow trout thymus is a single epithelial layer in contact with the water. It has been suggested that this thin layer offers very little protection for the thymus and may represent a source of entry of antigen from the environment (Grace, 1981; Chilmonczyk, 1983).

The histogenesis of the lymphoid organs in rainbow trout, *Salmo gairdneri* (Rich 1836), has been studied by Grace and Manning (1980) who concluded that the embryo of the trout already possesses the thymic rudiments 5 days pre-hatch at $14^{o}C$ (Stage 28) when haemopoietic foci are also present in the developing kidney. During the next week, active lymphopoiesis takes place in the thymus and by stage 33 (5 days post hatch) the thymus is clearly a lymphoid organ. In carp at $22^{o}C \pm 1^{o}C$, the thymus first appears at 2 days post hatching and becomes actively lymphopoietic by day 5 (Botham and Manning, 1981). In *Barbus conchonius* at $23^{o}C$, the first appearance of lymphocytes in the thymus was noted at 4 days post hatch (Grace, 1981), and Sailendri (1973) observed the appearance of thymic lymphocytes from 6 - 8 days in *Tilapia mossambica*. In contrast in *Salmo salar* at 4 - $7^{o}C$, the first appearance of lymphocytes in the thymus was at 22 days pre-hatch (Ellis, 1977). Whilst the thymus of teleosts may be acting as a primary lymphoid organ, a number of authors have demonstrated the presence of plaque forming cells in the thymus

of different fish. This suggests that in teleosts the thymus may also have the functional characteristics of secondary lymphoid tissue (Ortiz - Muniz and Sigel, 1971; Sailendri and Muthukkaruppan 1975).

In fish, the anterior portion of the kidney, which lies dorsal to the body cavity, is predominantly lymphoid. The pronephros, or head kidney, is of major importance as a lymphoid and haemopoietic organ. In *Rutilus rutilus, Gobio gobio;* and in rainbow trout, the head kidney can be distinguished from the middle and posterior kidney simply by the reduction in the number of nephric tubules and increased numbers of lymphoid cells (Zapata, 1979; Grace, 1981). The organ is well vascularized and the small blood vessels are lined with reticular cells. Other cells that may be present include granulocytes, plasma cells, and macrophages. Melanomacrophage centres are also present, as in spleen, but true differentiation into red and white pulp is not observed. Erythroblasts are also to be found in the pronephros, indicating its haemopoietic nature. There is considerable argument as to the homology of the pronephros with mammalian lymphoid organs but Zapata (1979) has shown a histological resemblance to bone marrow. The morphology of the various lymphoid organs in a range of fishes from cyclostomes upwards was studied in 1966, by Good et al., whose studies revealed that the development of immunological capacity is associated with the development of well organized lymphoid centres such as spleen or thymus, and also with the appearance of plasma cells (antibody secreting cells). Sailendri (1975) studied the origin, development and functional morphology of the lymphoid system in *Tilapia mossambica* and found that neither gut associated lymphoid tissues nor lymph nodes were present at this level of evolution. However in *Tilapia* as in other teleosts, there is good evidence that the spleen and head kidney are important sites of antibody synthesis (Smith et al, 1967; Chiller et al, 1969; Pontius and Ambrosius, 1972; Sailendri, 1973; Manning and Turner, 1976).

The spleen in teleosts lies along the left side of the stomach as an elongated organ in the body cavity. In early months it is an erythroid organ but gradually acquires a lymphoid cell population and begins to differentiate to red and white pulp areas. The spleen has a distinctive deep red colouration caused by erythrocytes which are its major cellular component. Generally, the erythroid and lymphoid elements of this organ are largely

divided into red and white pulp areas. However, carp has no such distinction into red and white pulp regions (Botham, 1982). In *Rutilus rutilus* and *Gobio gobio*, the erythroid and lymphoid elements are largely divided into red and white pulp areas respectively (Zapata, 1982). An intermediate stage is described by Grace (1981) for the rainbow trout, where lymphocytes tend to congregate around splenic blood vessels. Melanin is often associated with these accumulations and in *Cyprinus carpio* (carp), *Rutilus rutilus, Gobio gobio* and other fish (Agius, 1984) these are termed melanomacrophage centres. In *Rutilus rutilus* and *Gobio gobio* , one important feature of splenic lymphoid tissue is the relationship between macrophages and lymphoid elements. Large macrophages often form clusters with small lymphocytes blasts, and plasma cells where cellular junctions between lymphoid elements and macrophages have been reported (Zapata, 1982).

The spleen is also a site of antigen trapping. The generation of memory cells and the regulation of humoral immunity is thought to involve antigen trapping and retention in the spleen and head kidney. The thymus of fish is not a major phagocytic organ (Ellis, 1974; Ferguson, 1976; Grace, 1981), but fish are capable of trapping antigen in the spleen and the lymphoid areas of the kidney, mainly the pronephros (Secombes and Manning, 1980; Secombes et al., 1982). Soluble antigen can be detected in the ellipsoid sheaths which surround the splenic capillaries and appears to be trapped extracellularly. Cellular antigens appear to be engulfed by macrophages both in the ellipsoids and within the splenic red pulp. On secondary injection, antigen trapping is much more rapid in both spleen and pronephros and this may indicate that the trapping of antigen is dependent upon the presence of specific antibody. Recent studies by Secombes and Resink (1984) have shown that injecting antigen-antibody complexes results in good memory formation.

Clusters of pyroninophilic cells also appear in lymphoid organs during immune responses, and seem to be a prerequisite for good secondary responses. It is possible that these cell clusters are the analogues of the higher vertebrate germinal centres. These cells disappear with time to be replaced by cells which often contain pigments. Secombes and Manning (1982) thought that these may become melanomacrophage centres which can influence lymphocyte populations.

The histogenesis of the lymphoid organs in rainbow trout, *Salmo gairdneri* (Rich, 1836) has been studied by Grace and Manning (1980). They found that the splenic anlagen is first seen at 3 days post hatching but the spleen is slow to become lymphocytic. It remains erythroid with no division into red and white pulp, until 25 days post hatching. The spleen first appears at day 5 in *Cyprinus carpio* (carp) but it develops more slowly than the kidney and remains predominantly erythroid for several months (Botham and Manning, 1981). Studies on *Barbus conchonius* by Grace (1981) revealed that the spleen first appears at 7 days post-hatching, however, Sailendri (1973) found that lymphocytes appeared in the spleen of *Tilapia mossambica* between 30 to 80 days of age, and in *Salmo salar*, the spleen only becomes lymphoid at 42 days post hatching (Ellis, 1977).

Following antigenic challenge, cell-mediated immunity in mammals involves the clonal expansion of sensitized T-lymphocytes which have antigen specific receptor molecules on their surface. These T-cells (thymus-derived) are the effectors of cell-mediated immunity. This is manifested in a number of ways including the ability to reject tissue transplants from genetically dissimilar donors. Allograft rejection has been well studied in fish. The lower orders of fish show longer rejection times than do the more advanced teleosts, but second set grafts are rejected in an accelerated manner, indicating a true anamnestic response. The evolutionary trends in transplantation immunity in fishes have been reviewed by Good and Finstad (1964), Hildemann (1972b), Manning and Turner (1976). The ability to reject foreign tissue grafts is already present in the hagfish and lampreys of the class Agnatha, which are considered to be the most primitive living vertebrates (Hildemann and Thoenes, 1969). These rejected foreign grafts slowly, and second set grafts were rejected more quickly, showing this to be a true immunological response. The same picture of slow rejection of initial skin grafts is also characteristic of the sting rays and sharks (Chondrichthyes). Among the primitive bony fish, as examplified by the paddlefish and arowana, a transition from slow to rapid elicitation of transplantation immunity is evident (Finstad and Good, 1966). Advanced bony fish, without exception, all show acute rejection of first set grafts and accelerated rejection of second set grafts, with a vigorous cellular immune response equal to that of many

mammalian laboratory animals. This is suggestive of the presence of a major histocompatibility system coding for products analogous to mammalian class I MHC antigens.

The allograft response in fish has been studied extensively in a number of species. Most investigators have studied the response to transplanted scales (Rijkers & Van Muiswinkel, 1977; Hildemann, 1960, 1962; Hildemann & Cooper, 1963; Triplett & Barrymore, 1960; Sailendri, 1973) using the technique first described by Mori in 1931. Other workers have transplanted skin (Botham, Grace and Manning 1981; Tatner and Manning 1983; Hildemann and Thoenes 1969; Perey, Finstad, Pollara and Good 1968) and whole fin transplantation has also been carried out (Kallmann and Gordon 1958; Egami and Kukita 1969). In all cases, fish showed a typical immune response, they reject first set grafts at varying time intervals, which are thought to be dependent on the immune capabilities of the host, although the rejection time is also very temperature dependent (Hildemann 1956a, 1956b; Triplett and Barrymore 1960; Reid & Triplett 1968; Sailendri, 1973). Graft rejection in fish is similar to that observed in mammals. Typically, skin autografts and allografts are indistinguishable for several days after grafting. Host epidermis grows over the skin and revascularization takes place throughout the graft bed. Autografts continue to heal and retain full pigmentation. In allografts, lymphocytic invasion commences soon after grafting, breakdown of the dermal layer of the graft is seen after peak invasion, and melanin pigmentation is progressively lost from the graft. Complete rejection is assessed by the absence of melanin pigmentation from the graft.

Sailendri (1973) has shown that 1 1/2 month old *Tilapia mossambica* rejected grafts more slowly than did the adults, but by 4 1/2 months old, the rejection times were similar to those of adults. In 1980 and 1981 Botham, Grace and Manning studied the histological pattern of first and second set allograft rejection in the rainbow trout, *Salmo gairdneri* and the mirror carp, *Cyprinus carpio*. Their ontogenetic studies showed that trout as young as 14 days post-hatch, and carp at 16 days post-hatch were capable of eliciting an allograft response. However, in both cases the number of lymphocytes invading the graft area was fewer than that seen in older fish and rejection times were somewhat slower. The ability to respond to foreign tissue grafts was correlated with the presence of a morphologically

mature lymphoid population in the thymus and the kidney, and the presence of circulating lymphocytes.

The role of the thymus in transplantation immunity in fish has not yet been fully elucidated. The earliest experiments on thymectomy in fish were Sailendri's studies in 1973 on *Tilapia mossambica* and those of Jayaraman, et al., (1979), also on *Tilapia.* Sailendri's experiment showed that while adult thymectomy had no effect on allograft and antibody responses, the removal of the thymus in young adults at the age of two months completely abolishes the antibody response to SRBC. If thymectomy was performed at the age of 4 months, a prolongation of survival of scale allografts was observed (Sailendri and Muthukkaruppan, 1975). Thymectomy in adult trout had no effect on the allograft response, but thymectomy at 2 months of age gave rise to impaired graft rejection one month later (Grace, 1981).

In addition to their role in graft rejection, mammalian T-lymphocytes are also known to be involved in a number of other immunological activities. Thus, they are responsible for controlling the immune response in their capacity as helper or suppressor cells, and they also secrete a number of soluble molecules called lymphokines which influence the behaviour of other cells of the immune system (Dumonde, 1969). These effects include delayed hypersensitivity reactions such as the production of migration inhibitory factor (MIF) which prevents migration of macrophages in culture. Several authors have demonstrated cell-mediated immunity in fish by the degree of inhibition of macrophage migration *in-vitro*. Thus, levels of plaque forming cells and cell mediated immunity to sheep red blood cells (as assessed by the MIF assay) in *Tilapia mossambica* were found to be inversely proportional depending on the amount of antigen injected (Muthukkaruppan and et al., 1979). Cell suspensions from carp which contain relatively few macrophages also demonstrate inhibition of migration *in vitro*, and the magnitude of the response is antigen dose dependent (Secombes, 1981). A holostean fish, the gar, also exhibits macrophage migration inhibition (McKinney et al., 1981), and a cell free extract from stimulated lymphocytes was found to be capable of inducing morphological changes in salmon macrophages (Smith and Brown-Nesje, 1982). Grondel and Harmsen, 1985 have also reported that carp leucocytes can produce, and release growth factors with similar

characteristics to mammalian Interleukins. Such immunoregulatory factors may play an important role in the immune responses of fish.

The mixed leucocyte reaction MLR has been interpreted as an *in-vitro* analogue of the allograft response. In mammals, strong MLR's have been shown to be associated with major histocompatibility complex (MHC) incompatibility, and to obey the laws of transplantation (Bach, 1967) and Dutton (1966). Bony fish appear to possess strong histocompatibility antigens as evidenced by acute rejection of first set grafts (Hildemann, 1970; Borysenko, 1976). Positive MLR has so far been successfully demonstrated in a number of species including salmon, trout, blue gill and carp (Ellis, 1977; Etlinger, Hodgins and Chiller, 1977; Caspi and Avtalion 1984) . Negative results were obtained in catfish and snappers (McKinney and et al., 1976) as well as in the holostean gar, even though these bony fish also reject first set allografts in an acute manner (McKinney, McLeod and Sigel, 1981). In mammals strong MLR's are associated with Class II antigens coded for by the MHC (Klein, 1977). Another phenomenon associated with the products of this genetic region is the graft-versus-host reaction, which has also been described in fish by Wright and Cooper (1982).

Fish lymphocytes also undergo transformation when exposed to mammalian T and B cell mitogens. In mammals phytohaemagglutinin (PHA) and concanavalin A (Con A) activate T-cells, where as lipopolysaccharide (LPS) is known to stimulate B-cells. Lymphocytes from rainbow trout and plaice respond to both PHA and Con A (Bognor, 1975). Etlinger, Hodgins and Chiller (1976 a,b,c and 1978) found that in trout, cells from the thymus responded to Con A but not LPS in culture. Cells from pronephros responded well to LPS but splenocytes gave good responses to both mitogens - perhaps suggesting the presence of B and T-cell populations.

The humoral immune response involves the production and release of specific antibody into the blood and other body fluids following antigenic stimulation. In mammals, the antibodies are produced by host 'B' lymphocytes which on contact with their specific antigen, and appropriate 'help' from T-lymphocytes and antigen presenting cells, divide and differentiate to form a clone of antibody secreting plasma cells. Some lymphocytes became 'memory cells' following antigenic challenge, and the individual may

then be immune to further attacks. All vertebrate species studied so far are capable of producing specific antibody. Immunological memory is also evident (Manning, 1980).

In 1948 Bisset was the first to show the existence of specific agglutinating antibody in perch. He also demonstrated the temperature dependency of the antibody response in fish and showed that no antibody production occurred below a certain critical temperature. This temperature varied according to the species, and was related to the normal temperature range of their environment. More recently, Avtalion et al., (1973), have shown that it is the induction phase of the primary response which is temperature dependent, and not the antibody release from the plasma cells. The ectothermic nature of fish makes them dependent upon the water temperature for the speed of biochemical reactions. The immune response is so affected and lower temperatures delay the peak of antibody production from 8 days at 24°C, to 49 days at 12°C in *Cyprinus carpio*, but the magnitude of the response was unaffected (Rijkers, 1982). The route of administration of antigen is also important in determining the magnitude of the response; Rijkers et al. (1980a) have shown that intramuscular immunisation in *Cyprinus carpio* is more effective than the intravenous route.

Fish only possess antibody of the IgM class in contrast to mammals which have five classes of immunoglobulin. Such diversity as does exist amongst fish immunoglobulins is based upon differences in heavy chain length or the degree of polymerization of the basic four chain IgM unit. In elasmobranchs and chondrosteans 7S and 19S immunoglobulins occur; in holosteans a 19S form is predominant; and in teleosts both 7S and 19S forms occur. Serum IgM in the chondrichthyes is pentameric in form whereas in the Teleostei it is tetrameric (Ambrosius et al., 1982; Jurd, 1985). Ambrosius et al. (1982) demonstrated that the sIg on lymphocytes of carp is a 7S IgM. On thymocytes of the same species the membrane Ig also cross reacts extensively with the constant region of the IgM heavy chain. The possibility of novel Ig classes in fish has been raised by Clem (1971) who reported that the teleost *Epinephelus itaira* has an IgM molecule with a deleted M chain. Marchalonis (1969) also reported that the dipnoan *Neoceratodus forsteri* possesses an Ig molecule (IgN) with a sedimentation coefficient of 5.9S.

Antibody production in mammals is dependent upon cellular co-operation between T-lymphocyte subsets and B-cells. Hapten carrier studies have been carried out on a number of fish species and would seem to indicate that helper cell populations may exist in teleosts as in mammals (Stolen and Makela, 1975; Ruben et al., 1977; Yocum et al., 1975). Whether helper cells are truly thymus dependent in fish remains to be established. Immunological tolerance is the inability of an animal to generate an immune response against a specific antigen whilst making normal responses against other antigens. From studies on mammals, it is clear that there are a number of different factors which affect the induction of immunity or tolerance. Amongst these are the state or age of the lymphon, antigen size and presentation and antigen dose (Roitt, Brostoff and Male, 1985). Recently, attention has turned to the question of whether suppressor cells exist in fish. Avtalion and co-workers have demonstrated immunosuppression or tolerance in fish given high doses of antigen and held at low temperatures (Serero and Avtalion, 1978; Wiskkovsky and Avtalion, 1982). Early exposure to antigen in young fish may also result in tolerance during later life (Secombes, 1981; Van Loon et al., 1981, Mughal and Manning, 1985). There have also been reports to suggest that tolerance may be induced in adult fish (Lopez et al., 1974; Serero and Avtalion, 1978; Avtalion et al., 1980; Manning & et al., 1982). Recently, Ruglys (1985) has demonstrated that immunosuppression may be brought about in adult carp following exposure to hapten (Trinitrophenol TNP) modified self erythrocytes. Whether a discrete category of (T) suppressor cells exists in fish is not yet known but it may be inferred that in teleosts, as in mammals, the immune response is controlled by interacting lymphocyte subsets. In mammals cellular cooperation in the immune response is associated with class II regions of MHS and the same may also be true in fish. The production of monoclonal antibodies specific for surface determinants of fish thymocytes provides new possibilities for the recognition of cell subpopulations in teleosts (Secombes et al., 1983a; 1983b).

In addition to their well developed adaptive immune responses, teleosts also possess a range of non-specific or innate defence mechanisms. Many may be of particular importance as a first line of defence against pathogenic intrusion e.g., mucous secretion by the skin may prevent the attachment of unwanted ectoparasites, bacteria and fungal spores

(Pickering and Willoughby, 1982). Many of these more primitive defence mechanisms may be especially important at low temperatures when the adaptive immune response is slow or suppressed, or may have an important role to play during early life before the immune system is fully developed (Grace et al., 1980).

Teleosts possess natural haemolysins in their serum which are capable of lysing foreign red cells (Chiller & et al., 1969), they also possess haemagglutinins (Bezkorovainy et al., (1971). The function of these serum components is not clear but they may react to common surface carbohydrates on foreign cells. A number of authors have described the presence of α-precipitins in the serum of teleost fish which seem to react non-specifically to fungal extracts of the pathogenic Saprolegniales (Davies and Lawson, 1982; Alexander, 1980 to 1982; Wilson, 1976; Hodkinson and Hunter, 1970). However, Ellis (1985) has questioned the significance of α-precipitins. His work suggests that they may be artifactual products caused by enzymatic breakdown of fish serum components by the proteases present in fungal extracts used as antigen. Other non-specific defence mechanisms found in teleost include phagocytes (Grace et al., 1980; Ellis, 1978; MacArthur and Fletcher, 1985) which are particularly important in the removal of particulate foreign matter. Besides lymphocytes, fish also possess most of the white blood cell types described in mammals. However, this classification is restricted to their staining characteristics and not necessarily their function. Cells with phagocytic activity in fish include monocytes, macrophages and neutrophils. The structure and function of fish leucocytes has been reviewed by Ellis (1977a).

Fish also possess lysozyme which is a hydrolytic enzyme with bactericidal activity found in mucus and serum (Fletcher and White, 1973; Fänge et al., 1976). Interferon has also been demonstrated in rainbow trout (Dorson and de Kinkelin, 1975) and may be involved in preventing viral replication in host cells. C-Reactive Protein (CRP) is a protein associated with inflammatory reactions in mammals and appears to bind to phosphocholine containing molecules which are widely distributed in nature. White et al., (1981) found that levels of CRP in plaice increase rapidly following exposure to bacterial endotoxins. Fletcher (1982) has suggested that the binding of CRP onto foreign material may provide some form of protection whilst specific antibodies are being synthesized.

Fish have a well developed complement system which is capable of lysing foreign cells in the presence of complement fixing antibody. There is evidence that complement in fish shows interspecific variation since Chiller et al. (1969) found that only salmonid complement was effective for rainbow trout antibody. This finding was borne out by Rijkers et al. (1980) who found that only complement from closely related species would lyse erythrocytes in the presence of carp antibody. Fish complement appears to be more heat labile than its mammalian counterpart and is inactivated at 45°C. Besides its lytic activity, mammalian complement is also involved in a range of other biological activities including opsonisation, chemotaxis, inflammation and anaphylaxis (Roitt et al., 1985), although it is not known to what extent these activities are represented in the immune responses of fish. In catfish, there is some evidence to suggest that the alternative pathway of complement activation is important in the lysis of *Salmonella paratyphi* (Ourth and Wilson, 1982).

In conclusion, it would appear that the teleosts have acquired an effective immune system which incorporates many of the features seen in those of higher vertebrates. The lack of sophistication seen in some facets of teleost immunity has clearly not provided an undue obstacle to their evolutionary success.

Ontogeny presents an opportunity to study and understand the generation of antibody diversity. These early developmental stages are also crucial to the establishment of "self and non-self" recognition and offer opportunities for the study of immunological tolerance and the generation of major histocompatibility complex (MHC) restricted interactions. Finally, knowledge of the primary differentiation of lymphocytes in normal individuals may be of importance in understanding the mechanisms at work in immunodeficiency. In many of these areas, studies utilizing amphibians have become increasingly important in recent years (Du Pasquier, 1982; Arnall and Horton, 1986). The free-living nature of the embryonic or larval stages of fish also allows them to be utilized for studying ontogeny, but in contrast to mammals and amphibians less is known about similar events in fish. Recent studies however, have begun to redress the balance at least as far as ontogeny of lymphoid organs is concerned, and one of the aims of the work presented here is to add to the knowledge of early development of the fish immune system.

To this end, Chapters 2 and 3 are concerned with examining the ontogeny and development of the major lymphoid organs in the teleost *Tilapia mossambica*. Although a similar study has been carried out by Sailendri (1973) it was important to establish whether or not the detailed events of ontogeny were similar in fish raised under different laboratory conditions. The structure of the developing lymphoid organs is compared to those of the adult fish, and an attempt was made to study ultrastructural details of these organs in Chapter 3. In Chapter 4, attention is focussed upon the immune response of *Tilapia* to a range of different antigens. These include cellular antigens in the form of xenogeneic erythrocytes and bacteria and a soluble antigen (Human gamma globulin) was also used. The primary and secondary responses to these antigens are studied, and a comparison made between the immune response of young (10 week old fry) and adult fish. The effects of early exposure to antigen on the immune response in later life are also examined. Chapter 5 takes the form of a general discussion of the work presented in this thesis and raises some possibilities for future studies.

CHAPTER 2

CHAPTER 2

ONTOGENY OF LYMPHOID ORGANS IN *TILAPIA MOSSAMBICA*

2.1 INTRODUCTION

In order to fully understand the processes involved in immunological maturation, it is necessary to acquire information about the development of different lymphoid organs and tissues. Thus, in recent years there has been increased emphasis on developmental aspects of immunity in lower vertebrates. Amongst reptiles, for instance, the maturation of immunological capacity in relation to lymphoid differentiation has been studied in the lizard *Calotes versicolor* (Kanakambika, 1971; Kanakambika and Muthukkaruppan, 1972, 1973). Of all the lower vertebrates, the amphibians have received the most attention and a detailed picture of immunological events during amphibian ontogeny is now available (Du Pasquier, 1982). In 1970 Du Pasquier studied the ontogeny of the spleen in *Alytes obstetricans*, and found that even if it contains as few as 500 - 1000 cells, there is always one cell able to react with a specific antigen. Amphibians are particularly attractive models for the study of ontogenic events since their larvae are free living and are amenable to a range of experimental manipulations during early life. Early thymectomy has a suppressive effect on allograft reactivity (Horton and Manning, 1972; Horton and Horton, 1975; Rimmer and Horton, 1977). Turner and Manning (1974) also reported that specific antibody responses of both IgM and IgG type were inhibited in *Xenopus laevis* following early thymectomy. Du Pasquier and Horton (1976) demonstrated the lack of MLR and PHA responsiveness in thymectomized *Xenopus* after early thymus removal.

In adult fish, the lymphoid organs of several species have been described in detail e.g. Sailendri and Muthukkarruppan (1975) for *Tilapia mossambica,* and Smith (1970) for the pronephros of the carp, *Cyprinus carpio*. Zapata (1979) has performed electron microscope studies, on the fish pronephros, thymus and spleen. Fänge (1968) gave a general account of the lymphomyeloid tissues and white blood cells of fish and concluded that whilst a thymus had not been found in adult *Myxine glutinosa* (hagfish), it is present in young

stages and involutes as a rule with age. However in certain elasmobranchs, in ganoids and in *Chimaera monstrosa* the thymus shows little or no age involution (Good et al. 1966, Fange and Sundell, 1968).

Until recently however, studies on the ontogeny of lymphoid organs and the emergence of immunocompetence in fish have been relatively scarce. This is perhaps surprising when one considers that the maturation of the immune system takes place post-hatching and, like amphibians, fish have free-living embryonic and larval stages which are readily available for experimental study during early development. Part of the reason for this neglect may be that fish are relatively difficult to thymectomize when compared to larval amphibians.

Studies over the past decade however, have now begun to provide information on the ontogeny of lymphoid organs in a number of fish species e.g. Denton and Yousef (1976) have plotted the growth of the spleen in the first 14 months of life in the trout. The growth of the lymphoid organs in rainbow trout, *Salmo gairdneri* from one to fifteen months of age was studied by Tatner (nee´ Grace) in 1983 and the earlier development of the lymphoid organs of the same species has been described by Grace and Manning (1980). Manning, Grace and Secombes (1982) have reviewed details of the functional development of the immune system in fish. They reported that, as in other vertebrates, the thymus is the first lymphoid organ to develop in fish. Studies on the ontogeny of immunocompetence in fish were also made by Sailendri (1973) on Tilapia (*Sarotherodon mossambica)*; by Ellis (1977) on Salmon (*Salmo salar);* by Rijkers and Van Muiswinkel (1977) on the rosy barb *(Barbus conchonius)* ; and by Bly (1984) on *(Zoarces viviparus)*. Details are also emerging of the ontogenic development of immune functions such as alloimmune reactivity (Botham and Manning, 1981; Sailendri, 1973; and Botham, Grace and Manning, 1980); mixed leucocyte reactivity (Ellis, 1977); humoral antibody production (Paterson and Fryer, 1974; Khalifa and Post, 1976; Secombes, 1981); and the appearance of surface immunoglobulin on lymphoid cells (Ellis, 1977; Van Loon, Van Oosterom and Van Muiswinkel, 1981). Other studies have charted the ontogenetic appearance of immunological reactivity to thymus-dependent and thymus-independent antigens (Secombes, 1981; Etlinger, Chiller and Hodgins 1979) and the influence of age and lymphoid organ development on tolerance

induction (Manning, Grace and Secombes, 1982; Mughal and Manning, 1985).

The present study seeks to extend and add to earlier observations on the histogenesis of lymphoid organs in teleost fish. In this chapter the sequential development of the major lymphoid organs of *Tilapia mossambica* are examined by light microscopic studies and a comparison is made with the fully differentiated structure of these organs in the adult fish in Chapter 3. Particular attention is given to the stages at which the thymus, spleen and head kidney first became lymphoid and the subsequent differentiation of these organs is described.

2.2 MATERIALS AND METHODS

Eggs were obtained from Stirling University, Institute of Aquaculture, and fixed in Bouin's fluid from the day of hatching to 10 days post hatching [Eggs of *Tilapia* take from 60 to 72 hours till hatching].

Fry of 10 days post-hatching were also obtained from the same place for rearing in the laboratory. Fry and eggs were maintained in 6 litre tanks and the water temperature maintained at 22^{o}C. They were fed with artificial diet, trout pellets (ground pellets) from 12 days post-hatching onward.

From day 11 post-hatching till day 16 post-hatching, 3 fry were sacrificed daily. Each fry was weighed, measured and fixed in Bouin's fluid for wax embedding and Haematoxylin and Eosin staining. Serial longitudinal sections were cut at 8 microns and were supplemented with transverse sections in some ages. From day 17 post-hatching onwards 3 fry were sacrificed 3 times a week for a further 8 weeks in order to cover the whole experiment.

In all, about 120 fry were sectioned and examined to investigate the appearance of thymus, pronephros (head kidney), kidney and spleen as lymphoid organs. [Details of mean weight, length and width of each fry are seen in Table 2.1].

2.3 RESULTS

Histogenesis and Development of the Lymphoid Organs

(a) Thymus

The thymic anlagen was first visible above the second and third branchial arches at 2 days post-hatch (Fig. 2.2). At this time it was seen to be more than one cell layer thick and closely attached to the branchial pouches. Although not obviously lymphoblastic, the cells did not appear to be typically epithelial and are close to the operculum (Fig. 2.3).

By day 4 post-hatch (Figs. 2.4 & 2.5) the number of cells present had markedly increased and became larger with lightly staining cytoplasm and darker cytoplasmic inclusions. Their overall appearance was suggestive of lymphoblast-like cells. The thymus did not possess an encapsulating membrane at this time, but fibroblast-like cells observed in the vicinity of the organ appeared to form a capsule around it later on. The thymic rudiment increased rapidly in size and cell number and, in time, became detached from the pharyngeal epithelium.

At 8 days post-hatching (Fig. 2.6) the thymus had extended anteriorly so that it could be seen to extend in front of the opercular flap. At this stage the individual cells were more easily distinguished and more loosely packed. The thymus appeared to leave a clear passage at its border with the gill pouch at 9 days post-hatching (Fig. 2.7) and it became much larger and covered by a single layered epithelial capsule. Here, cells in the centre of the organ were smaller and more darkly staining, although they were still immature lymphocytes. Cells at the periphery of the thymus were obviously larger than those nearer the centre, and this larger cell type was also visible in the extra-thymic tissue.

Dorsally, there was a small break in the centre of the organ and here large thymoblasts which were in direct contact with the surrounding tissue were seen. After this time, the organ increased more and more in size due to division and active mitosis, and in the dorsal part of the organ connective tissue was clearly visible. Separation of the thymus from the gill pouches took place from about 8 days onwards. From four to five weeks of age lymphocytes of the thymus appeared to be clumped into groups separated by connective tissue. At this stage no clear division into cortex and medulla could be seen, although such

a division was noticed in older animals of 5 weeks of age. No distinct change in the structure of the thymus was seen after this time, although the overall size of the organ and the number of different cells, including lymphocytes, increased during the first months of life .

(b) Kidney

Kidney tubules were present as early as 48 hours after hatching (Fig. 2.8) at the same time they ramify in the pronephric region. At this stage, some cells could be seen between the tubules including mature erythrocytes and cells which were classified as haemopoietic stem cells. By day 5 post-hatch (Fig. 2.9) the tubules in the pronephric region had become more noticeable and an increase in the amount of intertubular tissue was also observed. Undifferentiated stem cells were still present in the kidney 6 days after hatching (Fig. 2.10) but together with these, smaller, more darkly staining cells were seen, which may have been differentiating cells of the lymphoid line.

A noticeable increase in intertubular haemopoietic tissue occurred by 7 days post-hatch (Fig. 2.11) much of this consisting of differentiating lymphoid cells. Mature lymphocytes were seen in the blood vessels of the kidney and in the intertubular tissue itself at this time. The pronephric region exhibited perhaps the greatest increase in intertubular tissue and the tubules were displaced laterally by its expansion.

By day 30 post-hatch or between four and five weeks old, the intertubular tissue in the pronephric region had increased and surrounded the blood sinuses (Fig. 2.12). Many mature lymphocytes were seen both in this area and in the intertubular area of the mesonephros, thereby marking the stage at which the pronephros becomes modified into a lymphoid head kidney (Fig. 2.13).

As the fish developed, the pronephric tubules were lost, leaving only those of the mesonephric region. However, the pronephros retained its haemopoeitic and lymphoid tissue, which was also found in the intertubular area of the mesonephros. Both thymus and head kidney are shown in (Fig. 2.14). Small numbers of lymphocytes were sometimes seen in the connective tissue between the two organs.

(c) Spleen

The spleen was first seen 5 days post-hatching (Fig. 2.15) when it appeared as a small, encapsulated organ still closely attached to the dorsal mesogastrium.

By 6 days post-hatch (Fig. 2.16) reticular cells were present and the cells appeared to be more tightly packed than at earlier stages of development. Connective tissue was also visible between the cells. At earlier stages of spleen development more than one cell type could be distinguished, erythrocytes were clearly visible as were large lightly staining cells similar to those seen in the developing kidney (Fig. 2.17). Mature lymphocytes were first visible in the spleen from 35 days post-hatch although organization of the cells into red and white pulp areas was not seen. The organ itself became more elongated in shape at this time. After this stage the spleen continued to increase in size, but even in the adult fish, it remained largely erythroid.

In late post larval stages (70 - 80 days) future red and white pulp were distinguished, the latter being lighter staining. From this stage onwards the spleen appeared to be a mature lymphoid organ with well defined red and white pulp. More details on adult spleen are given in Chapter 3.

2.4 DISCUSSION

All species of fish studied by previous researchers showed the same sequence of development, the thymus developing first followed by the pronephros and then the spleen. The thymus and the kidney are the most important lymphoid organs in young fish, and moreover (as in other vertebrates) the thymus is the first organ to become lymphoid. (Manning 1982). In 1980, Grace and Manning observed that the thymus of *Salmo gairdneri* starts its development at 3 days post-hatching as a thickening of the epithelial tissue in the dorso-anterior part of the pharynx and also that the thymus was separated from the pharyngeal cavity by only a single layer of epithelial cells at 5 days pre-hatching at 14°C. In rainbow trout, the embryo already possesses the rudiments of a thymus, and at 5 days post-hatching the thymus is clearly a lymphocytic organ. In the present study the

thymic anlagen is first visible at 2 days post-hatching and at this time, haemopoietic foci are present in the kidney. As the thymus increases in size, so its cell number increases and individual cell size decreases, presumably due to maturation division, by day 8 mature lymphocytes are the predominant cell type. These findings differ from those of Sailendri (1973) who noticed a pair of thymic rudiments at 1 to 2 days before hatching - slightly earlier than in the present study.

This apparent difference in the timing of thymic development may be explained by temperature differences. It is possible that Sailendri's fish were maintained at a higher temperature than those used in this study. Although she states that fry were maintained at room temperature (Sailendri, 1973) this may be quite high in India where the work was carried out.

The number of thymic lymphocytes were very small in sections of fry studied at 8 days but they are quite clear and the thymic rudiment is still in contact with the third and fourth branchial pouches, this coincides with Sailendri's result in 1973. From 9 days post-hatching onwards there is a gradual increase in the numbers of lymphocytes and differentiation of cortex and medulla was observed in both my study and that of Sailendri's. From 6 days post-hatching the thymus appeared to contain fully differentiated lymphocytes and at all stages of the life history of the thymus it remains separated from the pharyngeal cavity by only a single layer of cells. In other oviparous species the thymus becomes lymphoid from as early as 22 days pre-hatch in *Salmo salar* (Ellis, 1977b) to as late as 7→ 11 days post-hatch in *Astyanax mexicanus* (Hafter,1952). In contrast, differentiation of the thymus in viviparous species occurs much earlier - as early as 3 months pre-parturition in *Zoarces viviparus* where the gestation period is approximately 4 months (Bly,1984). In carp at 22 ± 1°C, the thymus first appears at 2 days post-hatching and becomes actively lymphopoietic by day 5.

Sailendri and Muthukkaruppan (1975a) noticed that the cells at the periphery of the thymus tended to be larger, whilst those located centrally were smaller. This observation was confirmed in the present study and furthermore, studies in Chapter 4 using tritiated thymidine would suggest that these larger peripheral cells are actively dividing. Similar findings have been reported for other vertebrate groups including mammals (Clark, 1968)

and amphibians (Charlemagne, 1974; Rimmer, 1977). The thymus retains its initial superficial position throughout development. A similar situation has been noticed in young trout, (Grace & Manning 1980) and in Salmon, (Ellis 1977b) thymocytes are seen outside the thymic capsule whilst the thymus is still actively lymphopoietic and the majority of the cells are immature.

The origin of thymic lymphocytes in fish has been of interest for many years. The idea that stem cells from other sources moved to the thymus and ultimately gave rise to lymphocytes was put forward by earlier workers (Hill, 1935; Hammar, 1909). However, others concluded that lymphoid cells were derived by division of the pharyngeal epithelium. Beard (1902) and Deansley (1927) suggested that, in *Salmo fario,* lymphoid elements were derived by division of the pharyngeal epithelium whilst other constituent tissue of the thymus was obtained by immigration from the surrounding tissue. The present study did not provide any experimental evidence to support either argument. Although 'thymocytes' are seen outside the thymus during development, their place of origin and ultimate destination cannot be ascertained.

As haemopoietic tissue is present in the kidney at a very early stage of development, the suggestion of Ellis (1977b) that haemopoietic stem cells migrate from the kidney to the thymus could hold true in this case. Indeed, Sailendri (1973) has suggested that a physical bridge between the pronephros and thymus may exist in *Tilapia mossambica*, allowing the direct migration of cells from one organ to another, but such a bridge was not seen in carp nor was it seen in the present study. However, small numbers of lymphocytes were observed in the connective tissue between thymus and pronephros.

Pronephros differentiation in oviparous species may occur as early as 14 days pre-hatch, as in *Salmo salar* (Ellis, 1977b) or as late as 6 - 8 days post-hatch as in *Salmo gairdneri* (Manning et al. 1981). In the present study, a pronephros with some tubules was seen from 6 - 9 days post-hatching but lymphocytes were not seen in any numbers until 14 days. Head kidney in sections cut from 17 - 20 day old fry was well differentiated with lymphocytes and urinary tubules. As with the thymus, the timing of pronephros development is delayed when compared to Sailendri's (1973) study. She found a pair of pronephros with pronephric tubules at one day post-hatching and she added that from 2 - 5

days post-hatching the pronephros of *Tilapia* became erythroid and lymphocytes first appeared at 7 - 8 days of age. In zoarces, pronephros tissue was first observed by Bly (1984) at 3 months preparturition and, at this stage consisted of a few kidney tubules surrounded by a small number of erythrocytes. By one month preparturition, the pronephros contained recognizable lymphoid tissue.

There were no major difference between this and earlier studies of the ontogeny of the spleen in *Tilapia* . In this study, sections from fry of 5 days post-hatching showed the first appearance of the splenic anlagen and a few splenic lymphocytes were clearly seen at 35 days post-hatching. In Sailendri's study of 1973 she noticed mesenchymal thickening very close to the pancreas at the "late prelarval stage" (6 - 8 days) and at the "early post-larval stage" (9 - 12 days) the spleen became erythroid with elongated erythrocytes and by the "mid-post larval stage" (13 - 16 days) definite sinuses were formed, however the appearance of large lymphocytes was recorded at the "late post-larval stage" (17 - 22 days). In the "juvenile stage" (30 - 80 days) and in the "young adult stage" (80 days) the spleen was well differentiated into red and white pulp areas and had become a mature lymphoid organ. These findings are generally in accordance with our observations for *Tilapia* fry except that in the present study lymphocytes were not seen until 35 days post-hatching. Thus although the early development of the spleen was the same in both studies the first appearance of lymphocytes was delayed in the present study. This apparent delay in splenic development in my study was not surprising when compared to the development of both thymus and head kidney which also developed later than that recorded by Sailendri.

In another study by Manning (1981) the spleen first appeared at 3 days post hatching in *Salmo gairdneri* and at 6 days post-hatching it contained mature lymphoid cells. The spleen was still erythroid with no obvious demarcation of red and white pulp at 28 days. In contrast, Bly (1984) showed the first appearance of a distinct organ 2 months preparturation in *Zoarces* and at 2 months preparturition, the spleen was evidently lymphoid and two zones were apparent: lightly - stained lymphoid areas aggregated around the trabeculae, and darkly staining areas composed mainly of erythrocytes. These zones may be analogous to the red pulp and the white pulp of mammals. Ellis (1977b) found that the spleen of *Salmo salar* does not develop until 42 days after hatching, approximately 56 days after circulating

lymphocytes are first seen. In contrast Botham (1982) found that carp, *Cyprinus carpio* had a spleen rudiment as early as 5 days after hatching, but it developed more slowly than the kidney and remained predominantly erythroid for several months.

The first occurrence of lymphocytes does not necessarily herald immunological maturity. In carp, although a few cells bearing surface immunoglobulin were found in suspensions taken from whole fish on day 7, such cells were not detected in the thymus or pronephros until day 14 (at 21°C). White cells with cytoplasmic immunoglobulin first appeared at day 21 in the pronephros (Van Loon et al., 1981). The appearance of the ability to reject allografts and respond in mixed leucocyte reactions (MLR) can be correlated with the development of the lymphoid organs. In the salmon at 4 - 7°C, the presence of surface immunoglobulin on lymphocytes and the ability to give a proliferative response in mixed cell cultures did not occur until long after the lymphoid thymus and kidney had differentiated (Ellis, 1977a).

The major findings presented in this chapter are summarised in Table 2.1 and these maybe compared with the summary (Table 2.2)which shows the histogenesis of lymphoid organs in other species of teleost fish (Manning et al., 1982b).

The functional development of humoral immunity in *Tilapia* is examined in a later chapter.

Fig 2.1 General view of a 5 day old Tilapia mossambica showing the caudal and head regions with yolk sac attached.

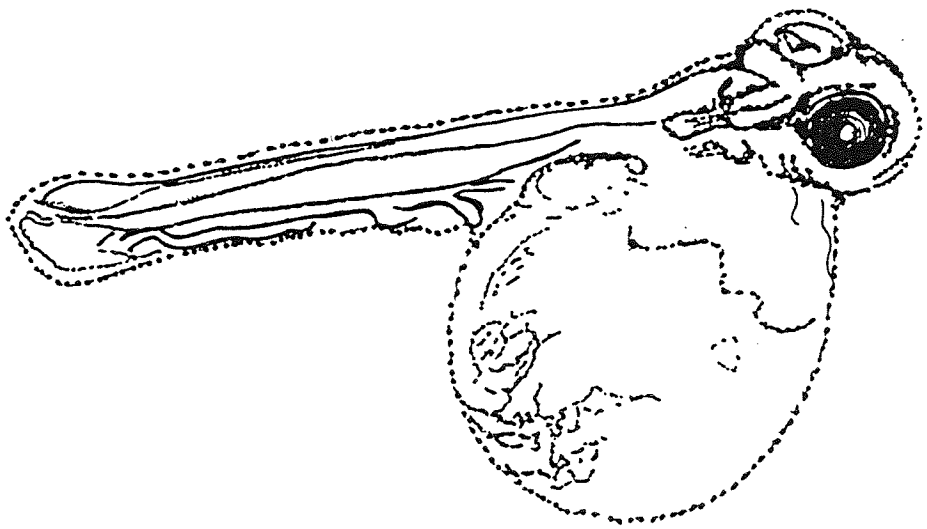


Table 2.1

Summary of the Histogenesis of the Major Lymphoid Organs in *Tilapia mossambica* (Temperature from 20 - 27°C)

Stage and Length of Fry	Width	Weight	Age in Hours	Age in Days	Lymphoid Differentiation
(1) Stage (14 → 25) (0.1 - 0.15 cm)	0.1 cm	8 mg	-	1 day before hatch	Nothing can be observed
(2) Stage (25 → 27) (0.25 - 0.3 cm)	0.15 cm	10 mg	24 hours	1 day post-hatch	- - -
(3) Stage (27 → 31) (0.3 cm)	0.15 cm	10 mg	48 hours	2 days post-hatch	(a) First appearance of the thymic anlagen attached to branchial pouches. (b) Few kidney tubules in pronephros (c) Haemopoietic tissue in kidney (mesonephros) (d) No indication of spleen as an organ.
(4) Stage (31 → 33) (0.5 - 0.58 cm)	0.33 cm	10.4 mg	110 hours	5 days post-hatch	(a) Some thymocytes in thymic tissue but the thymus is still attached to the branchial pouches. (b) First appearance of spleen anlagen

Table 2.1 contd....

Stage and Length of Fry	Width	Weight	Age in Hours	Age in Days	Lymphoid Differentiation
(5) Stage (34→35) (0.6 - 0.75 cm)	0.3 cm	10.72 mg	134 hours	6 days post hatching	(a) Appearance of small lymphocytes in thymus which is encapsulated. (b) Head kidney contains numerous kidney tubules. (c) Many lymphoid cells in kidney. (d) Some mesenchymal cells of spleen very close to the pancreas.
(6) Stage (35→36) 0.8 - 1 cm	0.35 cm	20.6 mg	192 hours	8 days post hatching	(a) Small lymphocytes in thymus predominate. (b) Blood sinuses are differentiated in spleen. (c) Big increase in number of tubules in head kidney
(7) Stage 36 (1 cm → 1.2 cm)	0.28 cm	30.12 mg	(216 - 278 hrs)	9-12 days post hatching	(a) Differentiation of thymus into two zones (middle and inner zones). (b) Erythrocytes are observed at the periphery of spleen, and spleen becomes elongated in shape (c) Increase in number of tubules of head kidney.

Table 2.1 contd...

Stage and Length of Fry	Width	Weight	Age in Hours	Age in Days	Lymphoid Differentiation
(8) Stage 36 (1.1 cm)	0.3 cm	30.41 mg	336 hours	14 days post hatching	(a) H.K. is partially lymphoid (b) Spleen still erythroid.
(9) (1.38 cm)	0.42 cm	40.7 mg	480 hours	20 days post hatching	Lymphocytes in thymus are clumped into groups separated by connective tissue and thymus is completely detached from branchial pouches. Outer zone is formed.
(10) (1.5 → 3 cm)	0.5 - 0.6 cm	50 - 75 mg	840 hours	35 days post (5 weeks old)	(a) Mature thymus which is well differentiated into cortex and medulla (b) Reduction in number of kidney tubules with more lymphocytes in H.K. (c) Spleen contains small lymphocytes.
(11) (2.5 → 3 cm)	1 cm	1 gm	(1680 - 1920 hrs)	70 - 80 days post-hatching (10 weeks)	(a) Well differentiated red and white pulp of spleen. (b) Mature thymus (c) Mature head kidney (pronephros)

Table 2.2

Histogenesis of Lymphoid Organs in Fish

(Manning et al. 1982b).

Species	Temperature °C	First appearance of lymphocytes in Thymus	Kidney	Spleen	Reference
Salmo gairdneri	14	3 days post-hatching	5 days post-hatching	6 days post-hatch; spleen still erythroid at day 28	Grace & Manning (1980)
Barbus conchonius	23	4 days post-hatching	4 days post-hatching	7 days post-hatch; spleen still erythroid at day 28	Grace (1981)
Cyprinus carpio	22	3 days post-hatching	6 days post-hatching	8 days post-hatch; spleen still erythroid at day 28	Botham & Manning (1981)
Salmo salar	4—7	22 days pre-hatch	14 days pre-hatch	42 days post hatch	Ellis (1977)
Sarotherodon mossambica	room temperature	6-8 days (late pre-larval)	13-16 days (mid post-larval)	30-80 days juvenile	Sailendri (1973)

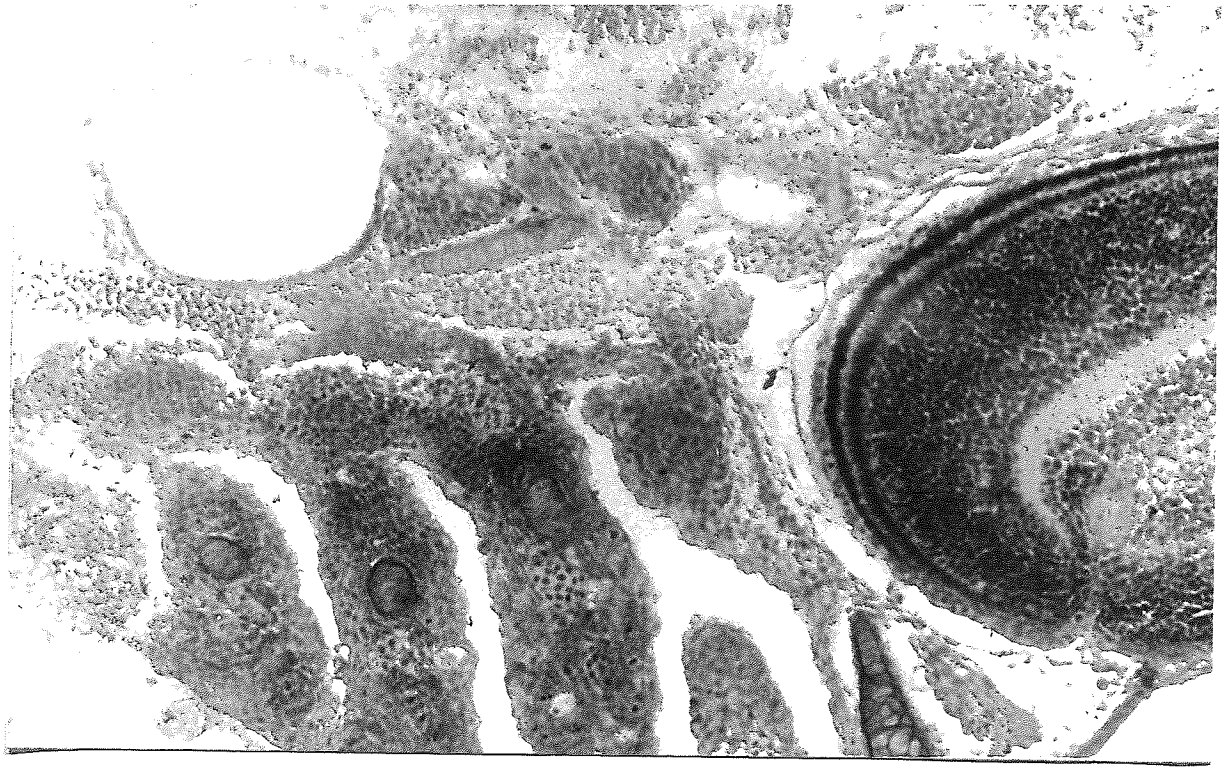


Fig. 2.2 Longitudinal section through head region of *Tilapia* fry 2 - 5 days post-hatching. (H & E stain - x 800).

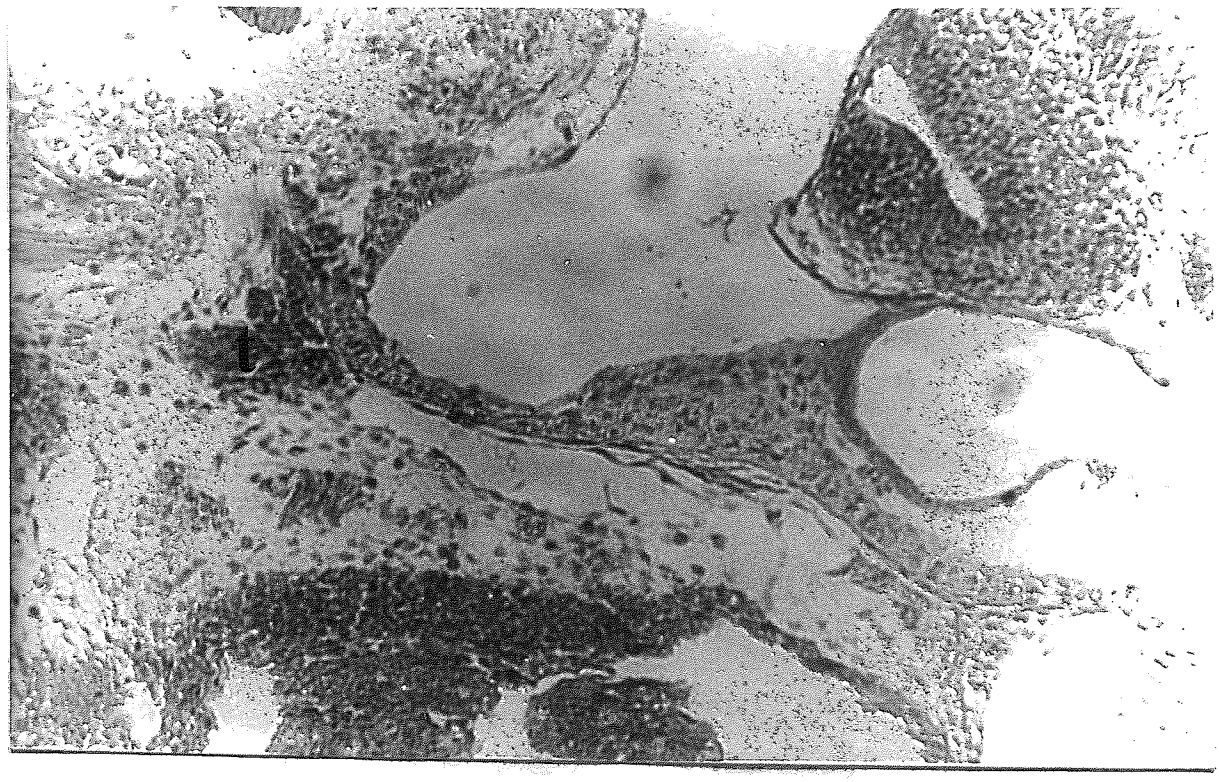


Fig. 2.3 Longitudinal section through developing thymus at 2 - 5 days post-hatching. The thymus (t) is attached to the pharyngeal epithelium and in close proximity to the operculum. (H & E stain - x 800).

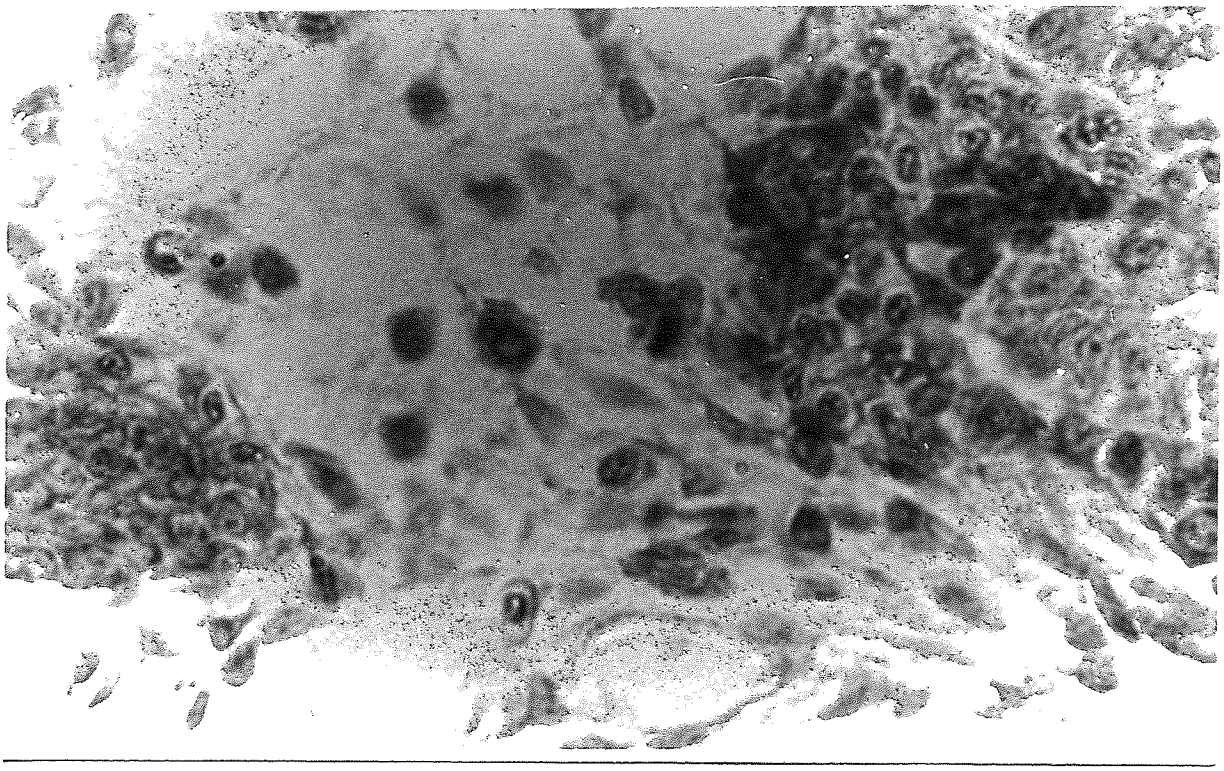


Fig. 2.4 Lymphoblast like cells in the thymus day 4 post-hatch. (H & E stain - x 3,200)

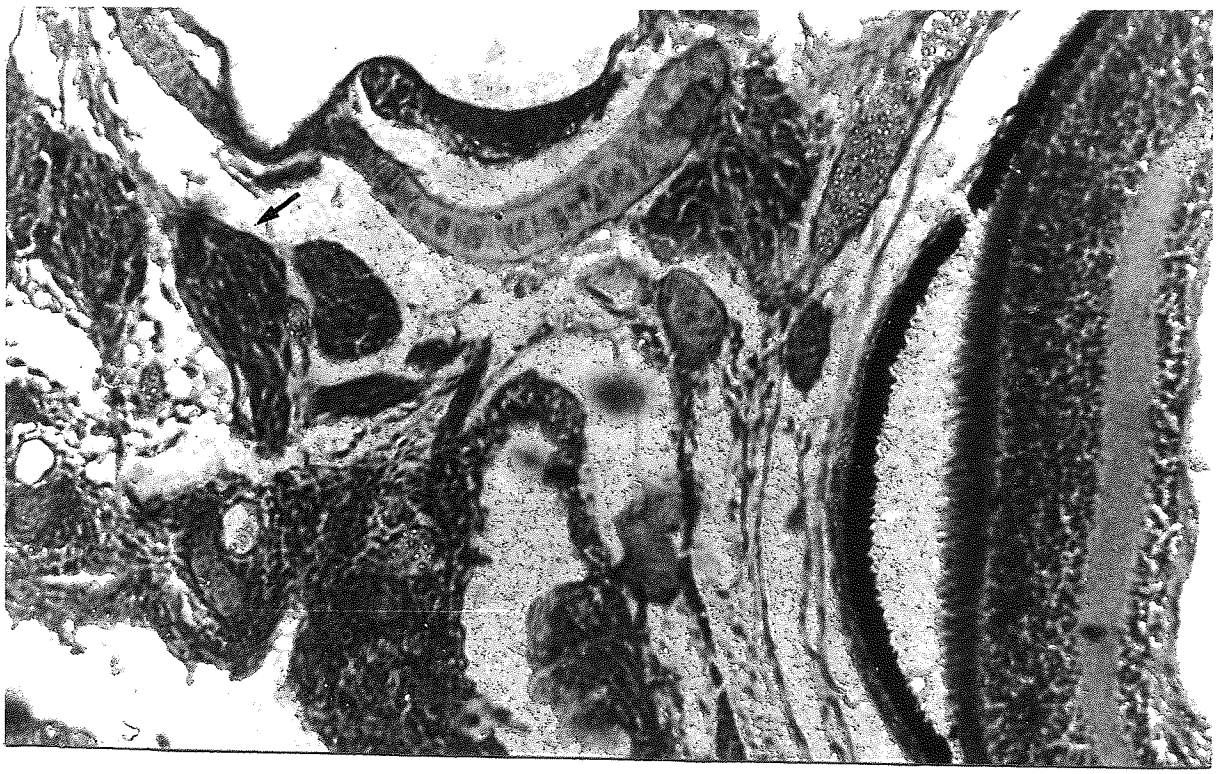


Fig. 2.5 T.S. Head region at 4 days post-hatch to show the position of the thymus (arrowed). (H & E stain - x 320)

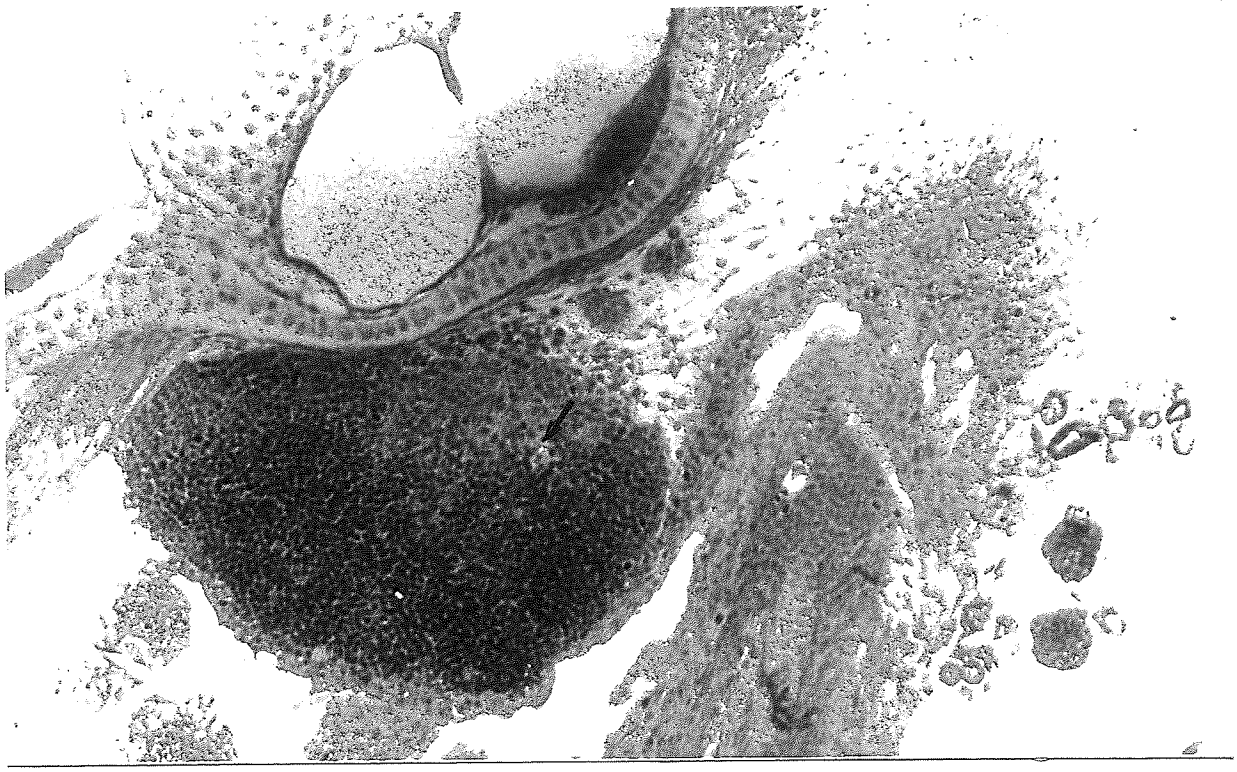


Fig. 2.6 The thymus [↓] at 8 days post hatching.
N.B. The capsule surrounding the organ which is still in contact with some of the gill arches.
(H & E stain - x 200).

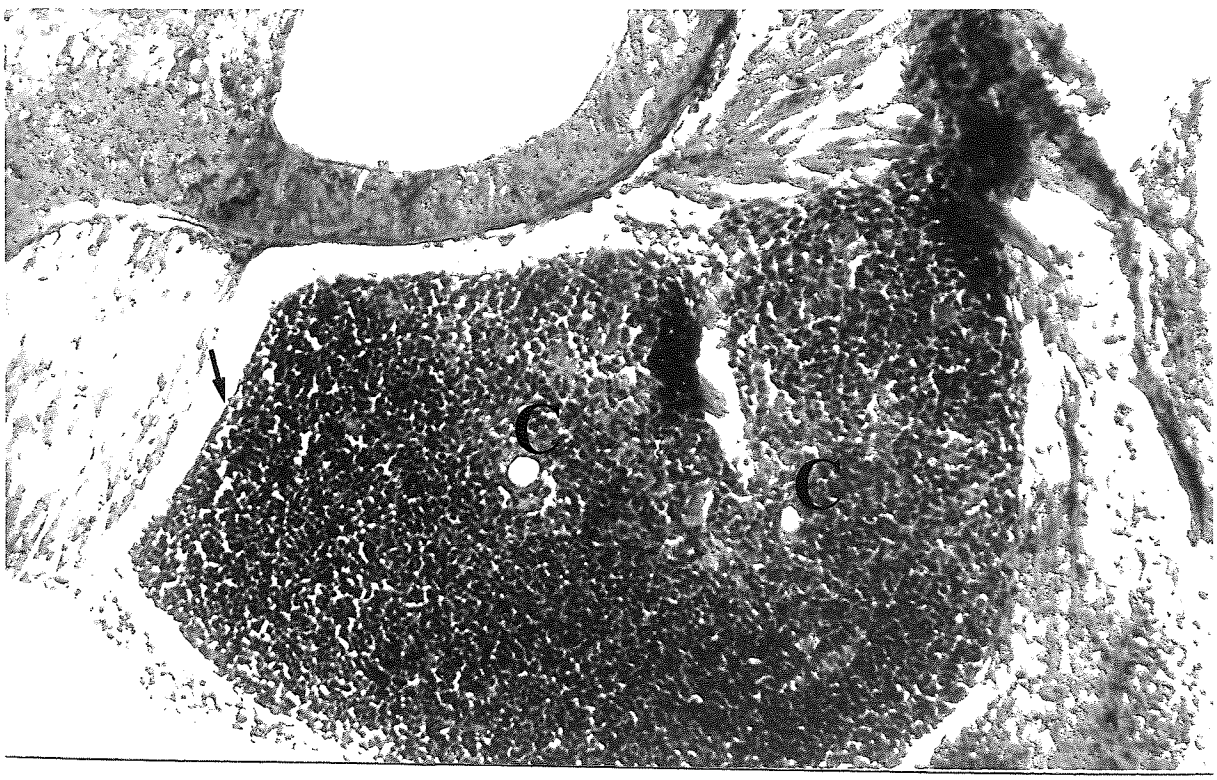


Fig. 2.7 Thymus at day 9 showing the appearance of thymic cysts (C).
The thymus is separated from the overlying operculum and appears to be bounded by a single epithelial cell layer (arrowed).
(H & E stain - x 3,200).

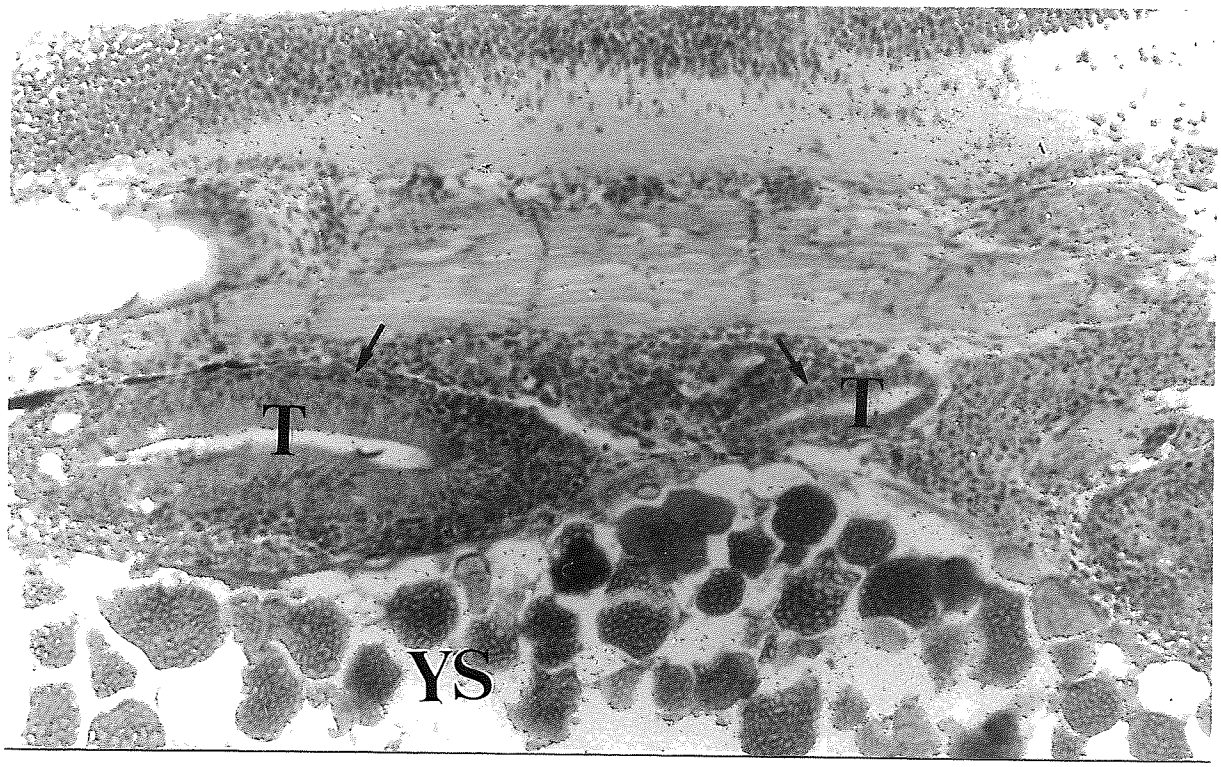


Fig. 2.8 Longitudinal section through trunk region of fry (2 - 5 days post-hatch) showing kidney tubules (T) in the mesonephros surrounded by haemopoietic tissue (arrowed). YS = Yolk sac. (H & E stain - x 800)

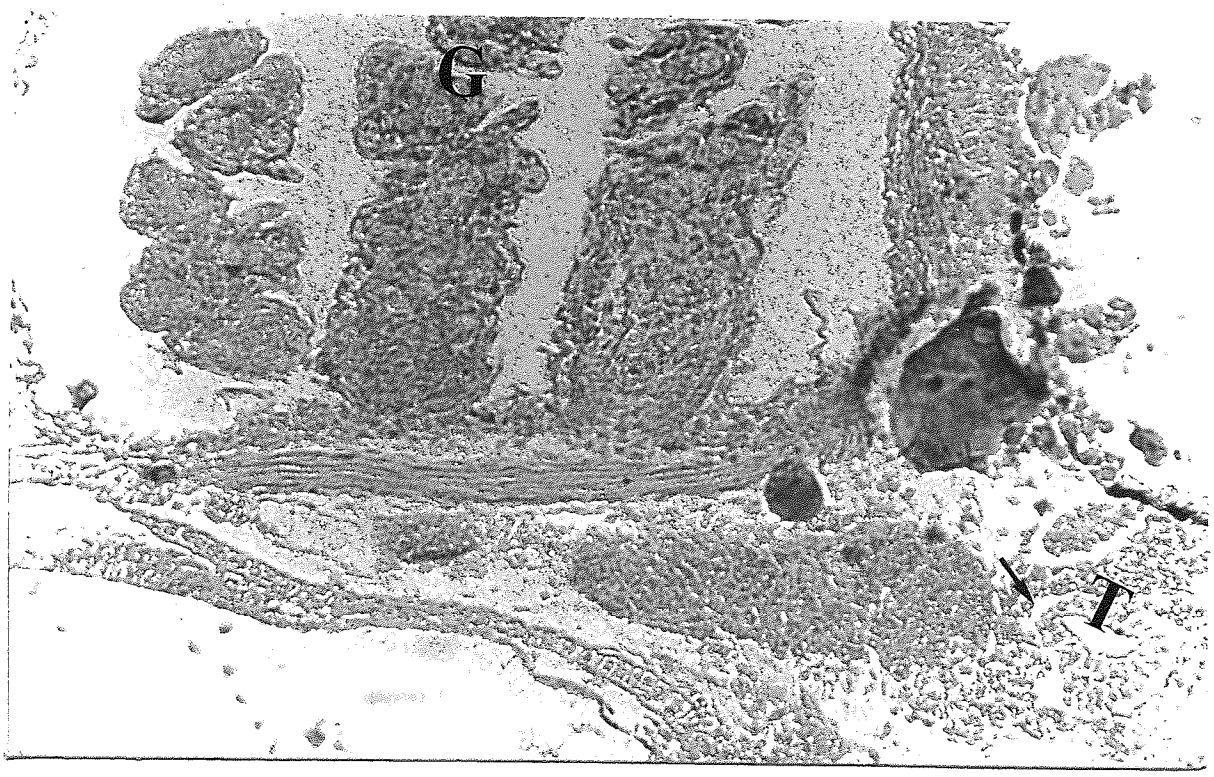


Fig. 2.9 Longitudinal section of *Tilapia mossambica* at 5 days post-hatching showing the tubules (T) of the pronephros and haemopoietic tissue (arrowed). G = Gill arches. (H & E stain - x 205).

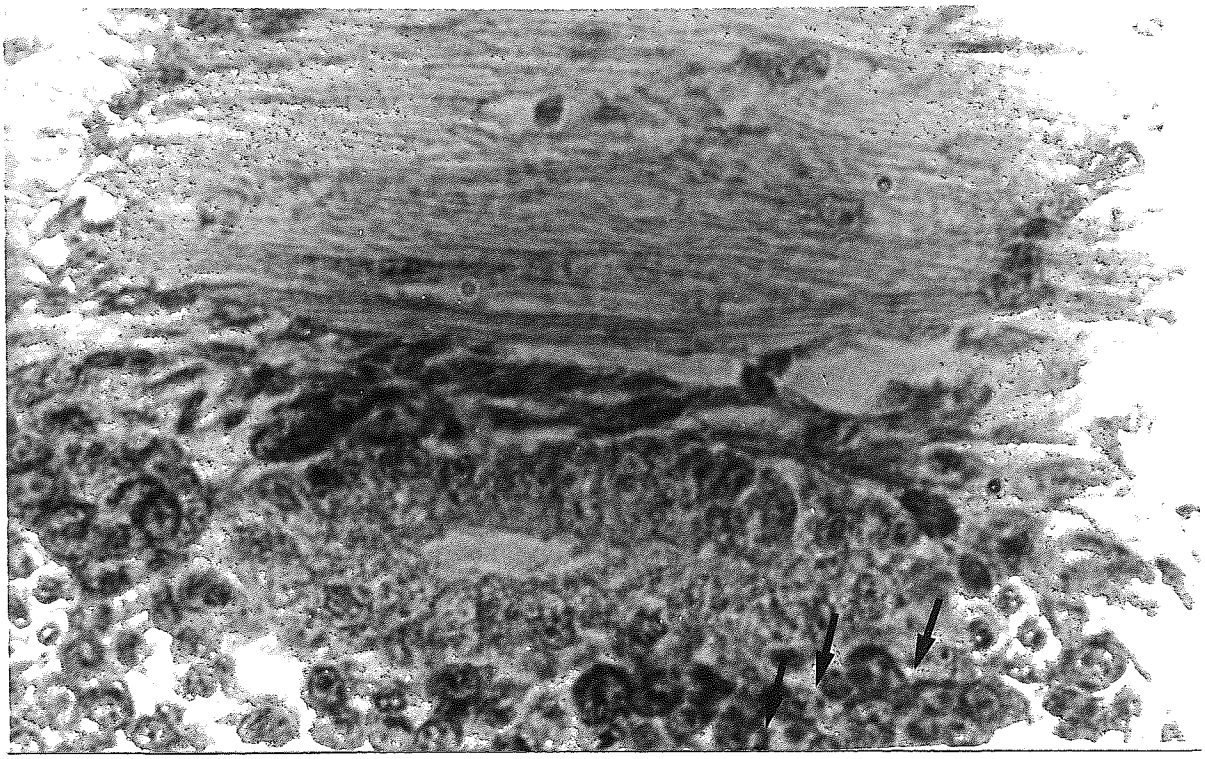


Fig. 2.10 Longitudinal section through the pronephros (head kidney) of *Tilapia mossambica* at 6 days post-hatching to show undifferentiated stem cells (arrowed). (H & E stain - x 3,200).

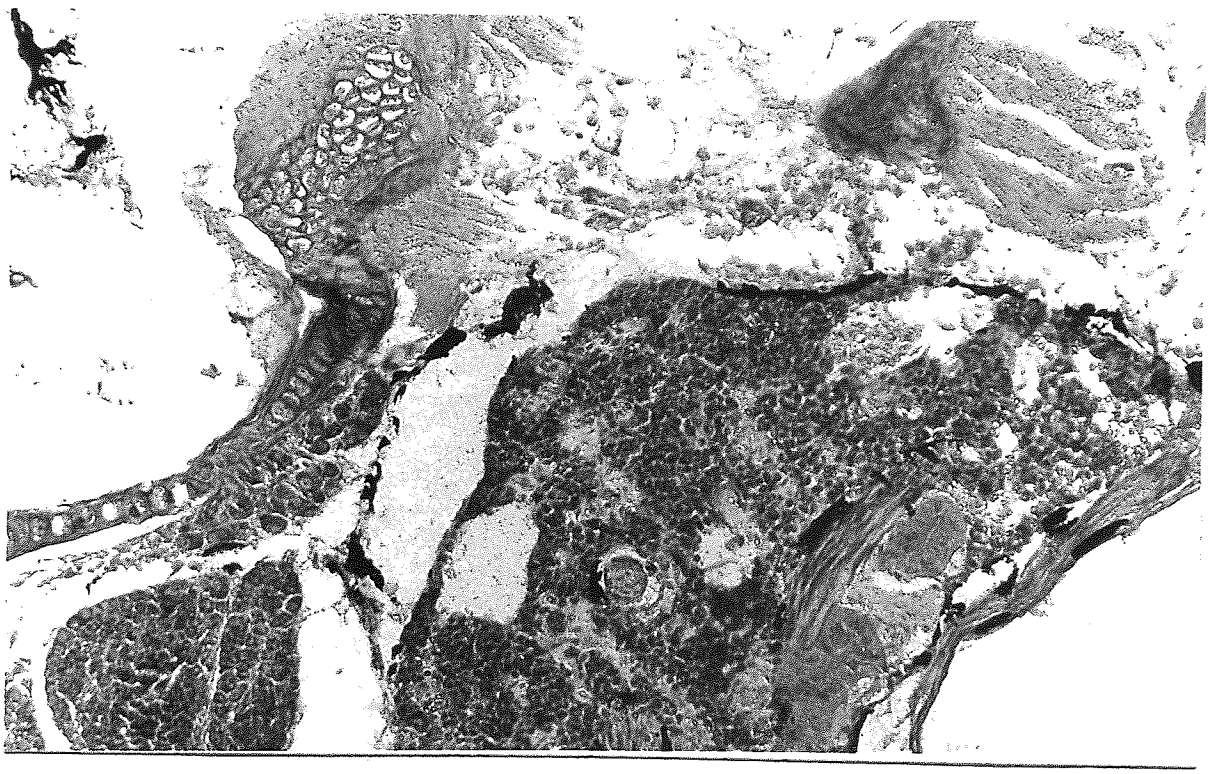


Fig. 2.11 Longitudinal section of pronephric region at 7 days post-hatching showing increase in the amount of intertubular haemopoietic tissue. (H & E stain - x 512).

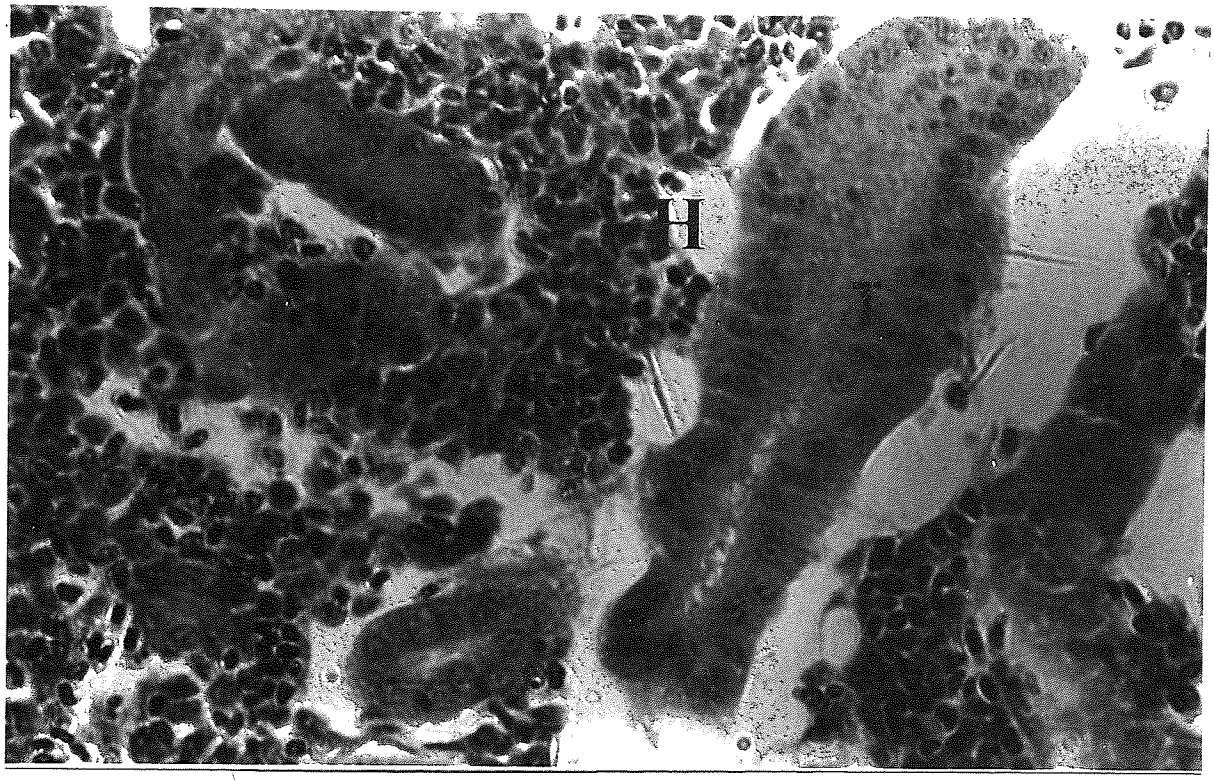


Fig. 2.12 Section through head kidney at 30 days post-hatch
T = Tubules, H = Haemopoietic tissue
(H & E stain - x = 2,000).

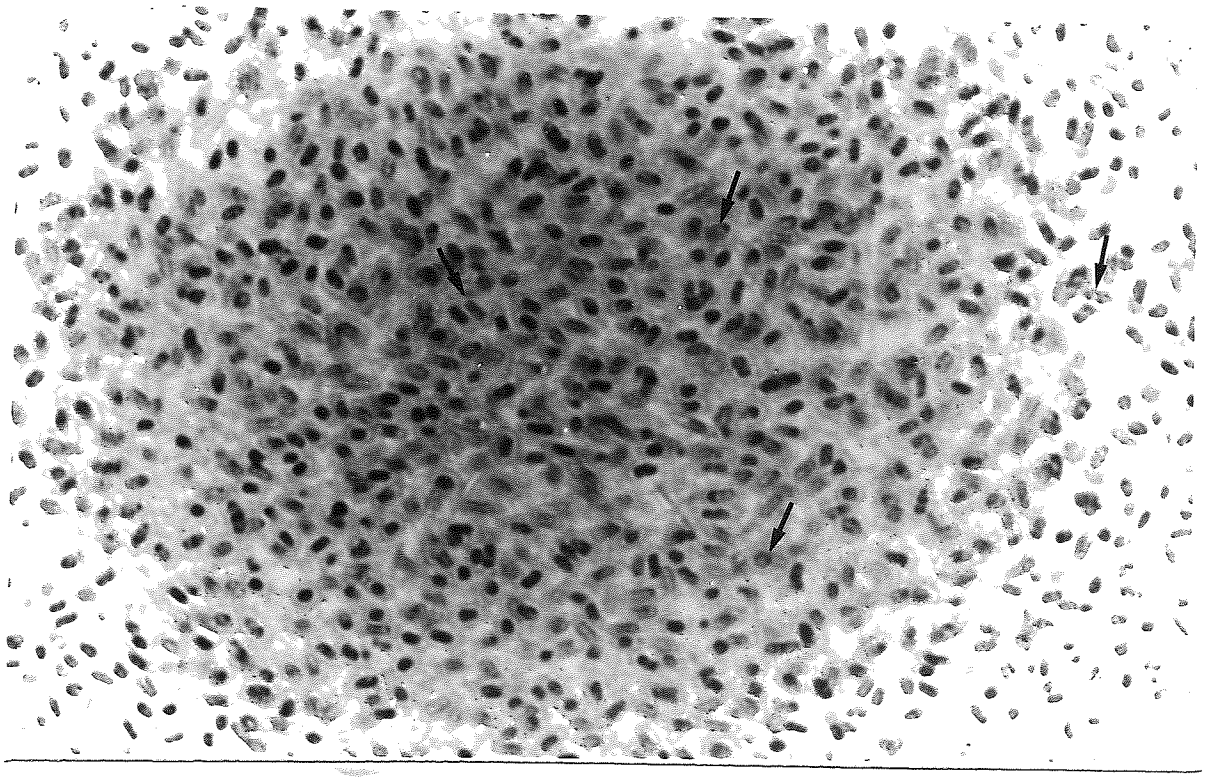


Fig. 2.13 Head kidney at 5 weeks showing erythrocytes and lymphoid cells (arrowed) (Part of a blood sinusoid).
(H & E stain - x 2,000).

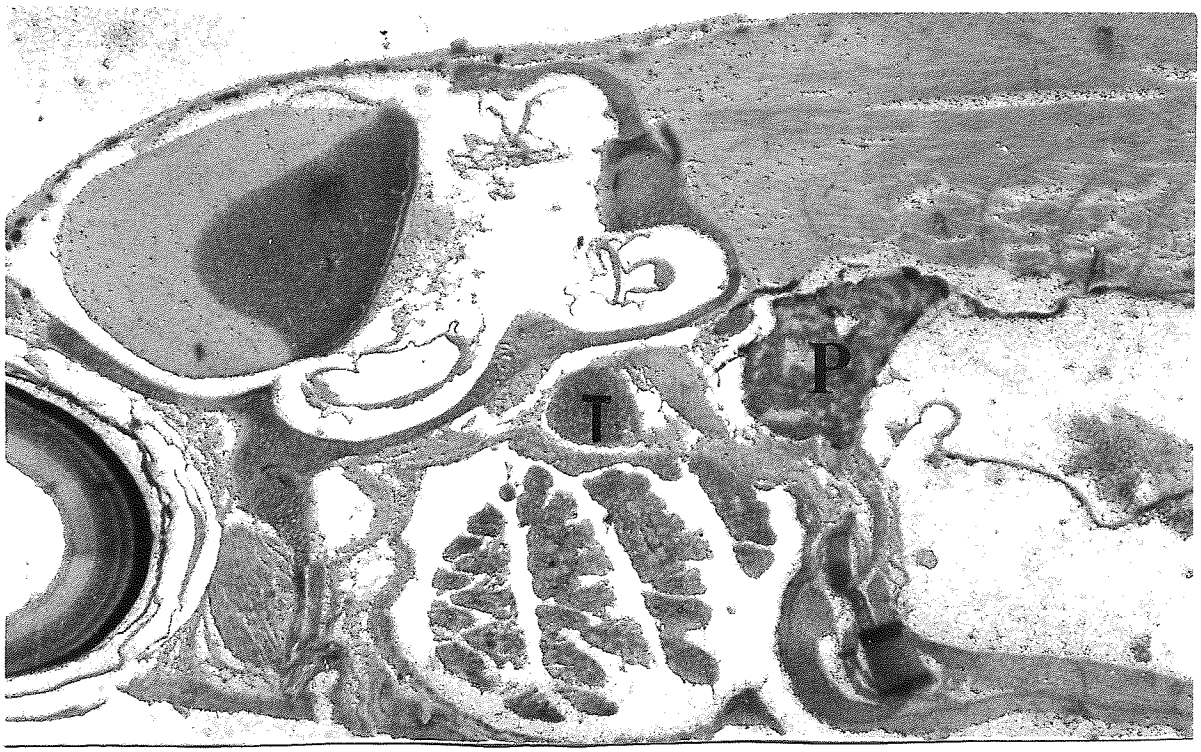


Fig. 2.14 Longitudinal section through 2 week old fry showing the relative positions of the thymus (T) and pronephros (P). (H & E stain - x 200).

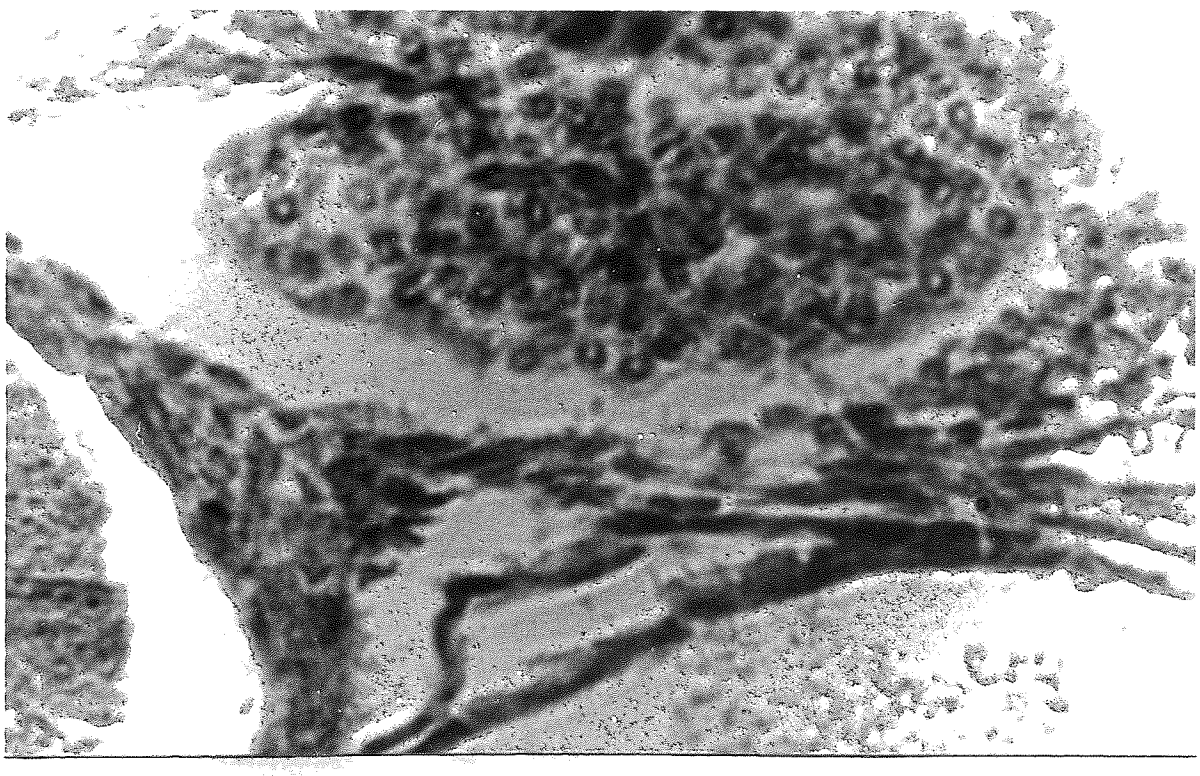


Fig. 2.15 Longitudinal section of spleen at 5 - 6 days post-hatch. Note the presence of large haemopoietic stem cells (H & E stain - x 3,200)

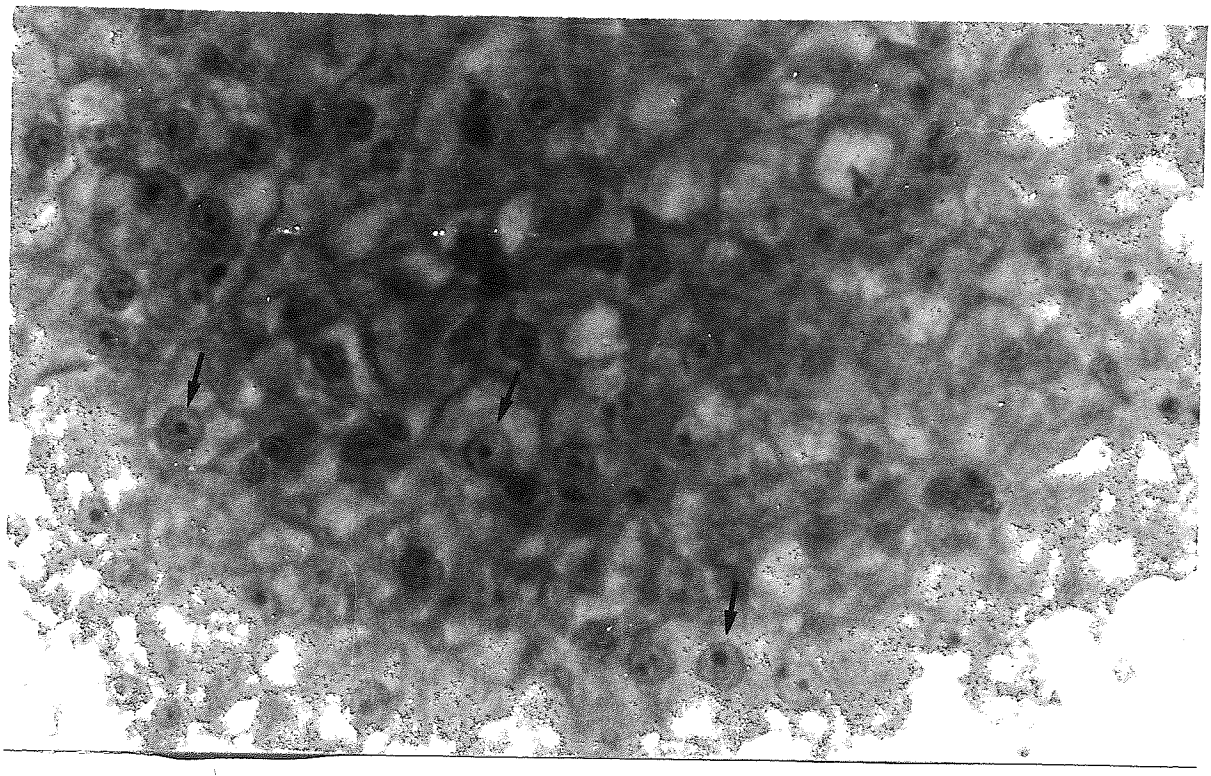


Fig. 2.16 Section through 6 day old spleen showing large stem cells (arrowed) scattered through the reticular framework. (H & E stain - x 3,200).

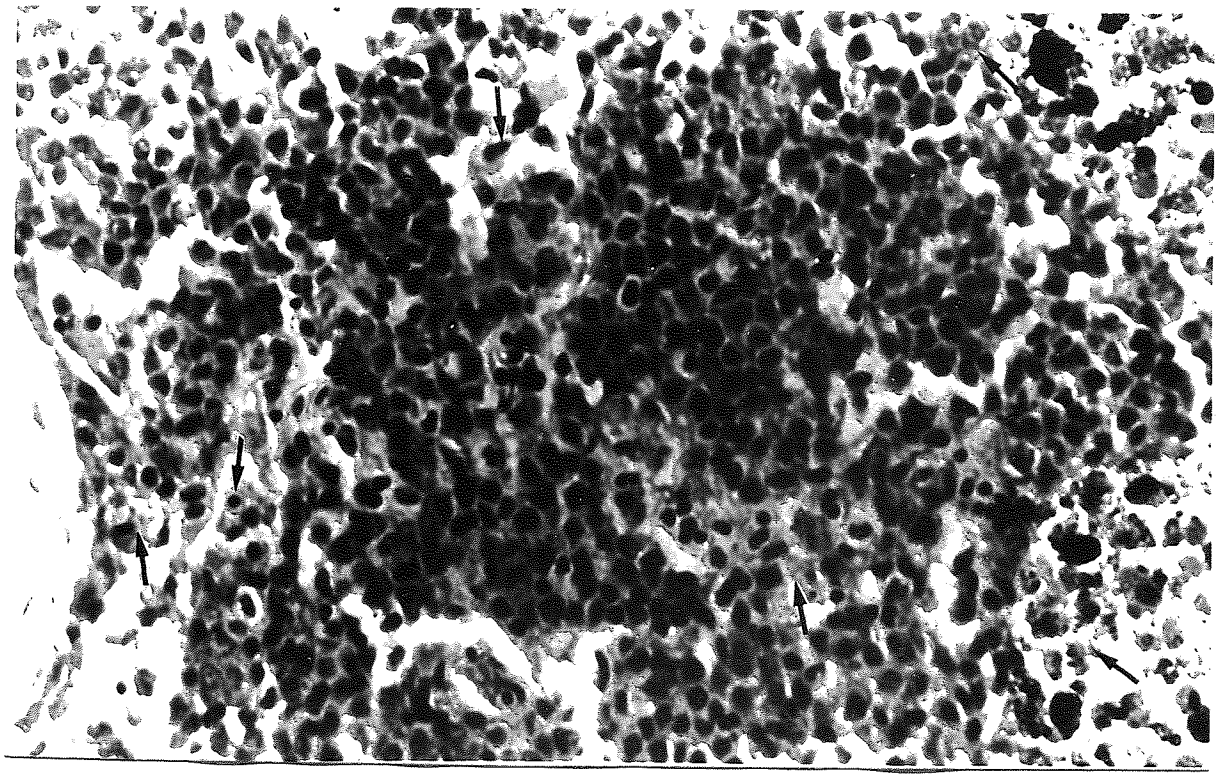


Fig. 2.17 Section through spleen in 10 day old fry showing the presence of erythrocytes with other lightly staining cells. (H & E stain - x 1,000).

CHAPTER 3

CHAPTER 3

LIGHT AND ELECTRON MICROSCOPIC STUDIES OF LYMPHOID TISSUES IN TEN WEEK OLD FRY AND ADULT *TILAPIA MOSSAMBICA*

3.1 INTRODUCTION

The work presented in the previous chapter has described the morphogenesis and ontogeny of the major lymphoid organs in fry of *Tilapia mossambica*. In the present chapter, attention is focussed upon the appearance of these organs in older fish. In contrast to mammals where the structure and function of lymphoid organs has been well studied (Miller and Osoba, 1967; Novikoff, 1971; Bearman & et al., 1975; Mandel, 1968); there has, until recently, been little detailed knowledge of the structure of teleost lymphoid organs. However, a number of authors have now carried out electron microscopic studies of lymphoid organs in a range of fish species (Zapata, 1979; 1981); Ferguson (1976b); Gräf and Schlüns (1979); Chilmonczyk (1983); Davina (1980); and Grace (1981).

Sailendri and Muthukkaruppan (1975) have investigated the lymphoid organs of adult *Tilapia mossambica* by light microscopy, but, to date, there has been no attempt to examine the ultrastructure of the major lymphoid organs in this species. The work in this chapter presents light microscopic studies of fully differentiated lymphoid organs for comparison with previous studies and the observations presented in chapter two. Furthermore, the present study also examines for the first time some aspects of the fine structure of thymus, spleen and head kidney in fry and adult *Tilapia*.

3.2 MATERIALS AND METHODS

Ten week old fry and adult (greater than one year old) *Tilapia mossambica* used in this study were obtained from the Aquaculture Institute of Stirling University, Scotland.

Fish were maintained at (27 - 30)^{o}C in tanks containing well aerated water and were fed with artificial food (trout pellets). The thymus, spleen and head kidney were examined in both fry and adult fish.

Fish were sacrificed by over - anaesthetisation in tricane methane sulfonate (MS 222 : Sandoz, Basle, Switzerland) and organs were removed for fixation immediately after dissection.

3.2.1 Fish Immunisation

Some fish were injected intraperitoneally with *Escherichia coli* type 0111 K58 (0.01 ml/gram body weight for each fish). This bacteria was obtained from NCTC, Central Public Health Laboratories, London and details of bacterial preparation and injection protocols are given in Appendix 1 and Chapter 4.

3.2.2 Light Microscopic Studies

Organs to be studied were dissected out from immunised and control *Tilapia mossambica* and fixed in Bouin's fixative overnight. The tissues were processed employing the routine outlined in Appendix No.5 at the end of this thesis. Following processing, the tissues were embedded in paraffin wax at 60^{o}C. Five to eight microns thick sections were cut on a rotary microtome, (Cambridge Rotary Rocking Microtome, Cambridge Instrument Co. Ltd., London & Cambridge) and mounted on chemically cleaned microscopic slides. Sections were stained with Harris' haematoxylin and counterstained with Eosin by following standard histological techniques. Optimal fixation of the spleen was achieved by removal of the major part of the blood within the organ by perfusion with formal saline, prior to immersion fixation. Perfusion for two to three minutes was accomplished by injecting fixative from a syringe, into the capsule of the divided organ, until the fixative emerging from the cut face was no longer discoloured with blood.

3.2.3 Preparation of Tissues for E.M.

(a) Spleen

Following sacrifice the spleen was quickly removed and cut into fragments of

approximately $1mm^3$. These fragments were fixed in 5 ml of 2.5% glutaraldehyde for 2.5 hours and then rinsed in 0.1M sodium cacodylate buffer (Ph 7.4) containing 7.5% sucrose. Specimens were post fixed in 1% OSO_4 (Osmium tetroxide) dissolved in 0.05M sodium cacodylate buffer (PH 7.4) containing 7.5% sucrose for two hours, or until the spleen fragments appeared completely black. Specimens were placed in 70% ethanol (if left overnight) and rapidly dehydrated through a graded series of ethanol dilutions. All steps were carried out at 0 - 4°C.

Specimens were embedded in Epon-araldite and cut on a Reichert Ultramicrotome (Shandon Scientific London Co.Ltd) using either a diamond or glass knife. Sections of (500 - 600 Å) floated on water, were collected onto uncoated diamond or square mesh grids. The sections were stained by floating the grids on drops of saturated uranyl acetate solution for 20 minutes. After washing in distilled water they were floated for 10 minutes on drops of lead citrate. The sections were finally rinsed on 0.02 molar sodium hydroxide solution and distilled water and after drying were examined on a JOEL 100 B electron microscope operating at 80 K.V.

(b) Thymus

The thymus of adult *Tilapia* was dissected out as a whole organ, whereas in ten week old fry the whole area containing the thymus was removed for processing. Tissues were fixed for E.M. in 4% glutaraldehyde buffered with 0.1M sodium cacodylate (PH 7.3) for 2.5 hours and specimens were then washed overnight in 0.1M phosphate buffer with 7.5% sucrose.

Following post fixation in 1% O_sO_4 with 0.1M phosphate buffer for 90 minutes, specimens were dehydrated in a graded series of ethanol dilutions. Following two changes of propylene oxide, specimens were transferred to an equal mixture of propylene oxide and Spurr's low viscosity resin. The propylene oxide was allowed to evaporate, leaving the tissues in the resin for a few hours. Tissues were embedded in pure Epon before sectioning and stained as described in section(a) for E.M.

(c) Head Kidney

Head kidney was dissected out of fish (*Tilapia mossambica*) and immediately fixed in 2.5% glutaraldehyde buffered to PH 7.3 with 0.1M sodium cacodylate. Specimens were post fixed in 1% osmium tetroxide in the same buffer until the organs became completely dark. They were then dehydrated in acetone and embedded in Araldite. Sections were cut and viewed as described in section (a) for E.M.

3.2.4 Preparation of thick sections (0.5 - 1 millimicron) for viewing under the light microscope

Tissues were fixed and embedded in Epon as described in (a), (b) and (c). 0.5 - 1 millimicron sections were cut and transferred to a hot 1% toluidine blue solution or methylblue (Hayat, 1970) where they were floated for 2 - 3 minutes before being transferred with a platinum loop, to a water bath. Here, excess stain was removed before the sections were dried onto a glass slide and mounted using a synthetic resin and a cover-slip. This method of preparing tissues provided a very useful link between paraffin sections viewed at the light microscope level and ultrathin sections viewed at the electron microscope level.

Photographs for all sections were taken using a Carl Zeiss microscope with 35mm camera attachment.

3.3 RESULTS

3.3.1 Thymus

Light Microscopic Observations

The thymus of 10 week old fry and that of the adult seemed to be similar in structure. The thymus of *Tilapia* is a paired, opaque, white, beanshaped organ found directly under the operculum in the branchial cavity and attached to the branchial epithelium. The position of the thymus is illustrated in Figure 3.1. It lies in the angle made by the operculum and the dorsal body musculature and is closely related to gills. At the same level but medial to the thymus is the auditory capsule. The thymus gland is a simple unlobulated structure surrounded by a connective tissue capsule which is composed of reticular and collagenous

fibres. It measures about 6mm x 3mm x 1mm in fish weighing from 40 to 50 grams, and contains from eleven to thirteen million lymphocytes (Sailendri, 1975).

The thymus consists of three zones : outer, middle and inner zone. The outer zone encircles the other zones, the middle zone is more deeply stained and similar to the thymic cortex of higher vertebrates and characterized by dense, tightly packed, small lymphocytes. The inner zone resembles the thymic medulla of mammalian thymus. The boundaries between these three zones are not sharply delineated and no lymphatic vessels are observed in the thymus (Figs. 3.2 & 3.3). The inner thymic membrane, where the thymus attaches to the head, is a smooth, regular structure. The outer membranes which separates the thymus from the pharyngeal cavity contains huge cells with many cysts inside (not illustrated). These resembled goblet cells and give the impression of being secretory in function. The outer membrane of the thymus did not appear to differ from the epithelial lining of the rest of the pharyngeal cavity. No germinal centres were seen in the organ but blood vessels were observed.

Fig. 3.4 represents an autoradiograph of the thymus from a 10 week old *Tilapia* fry and illustrates the heavy labelling associated with the outer (cortical) region of the thymus and suggests that the outer zone of the fry thymus contains large numbers of proliferating thymocytes.

The inner zone or medullary region of the adult thymus is seen in (Figs. 3.2 and 3.3). The medullary region contain large thymic "corpuscles" with concentrically arranged nuclei and central aggregations of orange/brown pigment (Fig 3.3). These superficially resembled the myoid cells described by other workers, but there was no evidence of the striated muscle fibres normally seen in such structures. Other features of the medullary region included the presence of large groups of cells with black/brown pigmentation. These are illustrated in Fig. 3.2. Large numbers of thymocytes were also seen, scattered throughout the paler staining epithelial cells of the inner zone.

Electron Microscopic Observations

The results obtained for this section were compromised by a fault in the electron microscope and only three figures are presented. Fig. 3.5 is a section from the thymus of

10 week old fry and appears similar in appearance to the fenestrated endothelial cells cytoplasm reported in the thymus of rainbow trout (*Salmo gairdneri*) by Chilmonczyk (1983).

Figure 3.6, shows a thymocyte in the thymus of a 10 week old fry, note the rounded nucleus and the characteristic pattern of chromatin condensation. The cell depicted in Figure 3.7 is thought to be a granulocyte as evidenced by lobulated nucleus and the presence of electron dense granules in the cytoplasm.

3.3.2 Head Kidney (the pronephros)

Light Microscopic Observations

The term head kidney refers to the modified pronephros which lies dorsal to the body cavity, it is a bilaterally symmetrical organ and extends from behind the level of the thymus in the branchial region to the anterior end of the mesonephros. In adults weighing 45 - 50 grams, it measures about eight to fifteen millimetres in length, however in younger fish (fry) weighing one to 5 grams it measures about 2.0 - 3.5 millimetres in length. It is dark brown in colour and contains approximately nine to sixteen million white cells (Sailendri, 1975). The head kidney is encapsulated by thin strands of collagen fibres and includes lymphatic and blood vessels and sinuses. It is mainly a lymphoreticular organ and contains both deeply stained lymphoid zones and lighter staining non-lymphoid areas (Fig 3.8). In the same figure, at the periphery and in the centre of the follicle large vacuolated cells with brownish dark pigments and a few large lymphocytes are found. The follicle also encloses numerous blood vessels and blood sinuses. The lymphoid zone is represented by a number of lymphoid follicles of tightly packed small lymphocytes as seen in Fig. 3.9.

Immunised Head Kidney after Injection of 20% SRBC

Changes were observed in immunised sections. Large numbers of spherical bodies were found in the lymphoid zone of the head kidney, following immunisation. Each body consisted of clusters of pigmented cells or pigmented granules (Fig. 3.8). These bodies were not seen in control head kidney.

Electron Microscopic Observations

Fig. 3.10 shows a low power general view of the head kidney of *Tilapia* fry. Proliferating cells are clearly visible and in the section shown, they are located close to a blood vessel. Fig. 3.11, illustrates what is thought to be a phagocytic reticular cell in the head kidney of a 10 week old fry. The cytoplasm contains a number of small vesicles and some large electron dense inclusions. This figure also shows a large membrane bound vacuolated structure which appears to contain a few large electron dense bodies together with smaller darker staining granules. The significance of this structure is not known, but it may represent a stage involved in pigment deposition. Small lymphocytes are also visible in this section. In figure 3.12, a number of large pale staining cells connected by desmosomal junctions are seen. These cells show some condensation of the nuclear chromatin and are thought to be part of the haemopoietic element of the head kidney.

3.3.3 Kidney (the mesonephros)

The mesonephros in adult *Tilapia* is primarily an excretory organ. Fig. 3.13 shows a transverse section through the mesonephros and illustrates the predominance of excretory tissue and the absence of lymphoid and haemopoietic tissue.

3.3.4 Spleen

Light Microscopic Observations

In *Tilapia mossambica* the spleen is an elongated flattened structure, measuring about 12mm in length and 4mm in thickness in fish weighing 45 - 50 grams. It lies along the left side of the stomach continous with the mesenterium and in close association with the pancreas. The spleen is enclosed in a capsule which consists of a layer of mesothelium and squamous epithelium and is often disrupted in preparing the specimen. The greater part of the spleen appears reddish in colour when stained with hematoxylin and eosin. This area is the red pulp. Scattered through this reddish mass are small areas that appear white in the living state but stain purple with hematoxylin and eosin. These areas are called white pulp and comprise the lymphatic tissue of the spleen (see Fig. 3.14). Within the white pulp of the spleen, diffuse lymphoid cells, including plasma cells, are arranged around a central

arteriole (See Fig. 3.15). The white pulp is separated from the red pulp by a marginal zone which is seen in Figs. 3.15 and 3.16.

Figure 3.16 shows the appearance of the red pulp in an SRBC immunised adult. The red pulp is crowded with small lymphocytes which are thought to arise from proliferation *in- situ.*

Electron Microscopic Observations

Fig. 3.17 depicts an E.M. section through the spleen of a 10 week old fry. A number of different cell types are visible at this stage. These include immature lymphoid cells as judged by the relatively large amounts of cytoplasm and the incipient chromatin condensation in the nucleus. Part of the cytoplasm of a phagocytic cell is also present and contains a number of cytoplasmic inclusions. One of these vacuoles appears to contain the remnants of an erythrocyte. The figure also shows a section through the cytoplasm of a cell which contains a number of electron dense granules. Unfortunately the section does not include the nucleus of the cell, but the granular cytoplasm may form a part of a neutrophil. Fig. 3.18 is taken from a section through the spleen of adult fish which was injected 7 days earlier with *Escherichia coli* bacteria. The cell depicted in the figure contains a number of dark staining bodies in its cytoplasm which may represent phagocytosed bacteria. An alternative suggestion for the identity of this cell is that it is an eosinophil which in mammals are characterised by the presence of large electron dense granules in the cytoplasm. Only a small portion of the cell's nucleus is visible so it is not possible to categorize it further.

3.4 DISCUSSION

In *Tilapia* , the major functional lymphoid organs are the pronephros, mesonephros and spleen. The location of teleost thymus near the branchial cavity and its permanent continuity with pharyngeal epithelium has been confirmed by various authors, [Rizkalla (1969); Sailendri and Muthukkaruppan, 1975)]. The thymus in adult *Tilapia* is unlobulated and in this respect is similar to that of *Rutilus rutilus* (Zapata, 1981); however, the angler

fish, *Lophius piscatorius* (Burne, 1927); and the Nile fish, *Clarias lazela* (Rizkalla, 1969); are characterised by thymic lobulation. The structure of the thymus varies from species to species. In 1973, Sailendri noticed a clear division into outer, middle and inner zones. The middle zone resembling the mammalian cortex and the inner zone, the mammalian medulla. This organisation into several adjacent regions has been reported in other teleost fish : Three layers were described in *Astyanax mexicanus* (Hafter, 1952); in *Salmo fario* (Deanesly, 1927); in rainbow trout (*Salmo gairdneri*) (Chilmonczyk, 1983); and in *Clarias lazella* (Rizkalla, 1969). A lack of clear demarcation between cortex and medulla has been reported for other species of fish on [*Salmo salar* (Ellis, 1977b); *Lophius piscatorius* (Fänge and Pulsford, 1985) and *Salmo gairdneri* (Grace and Manning, 1980)]. Similarly, the thymus of carp shows no clear division into zones, although in younger fish larger lymphoblast - like cells accumulated at the periphery of the organ, and smaller and mature cells were found centrally (Botham 1982). Rijkers (1980) reported that there were possibly two zones in the thymus of adult carp, but they were more difficult to distinguish than in other species.

Electron microscopic examination of the thymus, revealed the presence of a continuous connective tissue similar to that found in *Rutilus rutilus* by Zapata (1981). The pictures obtained for electron microscopic observations on the thymus were limited by a technical fault on the electron microscope. However, the thymus was seen to consist of a reticular epithelial framework interspersed with thymocytes at various stages of development. The ultrastructural appearance of the thymocytes resembles those observed in the thymus of other vertebrate species including amphibians (*Rana pipiens*, Curtis and et al., 1972; *Xenopus laevis*, Nagata, 1976), reptiles (Bockman, 1970), and mammals (golden hamster, Ito and et al., 1966). A presumed granulocytic cell was also observed in the thymus at this stage, although whether or not this is a regular feature of thymus development in this species is not known.

The fenestrated endothelial cell cytoplasm observed here was also seen by Chilmonczyk (1983) in rainbow trout. The functional significance of this feature is not known, but it may be related to facilitating the entry of antigen into the thymus. The thymus of *Tilapia* contains plasma cells and also produces plaque forming cells in response to

challenge with foreign erythrocytes (Sailendri and Muthukkaruppan, 1973). Other morphological adaptations to facilitate the entry of antigen into the fish thymus have been reported by Grace (1981) who demonstrated that in rainbow trout, the thymus is only separated from the water in the pharyngeal cavity by an epithelial layer one cell thick. She concluded that the thymus of fish may still represent an evolutionary stage where the organ functions not only as a primary lymphoid organ, as in mammals, but is also involved in the adaptive immune response.

The presence of thymic corpuscles in adult *Tilapia* was also reported by Sailendri and Muthukkaruppan (1975) who referred to them as Hassall's corpuscles. These structures are not present in all fish and are not seen in the thymus of eel (Von Hagen, 1936), *Astyanax* (Hafter, 1952), and *Lepomis* (Smith et al., 1967). More recently, Zapata (1980) reported that Hassall's corpuscles were absent from the thymus of *Raja clavata* and *Torpedo marmorata* . Hassall's corpuscles are also present in mammalian thymus, but their function is poorly understood. They may be involved in the destruction and digestion of phagocyted thymocytes (Toro, 1966).

Another feature of the adult thymus reported here is the presence of pigment accumulations in the medullary region of the thymus. The presence of such accumulations has been widely reported in other fish lymphoid organs and their possible significance reviewed by Agius (1985). However, Agius did not mention the presence of pigment accumulations in the thymus of fish, and it is not known whether such pigmentation is widespread or peculiar to *Tilapia.* In previous studies on *Tilapia* (Sailendri, 1973; Sailendri and Muthukkaruppan, 1975) the authors did not report observing such pigments and the significance of these observations awaits further study. The presence of large numbers of proliferating cells in the outer zone of the thymus of 10 week old fry is in accordance with previous studies in mammals and amphibia (Mandel, 1968 and Rimmer, 1977).

The cortical area of the thymus, particularly a zone just below the thymic capsule, is a site of rapid proliferative activity in mammals. The fate of these cells is unclear. Many are known to die '*in situ*', but some are thought to pass to the medulla to undergo differentiation prior to emigration into the periphery (Scollay & et al., 1984). The fate of this rapidly proliferating population in fish is not known, but studies by Tatner (1985) have

shown that thymus cells labelled '*in situ*' by intrathymic injection of tritiated thymidine, subsequently migrated from the thymus into the blood and secondary lymphoid organs.

Studies presented here on the pronephros of *Tilapia mossambica* confirm its role as a major lymphoid organ in fish. In common with earlier studies (Sailendri and Muthukkaruppan, 1975) it was possible to distinguish two major types of area within the head kidney of adult fish. The lymphoid zones consisted of tightly packed lymphoid follicles and the non-lymphoid areas were predominantly erythroid with smaller numbers of leucocytes. Electron microscopic examination of the head kidney of 10 week old fry revealed the presence of haemopoietic tissue together with immature lymphoid cells. A number of mitotic figures were also noted suggesting that the immature head kidney is a site of active proliferation. As haemopoietic tissue is present in the kidney at a very early stage of development, the suggestion of Ellis (1977b) that haemopoietic stem cells migrate from the kidney to the thymus could hold true in this case. Indeed, Sailendri (1973) has suggested that a physical bridge between the pronephros and thymus may exist in *Tilapia*, allowing the direct migration of cells from one organ to another. Such a bridge was not seen in the present study but a number of lymphocytic cells were observed between the pronephros and thymus on some slides. The absence of a lymphocytic bridge was also reported in carp by Botham (1982). Stem cell origin is still uncertain in fish. In other vertebrates, it seems to be generally accepted that stem cells originate in the developing embryo, probably in the foetal liver of mammals (Owen et al., 1977) and in the developing aorta of birds. Evidence now exists that erythrocytes and lymphocytes develop in different areas of the larval kidney in amphibia (Volpe et al., 1982). It seems likely therefore, that thymocyte precursors also have an extrinsic origin in fish. However, further studies are required to locate the anatomical site at which stem cells first appear. Chiller et al., (1969) and Boomker (1979) found that plasma cells, mononuclear leucocytes and thrombocytes, as well as their precursors, were present in greater numbers in the pronephros than in other haemopoietic organs. It is therefore assumed that they are formed mainly in the pronephros.

In addition to its haemopoietic function the pronephros is also the site of antibody production and cellular reactivity to antigens. This is shown later in Chapter 4 where both

PFC and RFC are seen in the pronephros following SRBC challenge (see also Sailendri, 1973). The pigment granules seen in the pronephros of *Tilapia* are a common feature of fish lymphoid tissue (Agius 1980).

Their function is not fully understood but they are thought to be involved or associated with antigen trapping and antibody production. Ellis (1974), demonstrated that, in the kidney, uptake of bovine serum albumin appeared to be associated with aggregates of lymphocyte type cells adjacent to the melanin centres but not actually within them. Secombes and Manning (1982) suggested that in carp, pyroninophilic cell clusters observed during antibody responses were replaced by pigment containing cells. These might then become melanomacrophage centres and be important in influencing lymphocyte populations during future exposure to antigen. These pigmented regions increased in number following antigenic stimulation and the results seen have confirmed the earlier findings of Sailendri and Muthukkaruppan (1975) in this respect.

The structure of the spleen shows considerable variation between different species of fish. Most teleosts seem to possess a spleen with a high proportion of red pulp and a network of branching arteries and arterioles, the latter tending to be bound by thick walled ellipsoids. In many species, melanomacrophage centres are found, usually in the axils of branching ellipsoids. These consist of large, melanin-bearing macrophages and in some instances lymphocytes (Botham, 1982). Fish such as gar and paddle fish (Finstad et al., 1964); plaice (Ellis & et al., 1976); *Tilapia* (Sailendri and Muthukkaruppan, 1975); and perch (Pontius and Ambrosius, 1972) all showed a clear distinction between red and white pulp in the spleen, with the red pulp containing few leucocytes. However, Sailendri and Muthukkaruppan (1975), and Ellis et al. (1976) reported that white pulp accumulations, although present, were not extensive in *Tilapia* and plaice, were as Ferguson (1976) noted a relatively large lymphoid cuff in the turbot. In the rainbow trout (Grace and Manning, 1980) and in the carp (Secombes and Manning, 1980) no organized white pulp was noticed in the spleen, although in the carp occasional white cells did tend to accumulate around the ellipsoids and were found scattered in the red pulp.

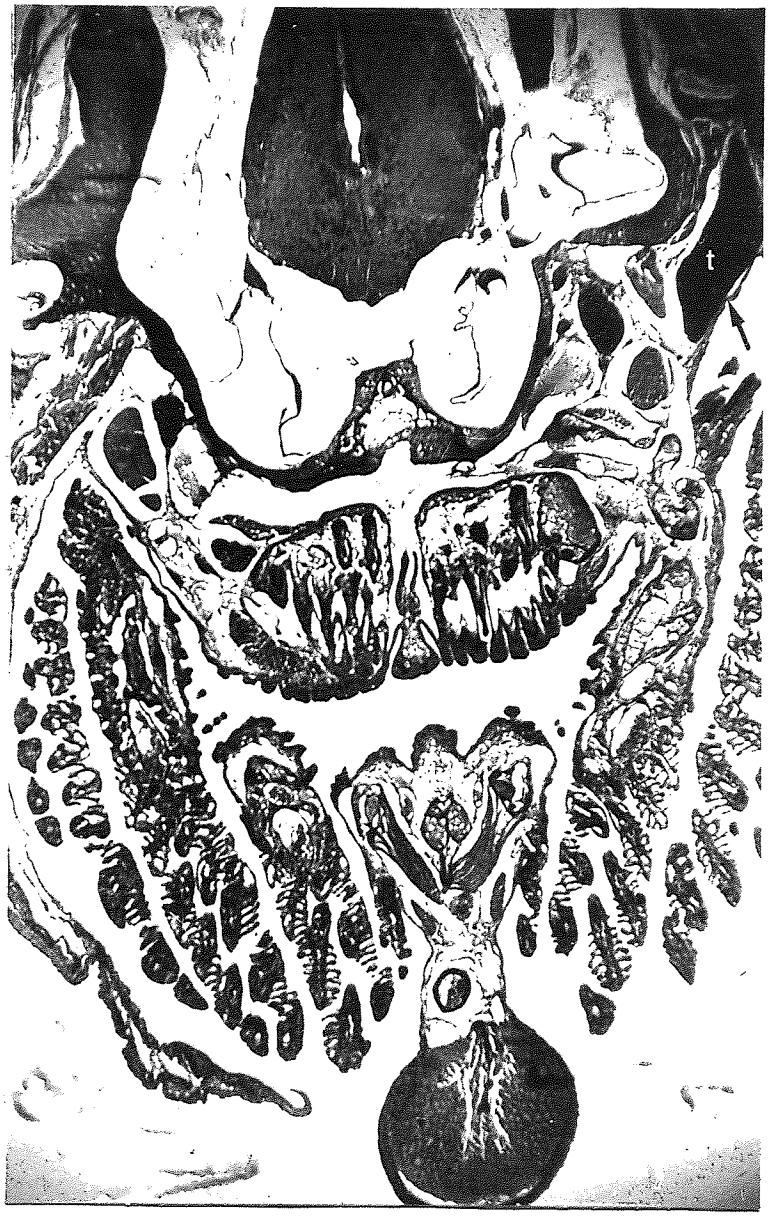
In the present study, the red pulp area became packed with lymphocytes following antigenic challenge with SRBC. This phenomenon was also reported in an earlier study on

Tilapia (Sailendri and Muthukkaruppan, 1975) and has also been observed in the blue gill (*Lepomis macrochirus)* by Smith & et al., (1967); in rainbow trout (*Salmo gairdneri*) by Chiller et al., (1969) and in perch (*Perca fluviatilis*) by Pontius and Ambrosius, (1972). Further work presented in Chapter 4, also serves to confirm that the spleen, like the head kidney, contains increased numbers of antibody producing cells and antigen reactive cells following antigenic challenge. Plasma cells were seen here in the white pulp areas of the spleen and have also been described in electron microscopic studies of the thymus of a marine teleost (Hullet, 1971, cited by Ortiz-Muniz and Sigel, 1971).

Electron microscopic studies of the spleen reveal the presence of lymphocytes and phagocytic cells in fry at 10 weeks of age when red and white pulp areas are differentiated. Problems were experienced in examining the ultrastructural details of the adult spleen, but following injection with *E. coli* bacteria it was possible to observe what may have been a phagocytic cell containing a number of engulfed bacteria. This phenomenon was similar to that reported by Bàch and et al., (1978) in the spleen of the channel catfish (*Ictalurus punctatus*) infected by *Aeromonas hydrophila.*

Thus far, the work presented in this thesis has examined the ontogenesis of the lymphoid organs in fry of *Tilapia mossambica* and compared these findings with those for adult fish. In the next section of the thesis, attention is focussed upon functional parameters of these lymphoid organs.

Fig. 3.1 Cross section of a 10 week old fish showing the dorsal position of the thymus in relation to the gills (H & E stain - x 40).



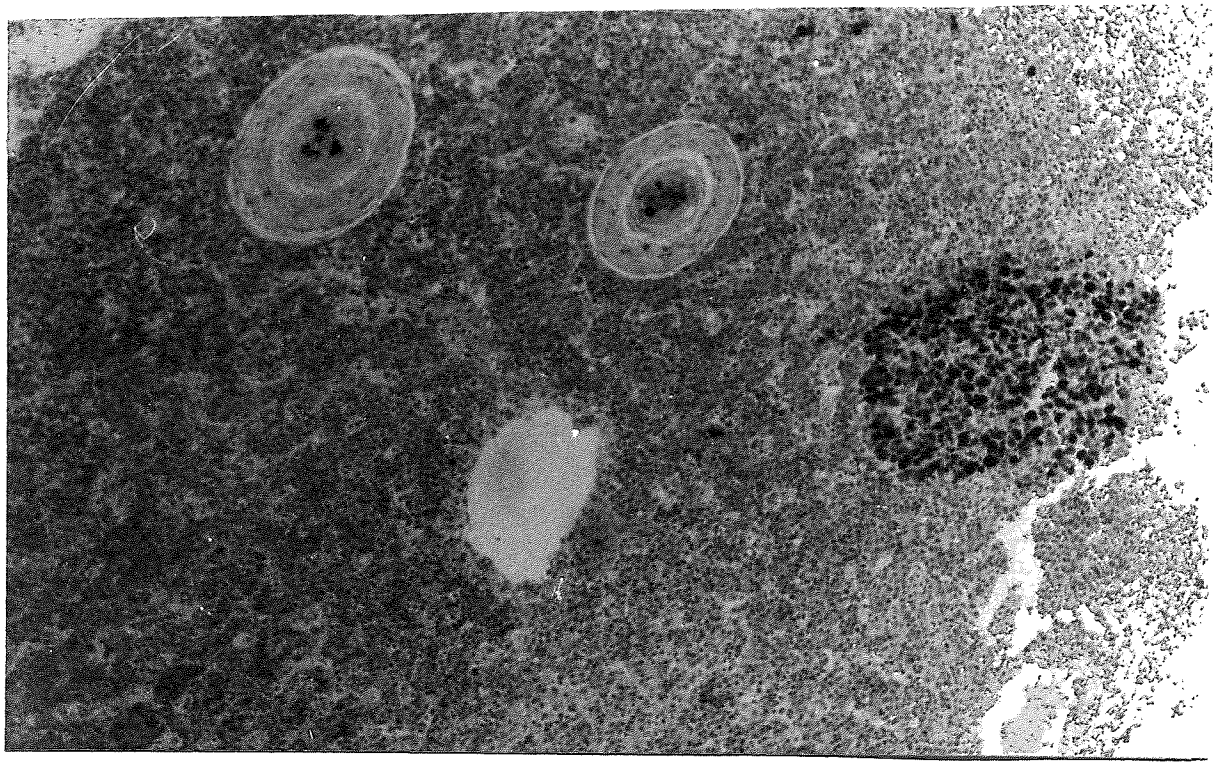


Fig. 3. 2 Section showing part of the thymic medulla in adult *Tilapia mossambica* (H & E stain - x 320).

(These are only found in adult fish).

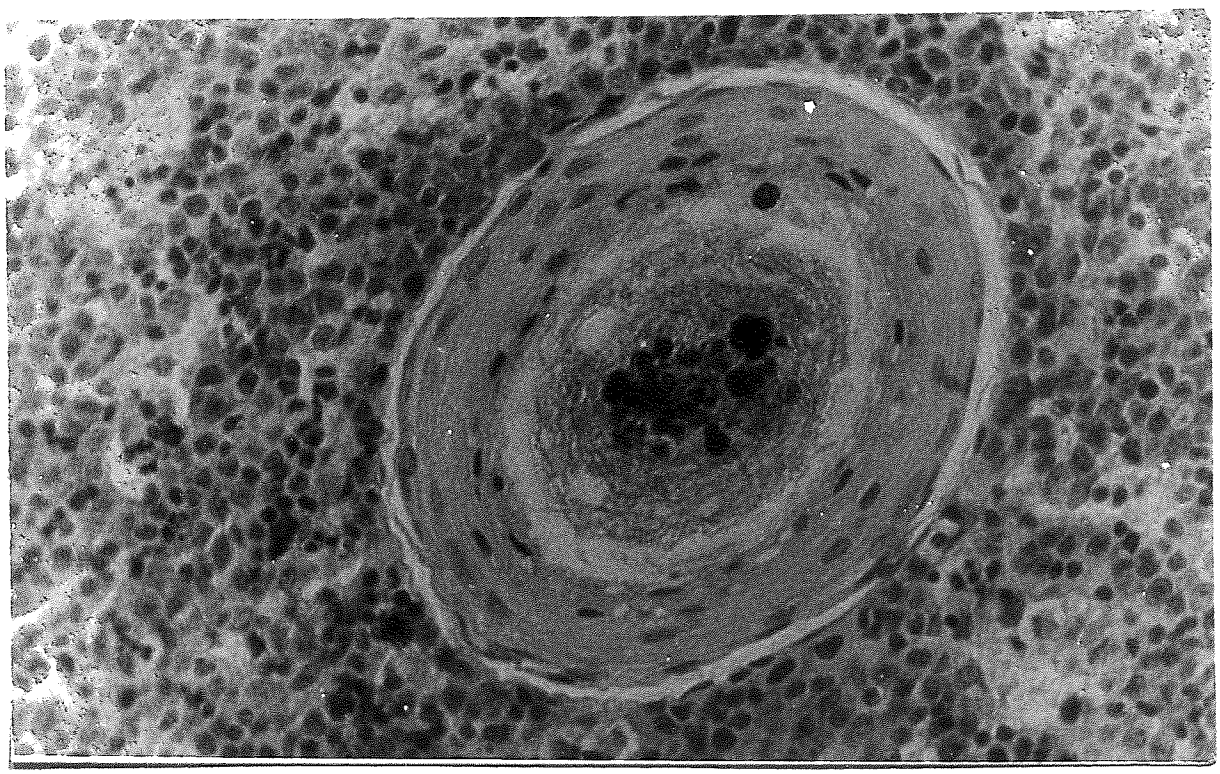


Fig. 3.3 Detail of medulla in thymus of *Tilapia mossambica* showing thymic corpuscle. (H & E stain - x 3,200).

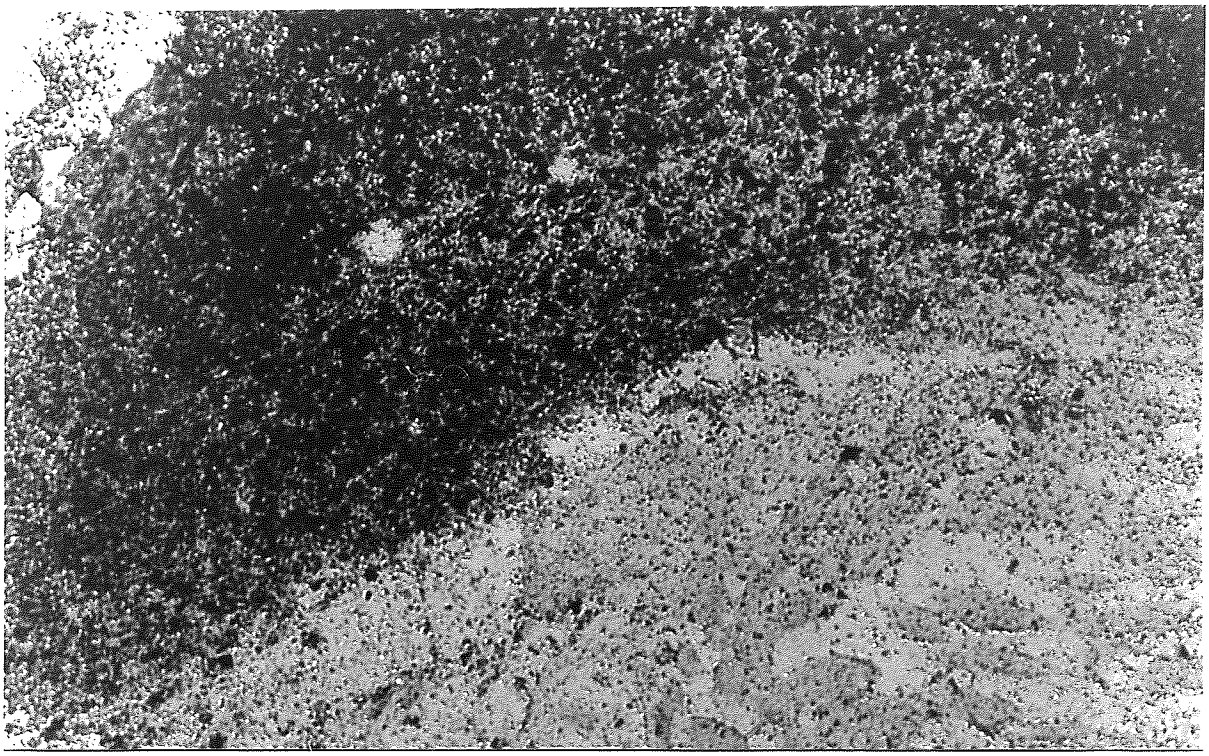


Fig. 3.4 Autoradiograph showing extensive proliferative activity in the thymus of 10 week old fry. Note the heavy concentration of silver granules over the outer region of the thymus. (Methyl green pyronin - x 800).

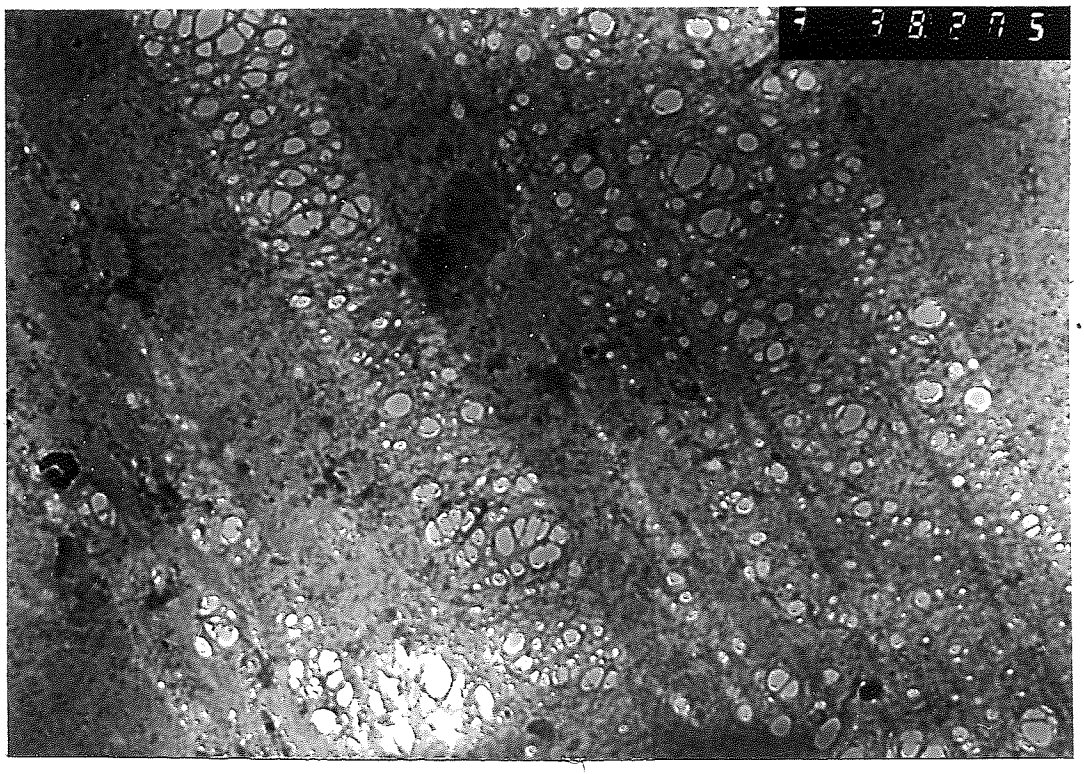


Fig. 3.5 E.M. of thymus showing fenestrated endothelial cell cytoplasm. (x 10,000).

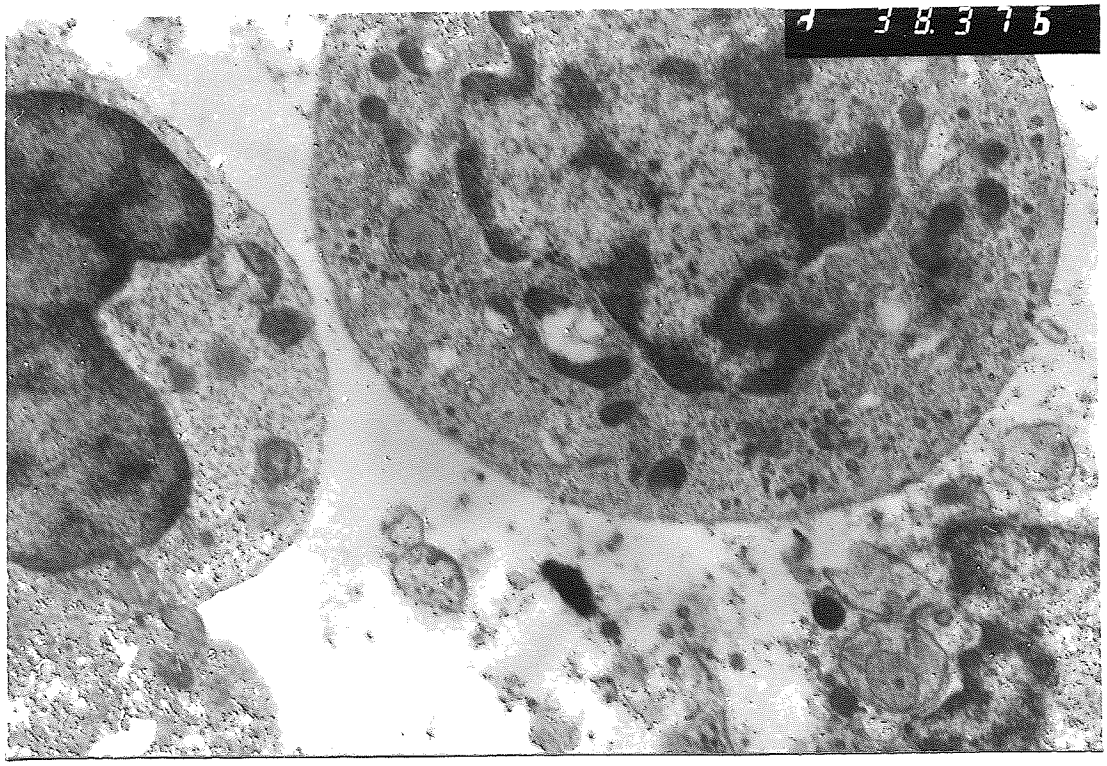


Fig. 3.6 E.M. of granulocyte (?) in thymus of 10 week old fry.(x 11,250).

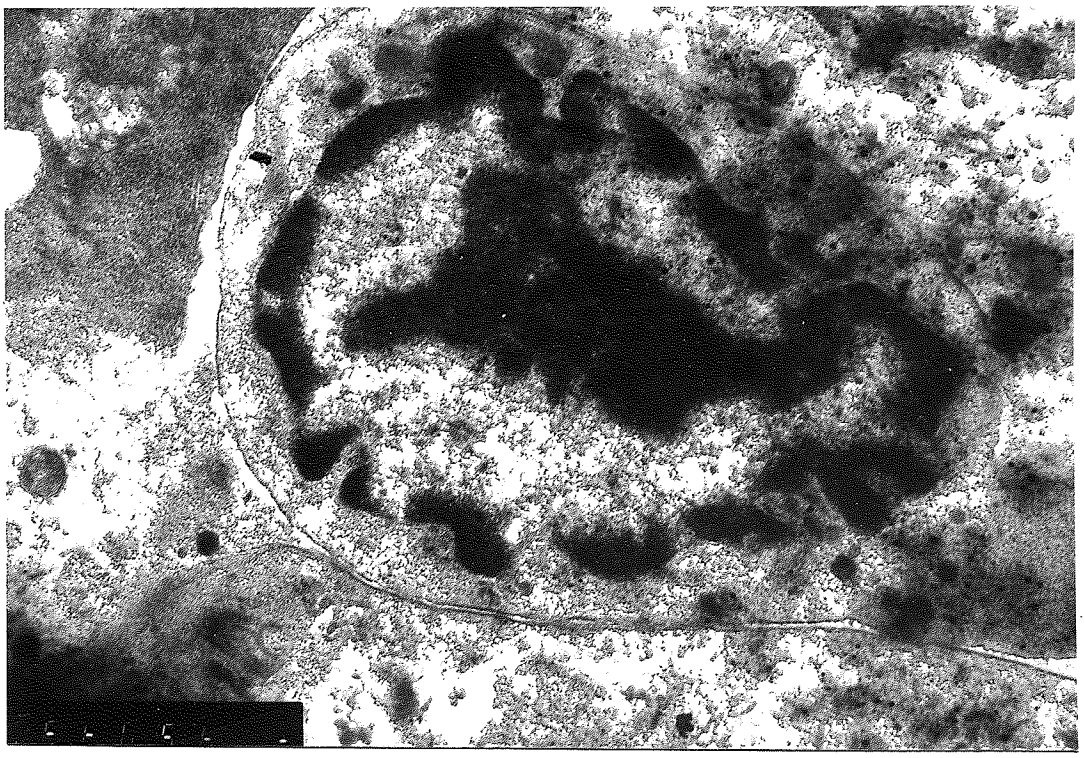


Fig.3. 7 Thymocyte in 10 week old fry thymus (E.M. - x 15, 000).

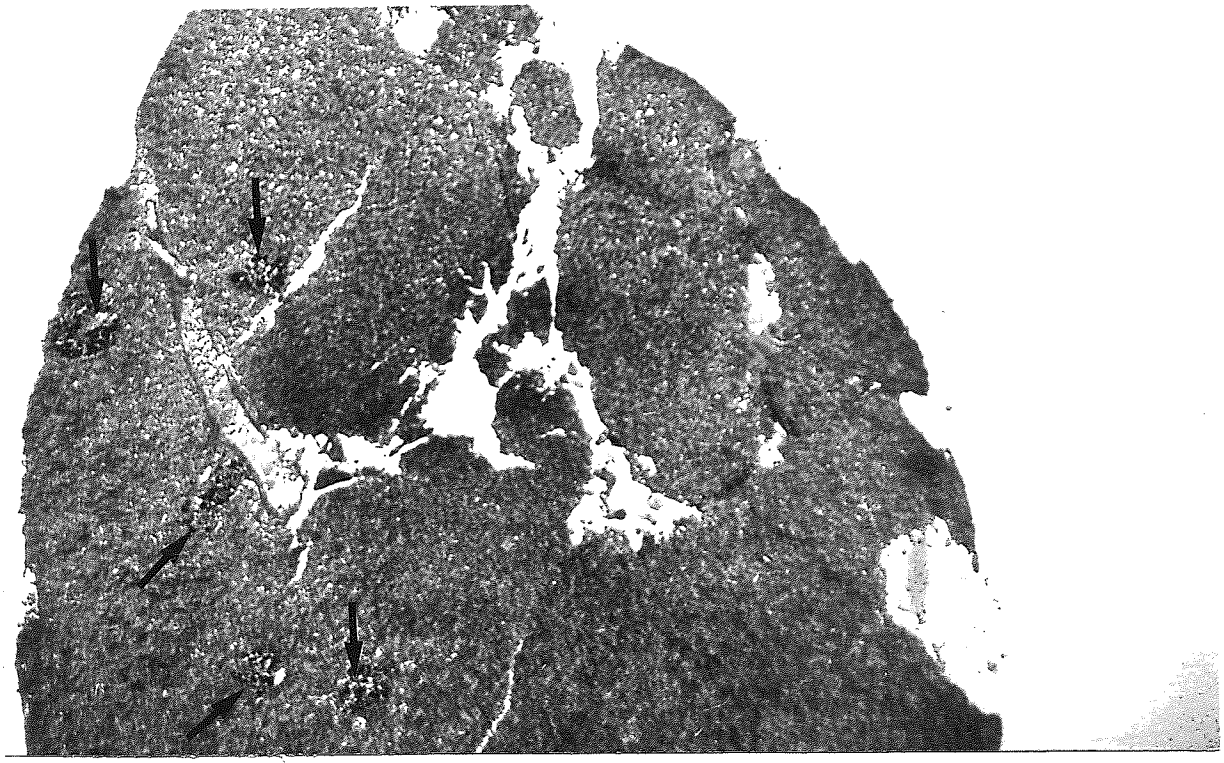


Fig. 3.8 Histological section of head kidney of *Tilapia mossambica*, 5 days after immunisation with sheep red blood cells. Notice the many spherical bodies containing pigment granules (arrowed). (H & E stain, - x 40).

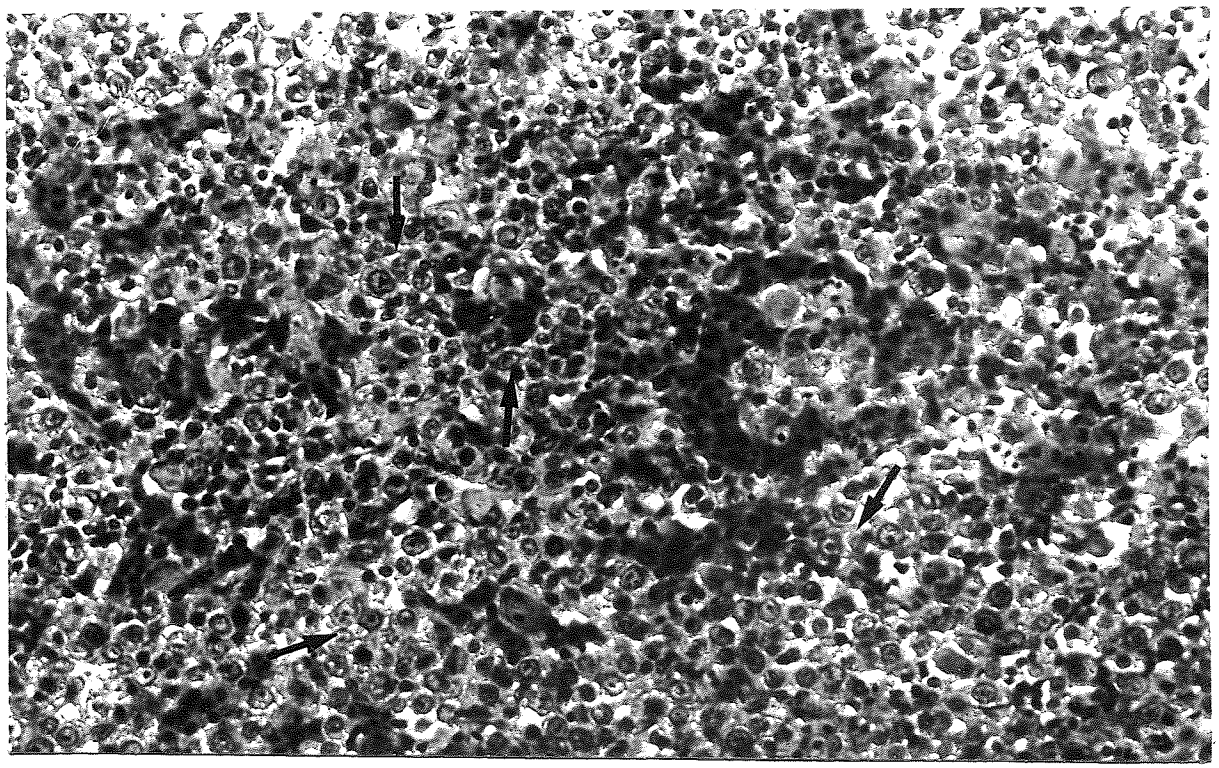


Fig. 3.9 Enlarged view of lymphoid area in the head kidney of adult *Tilapia* showing highly concentrated lymphocytes [▲]. (H & E stain, - x 250).

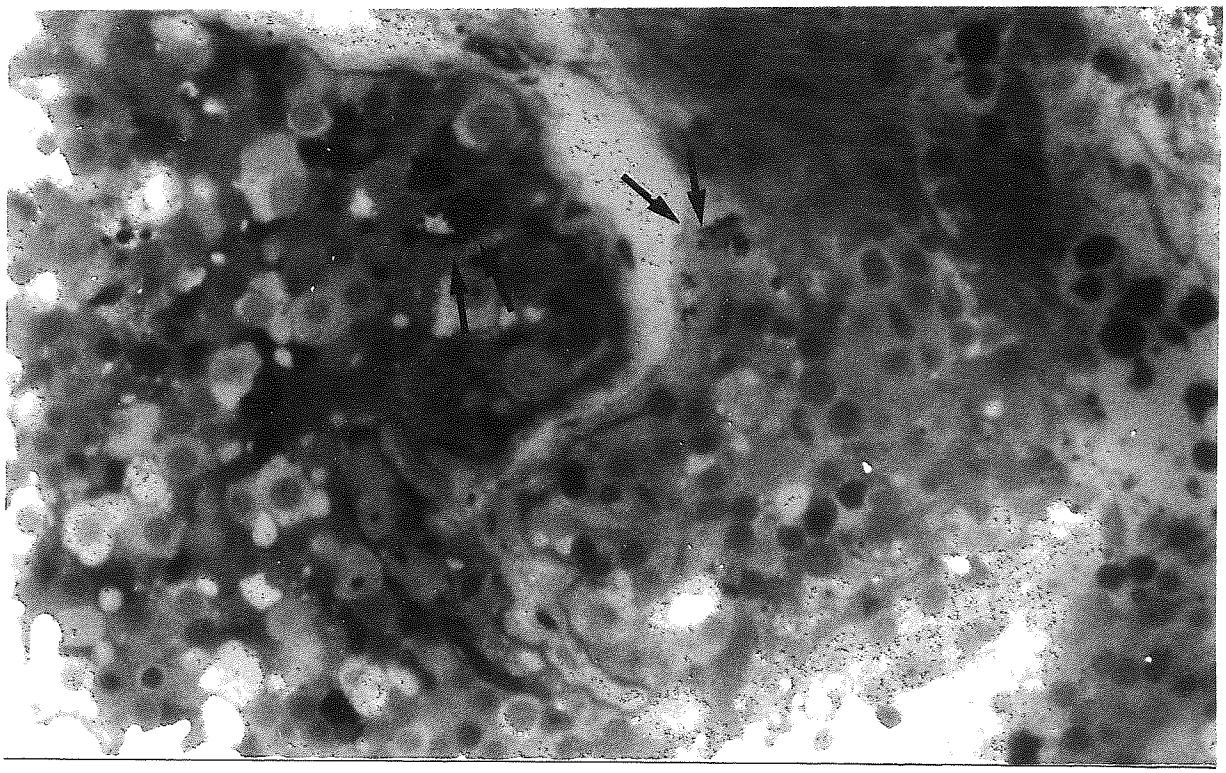


Fig. 3.10 Thick embedded section of head kidney of fry showing cellular proliferation. Two mitotic figures are indicated (see arrows) (Toluidine blue - x 2,000).

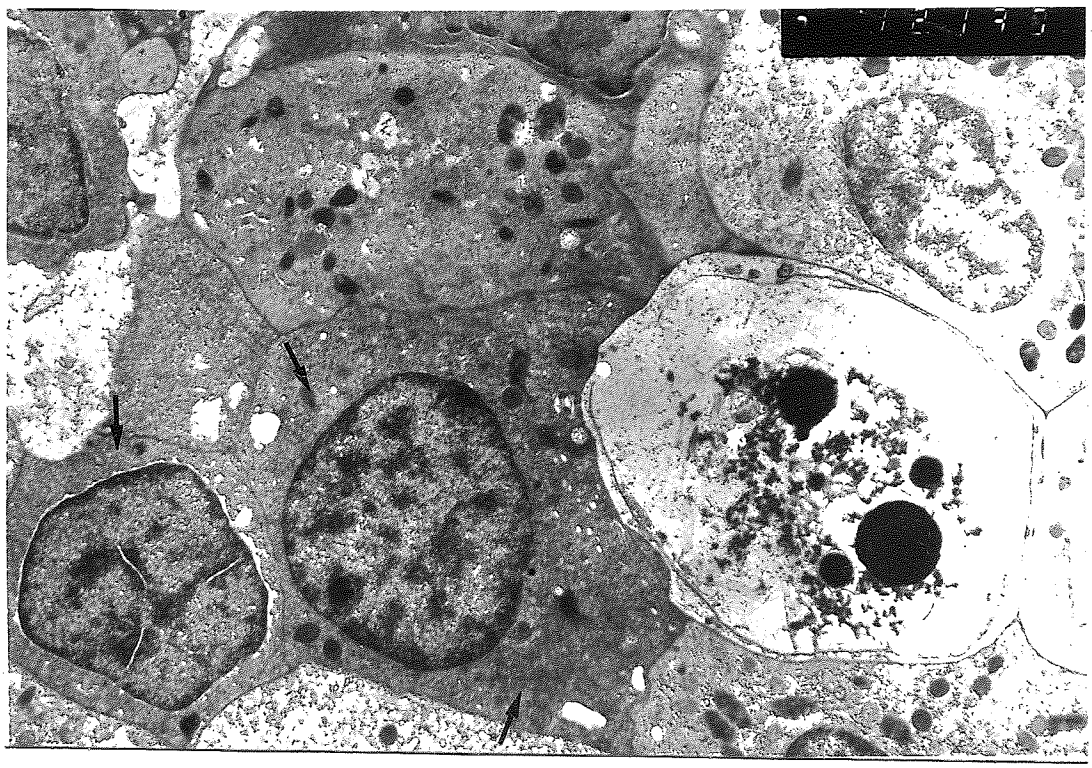


Fig. 3. 11 Electron micrograph of pronephros of 10 week old fry.(x 4,950) (Haemopoietic cells are arrowed).

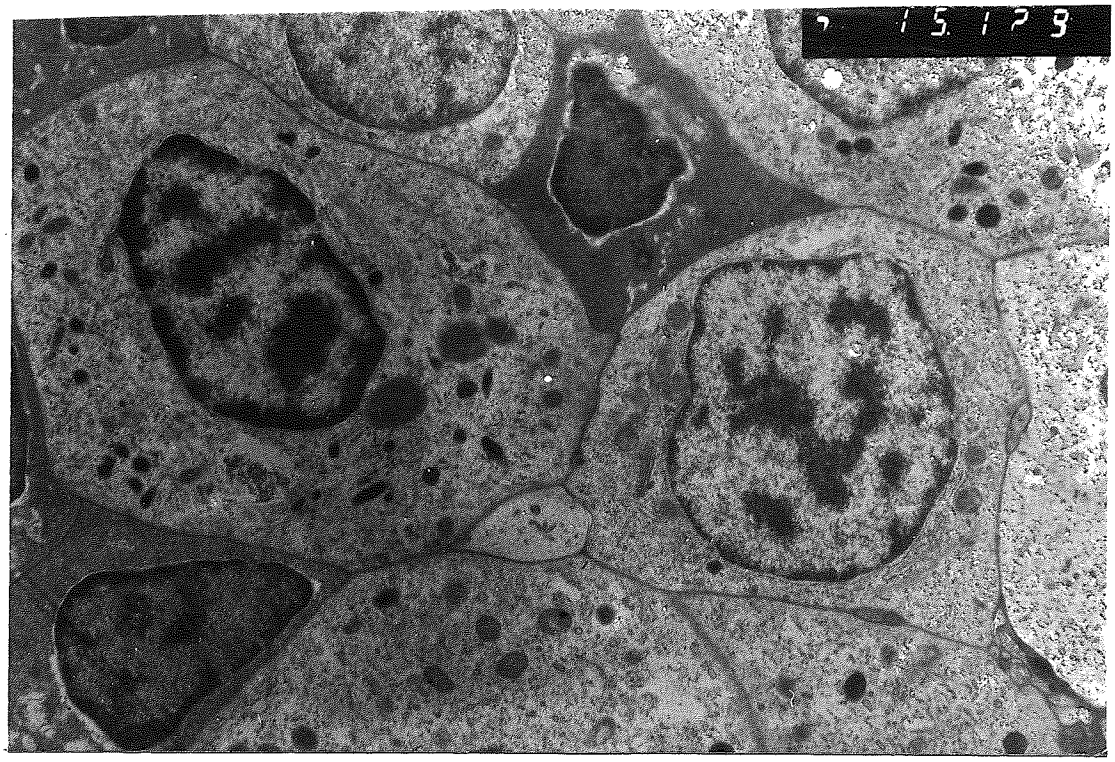


Fig. 3.12 The hematopoietic tissue in 10 week old pronephros. Note the desmosomal junctions between the reticular cells. (E.M. - x 4,950).

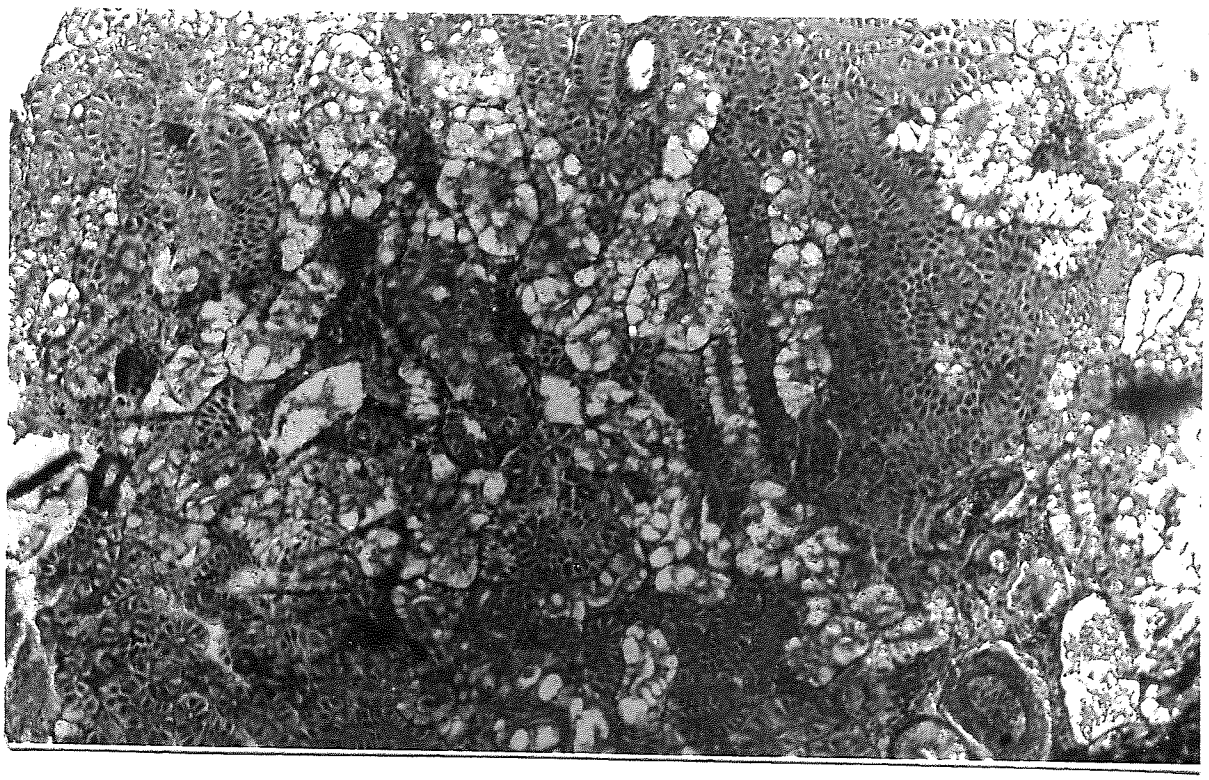
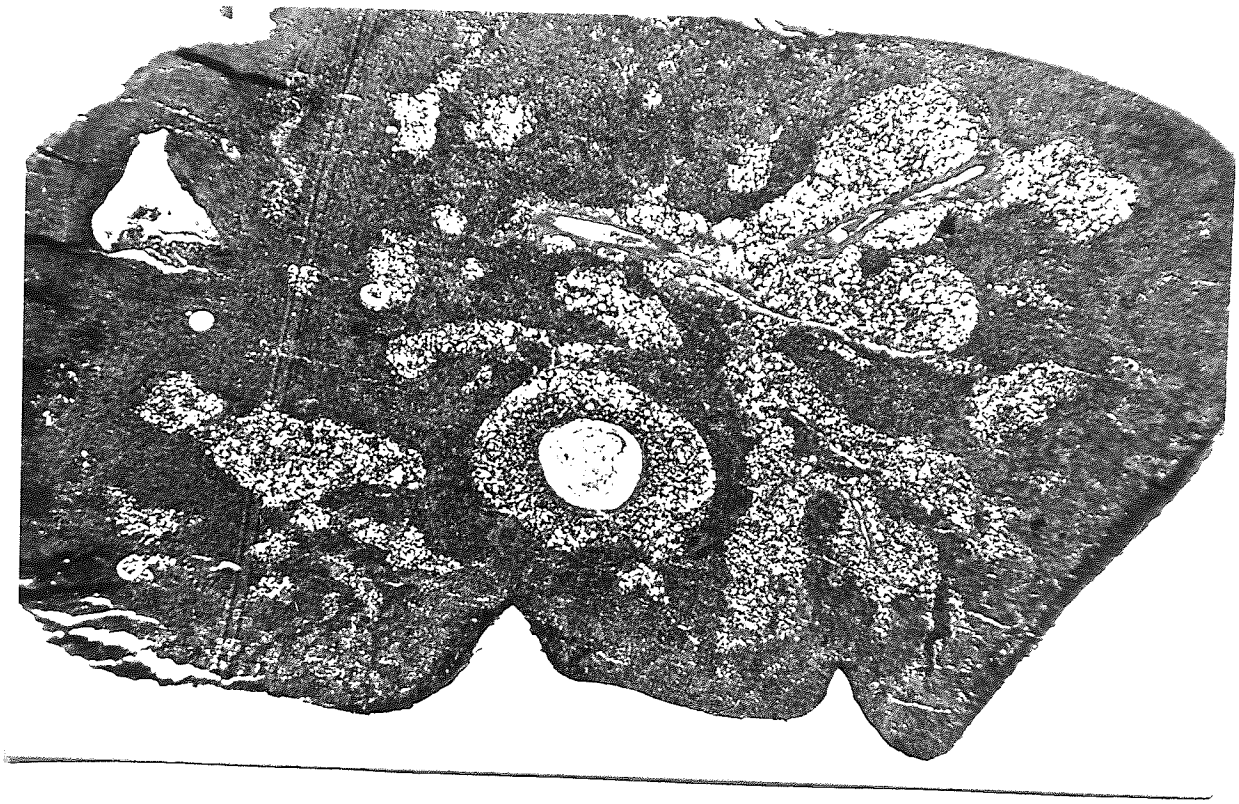


Fig. 3.13 Transverse section in the mesonephros showing kidney tubules and lack of lymphoid tissue. (H & E stain - x 320).

Fig. 3.14 Low power view of adult *Tilapia* spleen (H & E stain - x 250).



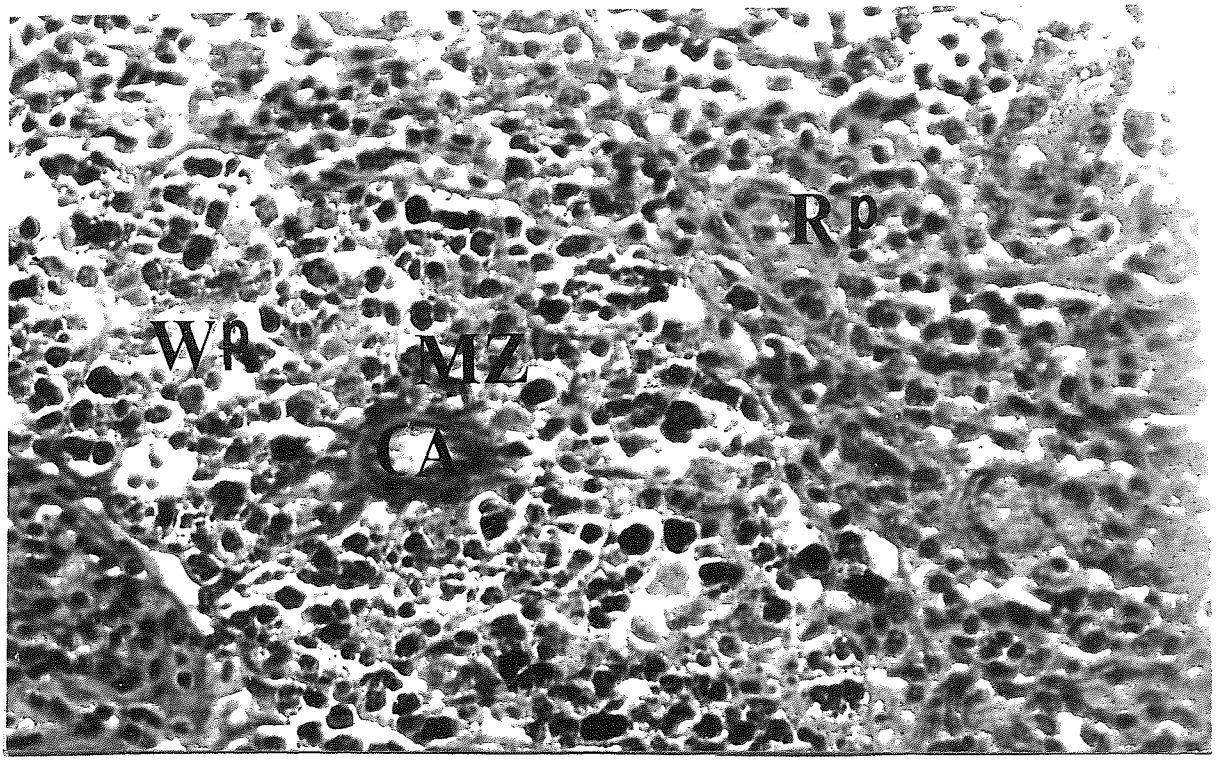


Fig. 3.15 Section of spleen of *Tilapia mossambica* showing white pulp (WP) and red pulp (RP) of control fish. (H & E stain - x 400).
(CA = Central Arteriole).
(MZ = Marginal Zone).

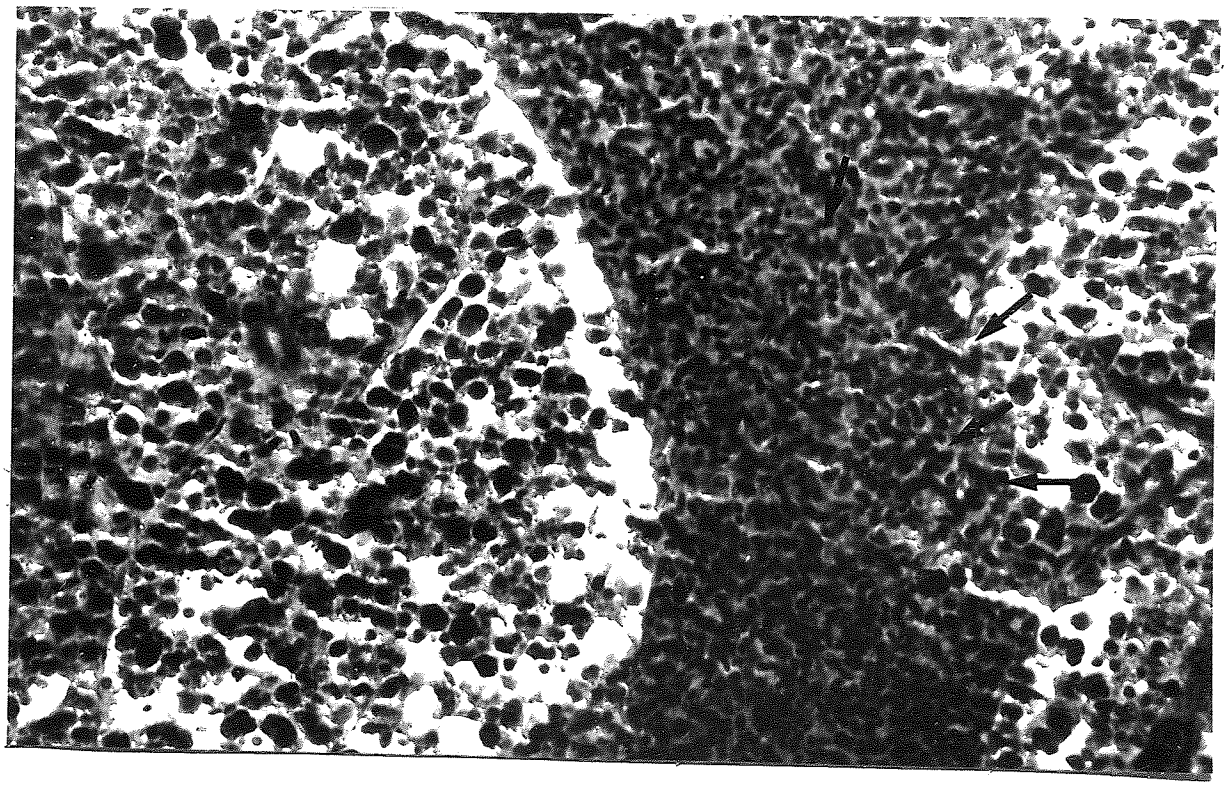


Fig. 3.16 Section of spleen from immunised fish 7 days after injection with 20% SRBC. The red pulp (arrowed) is overcrowded with lymphocytes (H & E stain - x 250).

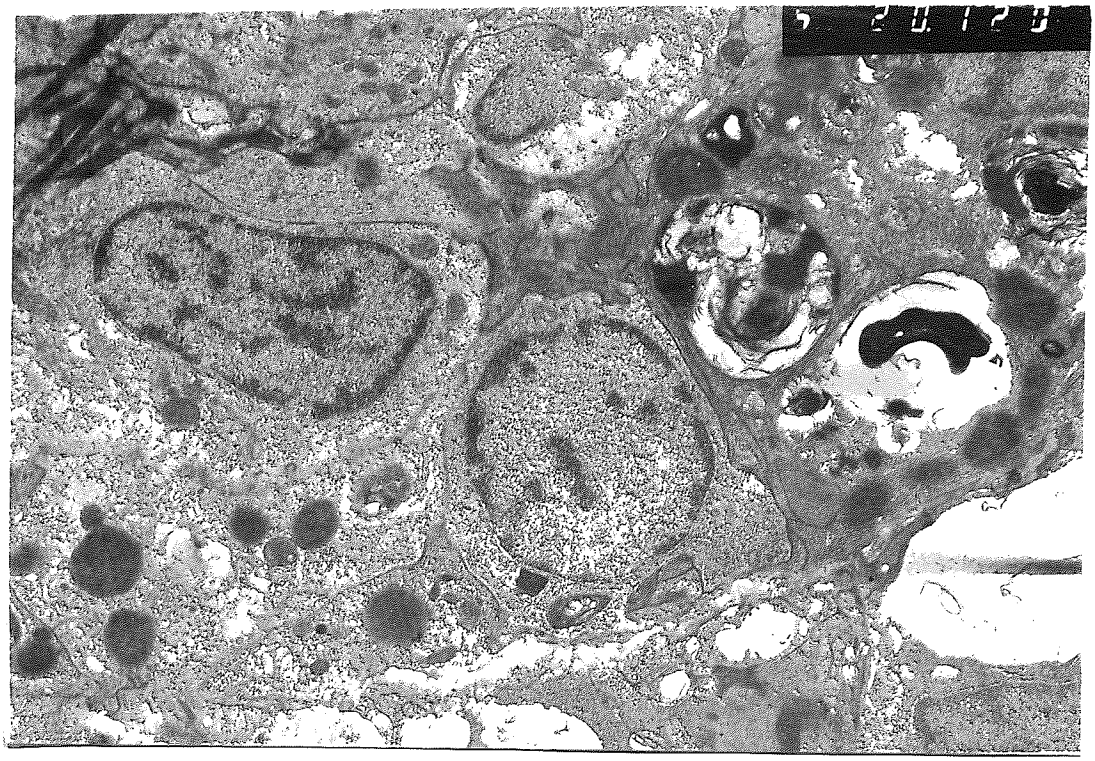


Fig. 3.17 Electron micrograph of spleen from 10 week old *Tilapia* fry (x 4,950).

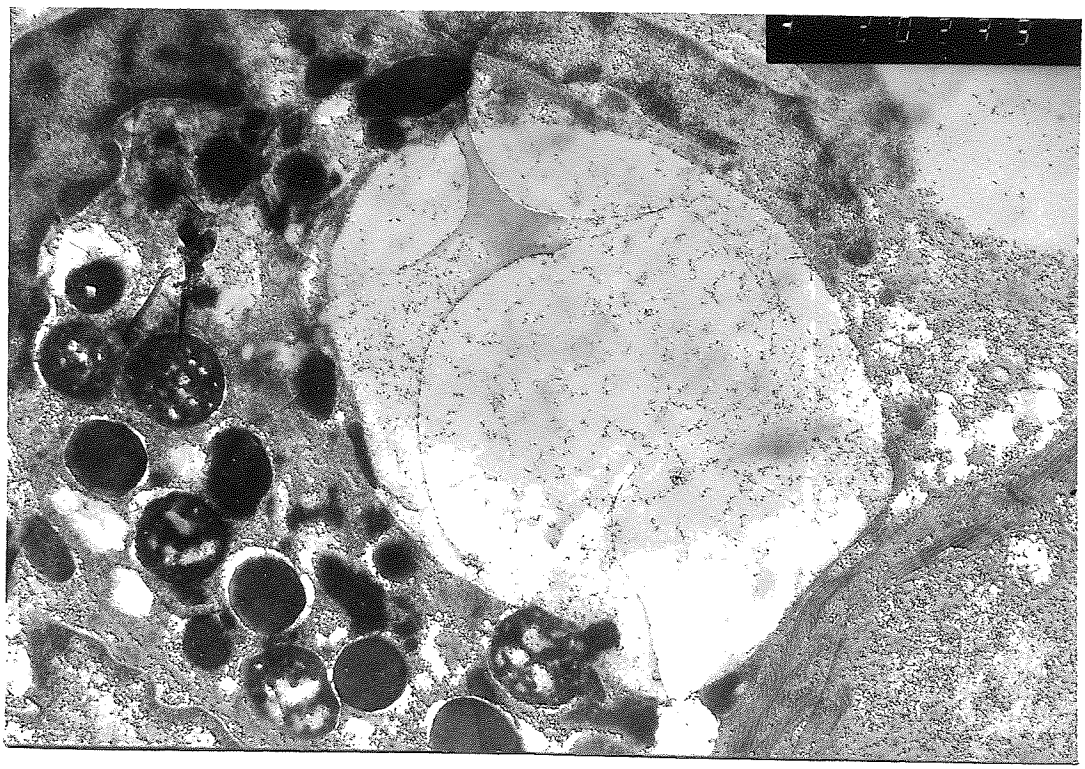


Fig. 3.18 Cell with electron dense cytoplasmic inclusions in spleen of adult fish injected with bacteria (*Escherichia coli*) 7 days earlier. (x 6,500).

CHAPTER 4

The effects of antigen dose and route of immunisation have also been studied and a comparison is made between the efficacy of intraperitoneal injection and the introduction of antigen using a hyperosmotic infiltration techniques (Antipa, 1976). Preliminary trials have also been carried out to assess the effects of adjuvant on the immune response.

For smaller fish where collection of serum samples was difficult, proliferative activity in lymphoid organs following antigenic challenge was assessed using histological and autoradiographical techniques. Antigen induced proliferative activity in lymphoid organs in adult fish was assessed by scintillation counting (Horton, Horton and Rimmer, 1977).

The potential tolerising effects of early exposure to antigen have also been examined. In view of the limited amount of information concerning cell mediated immunity in *Tilapia*, some preliminary studies presented here have examined the capacity for graft rejection in adult fish (Hildemann, 1957). In vitro cellular proliferation has also been assessed employing the mixed leucocyte reaction (Botham, 1982; Ruglys, 1985).

4.2 MATERIALS AND METHODS

4.2.1 Animals

Adult *Tilapia* of both sexes were obtained from the Aquaculture Institute, Stirling University and maintained as described in an earlier chapter. The mean weight of the adult fish used in this study was 60 ± 10 grams.

Ten week old *Tilapia* fry (mean weight 3.2 ± 2.1 grams) were also obtained from Stirling.

4.2.2 Antigens and Immunisation

Sheep Red Blood Cells

Sheep red blood cells (SRBC) in Alsever's solution (Flow laboratories) were washed three times in saline (0.85%) by centrifugation. The concentration of SRBC required was obtained by pelleting down the red cells and resuspending them in saline to a 10% or 20% v/v suspension.

For all injections fish were anaesthetised with a 50 p.p.m. concentration of tricaine methane sulphonate (MS 222 - SANDOZ). Each adult was given a single dose of 0.01ml

of 20% SRBC intraperitoneally per gram body weight using a sterile syringe fitted with a 26 - gauge needle. This protocol has previously been shown to give a high antibody titre in adult *Tilapia* (Sailendri, 1973).

The primary immune response was assessed at 2 to 3 day intervals for two weeks post-injection. In order to study the secondary response adult fish were given a further injection of SRBC 19 days after the primary immunisation, and responses were measured at 2 to 3 day intervals for another two weeks. Control fish received appropriate injections of 0.85% saline. The immune response to SRBC was assessed by measurement of haemagglutinating antibody in the serum (section 4.2.8), and by assessing the numbers of plaque forming cells and rosette forming cells in different lymphoid organs (see sections 4.2.7 and 4.2.10).

Bacteria

Two methods of bacterial immunisation were utilised. In the first, bacteria were injected intraperitoneally and in the second, bacteria were introduced by a hyperosmotic infiltration technique. The bacterial species employed in this study was *Escherichia coli* type 0111 K58 and was obtained from NCTC, Central Public Health Laboratories, London. Bacteria were maintained on nutrient agar plates for 36 hours before being washed off with sterile 0.85% saline.

The bacteria were collected and the cell concentration adjusted to 10^9 cells ml^{-1}. Further details of bacterial preparation are given in Appendix 1.

Intraperitoneal Injection

After adjusting the cell concentration, bacteria were kept in constant suspension with a magnetic stirrer prior to immunisation to ensure a constant dose. The temperature of the suspension was adjusted to ambient water temperature. All fish received an injection of 0.01 ml bacterial suspension per gram body weight (equivalent to 1×10^7 cells/gram fish). Fish were killed at weekly intervals after injection for study of the immune response.

A group of twenty adult fish (*Tilapia mossambica*) each received a single injection of *Escherichia coli* on day 0, and another twenty adult fish, each received two injections of

the same dose of *Escherichia coli* one on day 0 and the other on day 12.

The immune response to bacteria was studied using a slide agglutination technique (section 4.2.11). Control fish were each injected with the same dose of 0.85% saline only.

Hyperosmotic Infiltration

This method was applied in this study for adult fish only. Groups of four adult fish were placed in a ten fold concentration of Hank's balanced salt solution (10 x HBSS) for two minutes. The fish were then removed from the salt bath and immersed into a bacterial suspension containing 10^9 bacterial cells per ml. After 2 minutes immersion the fish were removed and placed in tanks containing fresh water (Antipa, 1976).

Human Gamma Globulin

Lyophilised human gamma globulin (HGG) (Sigma) was dissolved in 0.85% saline to a concentration of 2.5 mg/ml. The standard immunising dose used was 0.01 ml of HGG solution (i.e. 0.025 mg HGG) per gram body weight, delivered intraperitoneally. This dose was established as a result of preliminary trial experiments not presented here. A group of 15 adult *Tilapia mossambica* was immunised and the primary antibody response was assayed using passive latex particle agglutination. The same dose, given to each fish, was repeated 19 days after primary immunisation in order to study the secondary immune response. Control fish were injected with an equivalent dose of 0.85% saline.

4.2.3 Ontogenetic studies on humoral immunity in Fry

The ability of *Tilapia* fry to mount a humoral immune response against three different antigens was assessed. The antigens employed in these studies were sheep red blood cells (SRBC); the bacterium *Escherichia coli* type 0111 K58; and human gamma globulin.

Experiments were commenced when fry were 10 weeks of age, although in one study fry of five weeks old were used. Except where indicated, fry were injected in groups of 30 animals using a glass micrometer syringe fitted with a sterile 30 gauge needle (S.G.E.). Fish were anaesthetised for injection in a 50 p.p.m solution of MS 222.

Antibody production, plaque forming cells and rosette forming cells were monitored as outlined in the relevant sections 4.2.7 to 4.2.11.

4.2.4 Antigen Injection

Sheep Red Blood Cells (SRBC)

Three groups of fry were each injected with 10 µl/gram body weight of SRBC (20%) via the intraperitoneal route.

One group received a single injection of SRBC at 10 weeks of age, while a second group of animals was given a first injection at 10 weeks followed by a second injection seven days later. A further group of fry received one injection at 10 weeks of age and a second injection 12 days later. Controls received equivalent injections of saline only. Animals were sacrificed at intervals following the final injection and serum haemagglutination assays were performed.

Long term effects of early exposure to SRBC

In a second series of experiments to examine the long term effects on immunity of early exposure to antigen, one group of fry received a single injection of SRBC at 10 weeks followed by a second challenge with SRBC 56 days later. Controls received a saline injection in place of the first SRBC challenge.

A second group of fry was given two single injections of SRBC, one at 10 weeks of age and another 12 days later. This group was further challenged with SRBC 56 days after the first injection. Control fry were initially given two saline injections in place of SRBC and were challenged with SRBC 56 days after the first saline injection. Antibody (haemagglutination) responses were assessed at intervals following the last injection.

Bacteria

The bacterial species employed in studying the immune response of *Tilapia* fry was *Escherichia coli* type 0111 K58. Bacteria were adjusted to a concentration of 10^9 cells per ml. and were injected in 0.85% physiological saline at a dose of 10µl bacterial suspension per gram body weight via the intraperitoneal route. One group of fry received a single

injection of bacteria at 10 weeks of age, whilst a second group were given single injections at 10 weeks and 12 days later. Control groups received saline injections only.

Long term effects of early exposure to bacterial antigens.

The long term effects of early exposure to bacterial antigen were studied in a group of fry which received two injections of bacteria; one at 10 weeks of age and a second 12 days later. A third (challenge) injection of bacteria was given 56 days after the first injection, and the levels of bacterial agglutinins in the serum were measured at intervals after the final injection.

The immune response of fry to bacteria was studied using a slide agglutination test as outlined in a later section (4.2.11).

Effects of Freund's complete adjuvant (FCA)

To study the effects of Freund's complete adjuvant (FCA) on the immune response to bacteria, two groups of fry were given 1 x 10^7 bacterial cells suspended in 10 microlitres of FCA/saline emulsion (50:50). One group received i.p. injections at 10 weeks and 12 days later.

The second group received three injections of bacteria in FCA : the first at 10 weeks, followed by two single injections, 12 and 56 days later. Control fry received saline and FCA only. Bacterial agglutinins were measured in the serum at intervals following the final injection using the slide agglutination technique described in section (4.2.11).

Human Gamma Globulin (HGG)

HGG (Sigma) was dissolved in 0.85% saline to a concentration of 2.5 mg/ml. Fry were injected intraperitoneally with 10µl of antigen solution (0.025 mg HGG) per gram body weight. One group of 15 fry was given a single injection of HGG at 5 weeks of age and received a second injection of antigen 12 days later.

One group of older fry were injected with HGG at 10 weeks of age and 12 days later. Controls received appropriate injections of saline only.

Effects of Adjuvant (FCA)

The effects of adjuvant were examined in one group of fry which received injections of HGG in FCA/saline emulsion at 10 weeks of age and 12 days later. Control fry received injections of FCA/saline only. Antibody titres to HGG were assessed by passive agglutination of HGG-coated latex particles (Section 4.2.9) at intervals post-injection.

4.2.5 Collection of Serum

Immunised and control fish were bled from the common cardinal vein using a sterile syringe. Serum was collected after leaving the blood sample to clot for one hour at room temperature and then removing the clot. The serum was then spun to remove any residual blood cells. Serum samples to be tested were incubated at 56°C for 15 minutes to inactivate complement components. In the case of fry, pooled blood was collected by cardiac puncture from 3 - 5 individuals in order to obtain enough serum for the antibody assays.

4.2.6 Complement

A 0.2 ml aliquot of normal fresh *Tilapia* serum was absorbed with 0.01 ml of packed sheep red blood cells for about one hour at 5°C. After absorption, the serum was centrifuged and the supernatant was collected to be used immediately or kept for twenty-four hours in the fridge. Smith et al. (1967) found that *Tilapia* serum, when used as a source of complement displayed a high level of haemolytic activity. Guinea pig serum (Flow Laboratories) was also employed as a source of complement in the present study and was found to give similar results to *Tilapia* serum.

4.2.7 Plaque Forming Cell Assay (PFC)

To detect the presence of plaque-forming cells (PFC) in lymphoid organs, the technique of Cunningham and Szenberg (1968) was followed with minor modifications (Kanakambica and Muthukkaruppan, 1972).

Animals immunised with SRBC were sacrificed and thymus, head kidney and spleen were dissected out. Cell suspensions from each of these organs were individually prepared by teasing the organs apart in small plastic dishes containing approximately 1 ml of medium

[1 x Iscove's modification of Dulbecco's medium (Flow Laboratories)]. The cell suspensions were transferred to tubes and large tissue clumps were allowed to settle out on ice for 4 - 5 minutes. The supernatant containing the single cell suspension was then collected and the concentration and viability of these cells was determined using Trypan blue dye exclusion. Viable white cells were counted using a haemocytometer (Neubauer counting chamber). For studies on fry it was necessary to pool spleen or head kidney cells from up to 15 individuals in order to achieve the desired cell concentration. A mixture consisting of 0.1 ml of the test cell suspension adjusted to(5×10^6 - 6×10^6 cells/ml), 0.01 ml of fish complement and 0.01 ml of 20% SRBC was prepared and incubated as a monolayer between two sealed glass slides for between one and two hours at (26°C - 30°C) and the preparation was scored for the number of haemolytic plaques. The number of plaque forming cells per million leucocytes was calculated. In one experiment 20% chicken red blood cells (CRBC) were used in place of SRBC to test the specificity of fish anti-sheep red blood cell antibody.

4.2.8 Haemagglutination assay

Agglutinating antibody titres to SRBC were assessed by standard haemagglutination techniques (Hudson and Hay, 1980). The serum was collected from fish killed at weekly intervals post injection. The specificity of anti-SRBC activity in the serum was examined using 1% suspensions of chicken red blood cells, rat red blood cells or mouse red blood cells to test for cross reactivity.

4.2.9 Assessment of the Immune Response of *Tilapia mossambica* to Human Gamma Globulin (HGG) using the Passive Haemagglutination Test.

800 µl of latex particle (Difco) suspension was washed twice with 40 ml of 0.054 M glycine saline and centrifuged at 12,500g for 15 minutes. The latex was resuspended in 20 ml of 0.054 M glycine-saline and mixed with 300 µl of a 10 $mgml^{-1}$ solution of HGG for 30 minutes at room temperature. The latex was washed twice by adding 40 ml of 0.054 M glycine saline, mixed and centrifuged at 12,500g for 15 minutes. The latex was

resuspended in 20 ml of 0.27 M glycine-saline containing 0.1% of bovine serum albumin to block any remaining latex protein binding sites.

For the slide agglutination test, 25 µl aliquots of doubling dilutions of test antiserum were added to 25 µl aliquots of coated latex on a glass slide and after rocking gently for 2 minutes to mix the two droplets, agglutination was read visually by illuminating the slide from the side and viewing against a dark background.

4.2.10 Immunocytoadherence (Rosette Forming Cell) Assay

Immunised and control fish were killed as described previously and cell suspensions were prepared for different lymphoid organs as described for the PFC assay. For each adult fish studied (or groups of 3 - 5 fry) 0.2 ml aliquots of the appropriate lymphocyte suspension were added to two plastic tubes containing 0.2 mls of the 1% SRBC suspension. The tubes were incubated at 23°C for two hours during which time the erythrocytes and white blood cells sedimented to the bottom of the tube. The cells were gently resuspended on a vertical rotor turning at 8 to 10 revolutions per minute for five minutes.

The number of rosettes in each suspension was counted using a haemocytometer (at least four samples per tube were counted). A rosette was scored if a central lymphocyte was observed with three or more adherent SRBC. Rosettes with more than one lymphocyte or clumped red cells without lymphocytes were neglected. The number of rosette forming cells per million leucocytes was calculated for each lymphoid organ.

4.2.11 Slide Agglutination Test for Bacterial Antigen

The test antigen (*Escherichia coli*) was a formalin killed suspension of bacteria (1 x 10^{10} cells/ml) suspended in physiological saline containing a 1:20,000 concentration of crystal violet and brilliant green. The bacterial cell suspension was diluted 1:5 in physiological saline. With a pasteur pipette, three drops from each tube of a 2 fold serial dilution of the serum to be tested were placed in separate sections of a glass microscope slide. (Klantz, Reddecliff and Heist, 1963). One drop of test antigen was added from a 26-gauge needle fitted onto a syringe. The needle was held level and horizontal to the glass

plate. Solutions were mixed with individual wooden applicator sticks and incubated at room temperature for ten minutes before being gently agitated for two minutes. Agglutination was read by holding the plate in a beam of light from a microscope lamp fitted with a ground glass. The specificity of the reaction was assessed by testing the serum against the bacterium *Klebsiella aerogenes*.

4.2.12 Histology

Pyroninophilia

In order to study the histological changes occurring in *Tilapia* lymphoid organs following antigenic challenge, 8 microns sections of head kidney, spleen and thymus were prepared at weekly intervals for up to 5 weeks post injection from *Tilapia mossambica* fry, immunised at 10 weeks and 12 days later. *Escherichia coli* and HGG were both used as antigens as outlined in section 4.2.2.

These sections were processed then stained with methyl green pyronin (M.G.P.) as in appendix 6 and estimates made of the numbers (low, moderate or high) of pyroninophilic cells. Control sections from fry injected with the same dose of 0.85% saline also examined.

Autoradiography

Spleen, thymus and head kidney were dissected from fry immunised at 10 weeks and 12 days later with 20% SRBC and sacrificed at weekly intervals after immunisation. Imprints and sections from these organs were processed for autoradiography as in Appendices (7 & 8) and investigated under the light microscope. Sections from immunised and control fish were examined for the presence of labelled lymphoid cells.

4.2.13 Scintillation Counting

To confirm the histological data on splenic activity following antigenic challenge, scintillation counting was employed to study proliferation in immunised spleen following antigenic challenge. Tritiated thymidine uptake *in vitro* was examined as devised below. The methodology follows that outlined by Horton et al.(1977).

Spleens of SRBC immunised and control saline injected *Tilapia mossambica* were removed aseptically one week after injection, and immediately washed in L-15 culture medium (Appendix 9), before being transferred to fresh medium . 50 µl of spleen cells (2.5×10^6 leucocytes) was then distributed into individual wells of sterile microtitration plates (M220-25 ARTL-Gibco) using an Oxford sampler.

The cells were immediately pulsed, with 1 µc ^{3}H thymidine (Amersham International, S.A = 5Ci /mmol) being added to each well. All cultures were set up at least in triplicate and following incubation for 4 hours at 28°C in a humidified atmosphere, cells were harvested with the aid of a Skatron cell harvester (Flow Laboratories) and filters containing the harvested cells were dried at 52°C for 30 minutes. Filters were then placed in individual scintillation vials. The cellular material collected on the filters was digested with 0.3ml protosol (NEM) at 56°C for one hour and, after cooling, 0.5 ml of scintillation fluid (Optiphase-I, Fisons) was added to each vial. Incorporated ^{3}H-thymidine was counted in a "Packard 2660" scintillation ß-counter and corrected for background radioactivity and quenching. ^{3}H-thymidine uptake in spleen cells from animals injected with saline only was taken as 'background' and a stimulation index (S.I.) was calculated as follows:-

$$\text{S.I.} = \frac{\text{Mean c.p.m. in SRBC injected animals}}{\text{Mean c.p.m. in saline injected animals as control}}$$

c.p.m. = counts per minute

4.2.14 Scale Transplantation

Preliminary studies were carried out on adult fish to develop graft techniques and investigate mixed leucocyte reactivity *in vitro*. The grafting procedure followed that described by Hildemann (1968) and improved by Rijkers (1977). Ten pairs of individually tagged adult *Tilapia* of similar size were used for allografting studies in this experiment. Individual fish acted as both scale donors and hosts in each pair. Fish were anaesthetised in MS 222 for about 20 minutes and three scale grafts were reciprocally transplanted between fish of each pair by plucking single scales from their attachment pockets in the skin with

fine forceps, and placing individual allografted scales in the empty pockets thus created. The overlapping adjacent scales were generally sufficient to hold grafted scales in place. Five control fish received scale and autografts. All grafted fish were maintained at(21 - 27°C)as described previously. Grafts were examined every two days post transplantation and end point rejection was assessed by noting the time at which the opaque hyperplastic tissue which overgrows scale transplants, became clear (Hildemann, 1957; Rijkers and van Muiswinkel, 1977).

Second set grafts were applied from the original donors 27 days after first set allografting.

4.2.15 Mixed Leucocyte Culture

The technique described is similar to that described by (Ruglys, 1985). Cells were collected aseptically from the pronephros of adult *Tilapia* and were resuspended in medium as described in section 4.2.13. To achieve a concentration of 10^6 cells/ml. For each experiment, 100 µl of cell suspension from one fish (A) was placed into each of three wells of V-bottom microtitre plates (Sterilin) and mixed with 100 µl of cell suspension from the other fish (B), this combination of cells was designated (AB).

Control wells contained 200 µl of cells from fish A (AA) or 200 µl of cells from fish B (BB). Medium was put into the wells surrounding those containing cells in order to reduce evaporative losses. The plate was covered and placed in an incubator at 26°C. Three days later 10 µl of tritiated thymidine (methyl-3H-thymidine, 2 Ci/mmol, Amersham-International) was added to each of the microculture wells (2 µc per well), and the plate put back into the incubator for 24 hours.

Cell harvesting was performed with a Skatron cell harvester and processed for scintillation counting as described under a previous section.

The index of stimulation for each set of cultures was calculated as follows:-

$$S.I. = \frac{\text{2x mean counts of AB}}{\text{mean counts of AA + mean counts of BB}}$$

4.3 RESULTS

4.3.1 Serum Antibody responses in Adult Fish

Cellular and Humoral Antibody production to sheep red blood cells (SRBC)

Figure 4.1 demonstrates that all the immunised adult animals consistently showed an increased antibody response to SRBC. Serum antibodies were detected from the second day onwards after immunisation with sheep red blood cells. In this and subsequent experiments all antibody titres are expressed as ($-\log_2$). A mean haemagglutinating titre equal to 0.66 was detected on the second day (Fig. 4.1). The peak haemagglutination response occurred on the eighth day after injection of SRBC. The antibody level declined slowly thereafter and no titre was detectable by the fourteenth day post injection. All saline injected controls were negative for haemagglutination. No cross reactivity was found when antisheep red blood cells sera from three immunised fish were incubated with chicken red blood cells (CRBC), rat red blood cells (RRBC) and mouse red blood cells (MRBC). Saline injected control animals also gave negative results with all the antigens used in testing cross reactivity (CRBC, RRBC and MRBC).

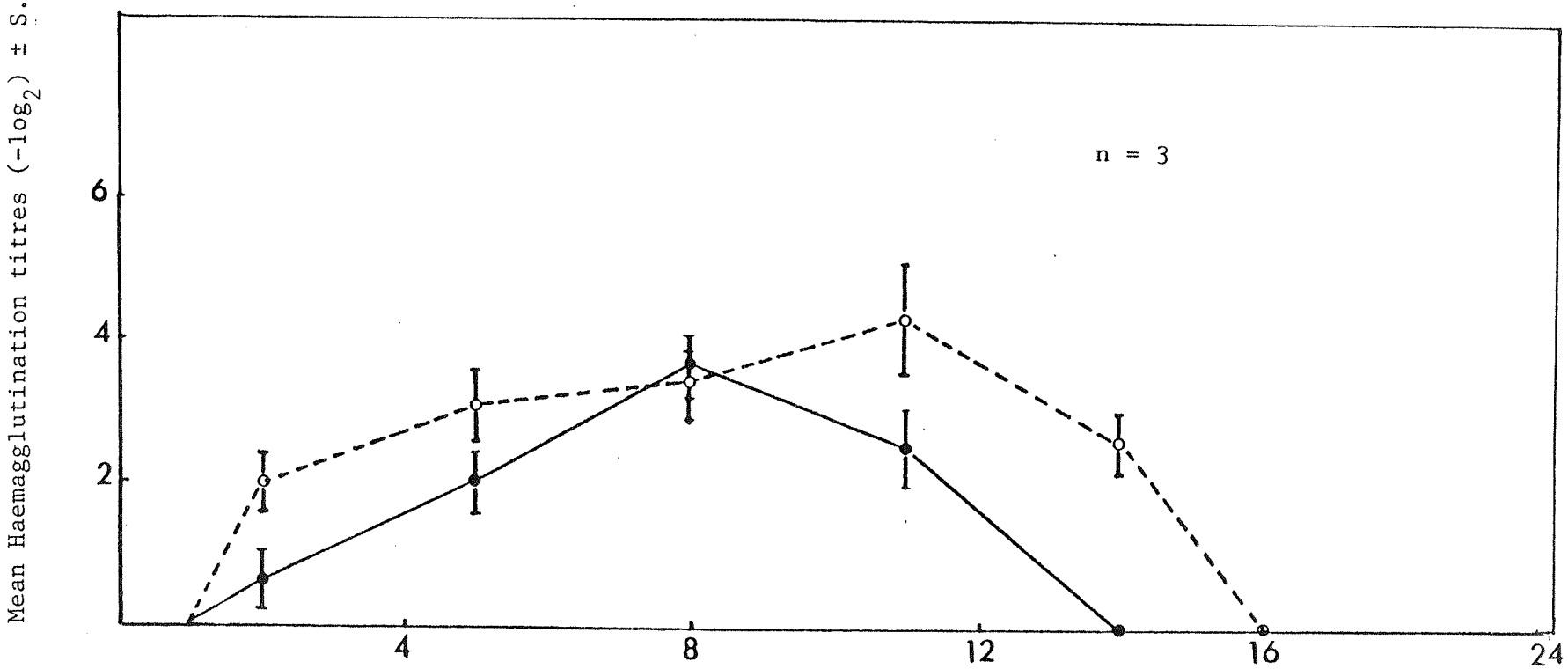
Secondary Response to Sheep Red Blood Cells

Following a second injection of SRBC nineteen days after primary immunisation, haemagglutinating activity was detected two days after secondary challenge. The antibody titres recorded at this and later stages of the secondary response were generally higher than those recorded during the primary response and also persisted for slightly longer (Fig. 4.1)

4.3.2 Plaque Forming Cells

Different groups of immunised fish were sacrificed at 2, 5, 8, 11 and 14 days after injection with sheep red blood cells for detection of plaque forming cells in spleen and head kidney. The results are shown in Fig. 4.2. Both large and small haemolytic plaques were formed by lymphocytes from these organs. No background plaque forming cells were observed in the spleen and head kidney of saline injected controls. As shown in Table 4.12 in Appendix 11, increased mean numbers of plaque forming cells (40 and 50

Fig 4.1



Primary and secondary humoral immune response of adult Tilapia mossambica to SRBC (20%).

PFC/10^6 white cells respectively) were detected in both the spleen and head kidney on the second day after injection with 20% sheep red blood cells, and 100 and 120 PFC /10^6 white cells were detected in the spleen and head kidney respectively on the fifth day after immunisation. This increase continued until maximum mean numbers of PFC were observed. These were 407PFC/10^6 white cells for spleen in the eleventh day, and 3,352 PFC/10^6 white cells for head kidney on the eighth day. Plaque forming cell numbers in both organs subsequently declined to zero by the fourteenth day post-injection (Fig. 4.2).

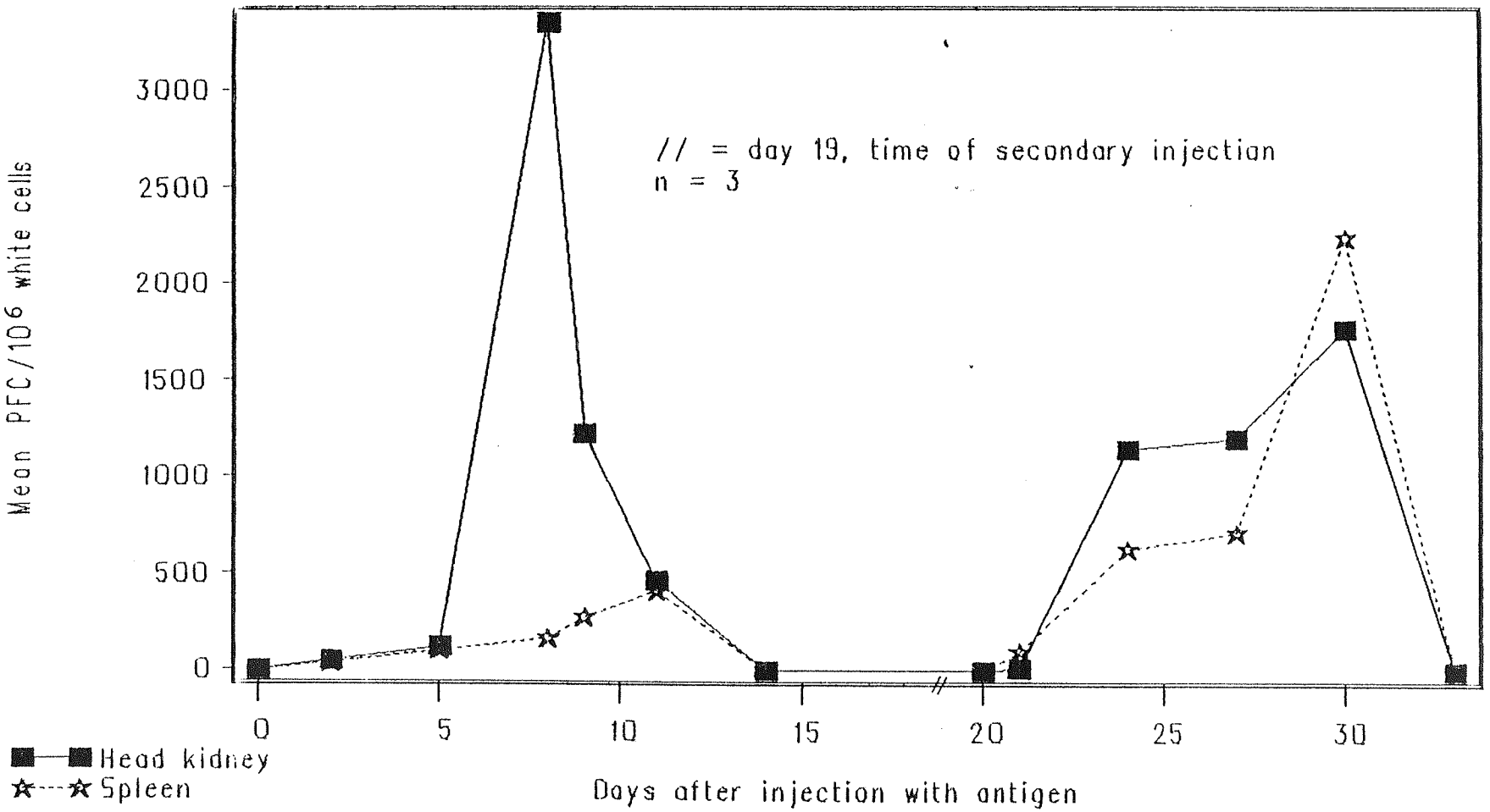
A second group of fish were subjected to a second injection of sheep red blood cells, nineteen days after primary immunistion, and were sacrificed on days 2,5,8,11, 14 and 16 after the second injection. As shown in Fig.4.2, the mean number of plaque forming cell was 628 PFC/10^6 white cells in spleen and 1,145 PFC/10^6 white cells in head kidney on the fifth day. The peak responses recorded were 2,240 PFC/10^6 white cells for spleen and 1,765 PFC/10^6 white cells for head kidney, both occurring on the eleventh day.

In these experiments there was a direct correlation between the time of appearance of plaque forming cells and serum antibody levels. The maximum number of plaque forming cells was observed in spleen three days after the peak serum antibody levels and on the same day in head kidney during the primary response. During the secondary immune response the maximum number of plaque forming cells was observed in spleen and head kidney on the same day as the peak serum antibody levels. The PFC response in head kidney after the first injection of SRBC was higher than that observed in spleen. However the opposite happened after the secondary injection of sheep red blood cells.

4.3.3 Production of Antigen Reactive Cells in Lymphoid Organs

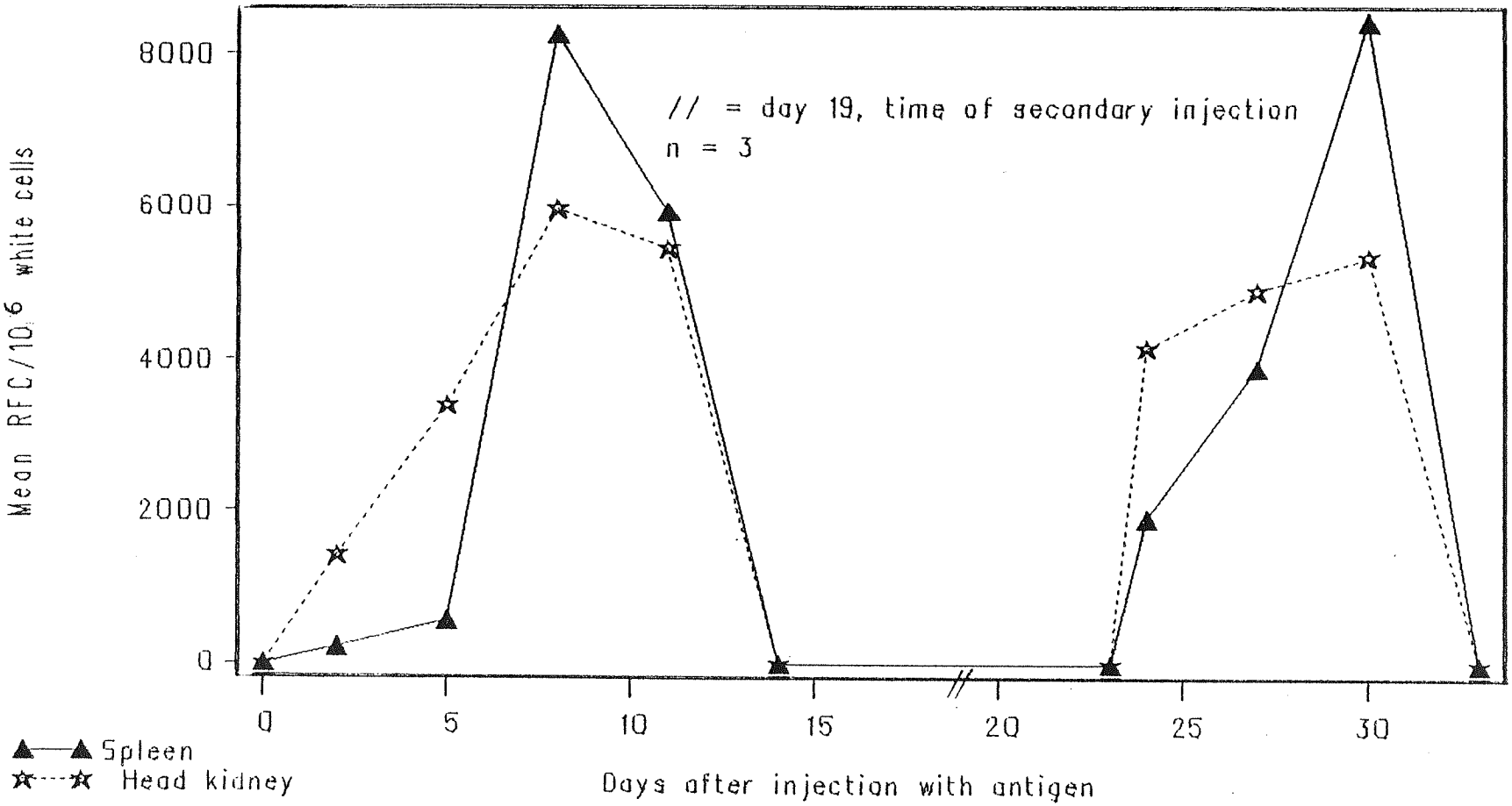
The sensitive immunocytoadherence assay was employed to detect total antigen reactive cells in lymphoid organs of immunised and control fish. In this experiment rosette forming cells were detected 2 days post immunisation, and the mean maximum number of rosettes observed during the primary response was 5,961 RFC/10^6 white cells in head kidney and 8,240 RFC/10^6 in spleen. The peak response for both organs was seen on the eighth day post immunisation (Fig. 4.3).

Fig. 4.2



Kinetics of plaque forming cells (PFC) in the spleen and head kidney of adult Tilapia mossambica following primary and secondary challenge with SRBC.
(Standard error is too small to be shown graphically).

Fig. 4.3



Kinetics of Rosette forming cells (RFCs) in the spleen and head kidney of Tilapia mossambica following primary and secondary challenge with SRBC.
(Standard error is too small to be shown graphically)

In the secondary immune response the mean maximum number of rosette forming cells was 8,406 RFC/10^6 white cells in spleen and 5,336 RFC/10^6 white cells in head kidney, and the peak response was evident on the eleventh day for both organs (Fig. 4.3).

4.3.4 Antibody Response to *Escherichia coli*

Figure 4.4 demonstrates the primary and secondary immune responses of adult *Tilapia mossambica* to *Escherichia coli*. Following primary challenge, the maximum titre (8) of bacterial agglutination for adult fish was seen in the 4th week post-injection and gradually declined to zero by week 6. In the case of the secondary immune response, the maximum titre achieved was 10 by week 3 post-challenge, thus giving a higher titre than that observed during the primary immune response. This titre gradually decreased to zero by week 6. There was no detectable reactivity of anti-*Escherichia coli* serum to *Klebsiella aerogenes*. Control fish injected with saline were all negative for bacterial agglutinins.

4.3.5 Hyperosmotic Administration of *Escherichia coli*

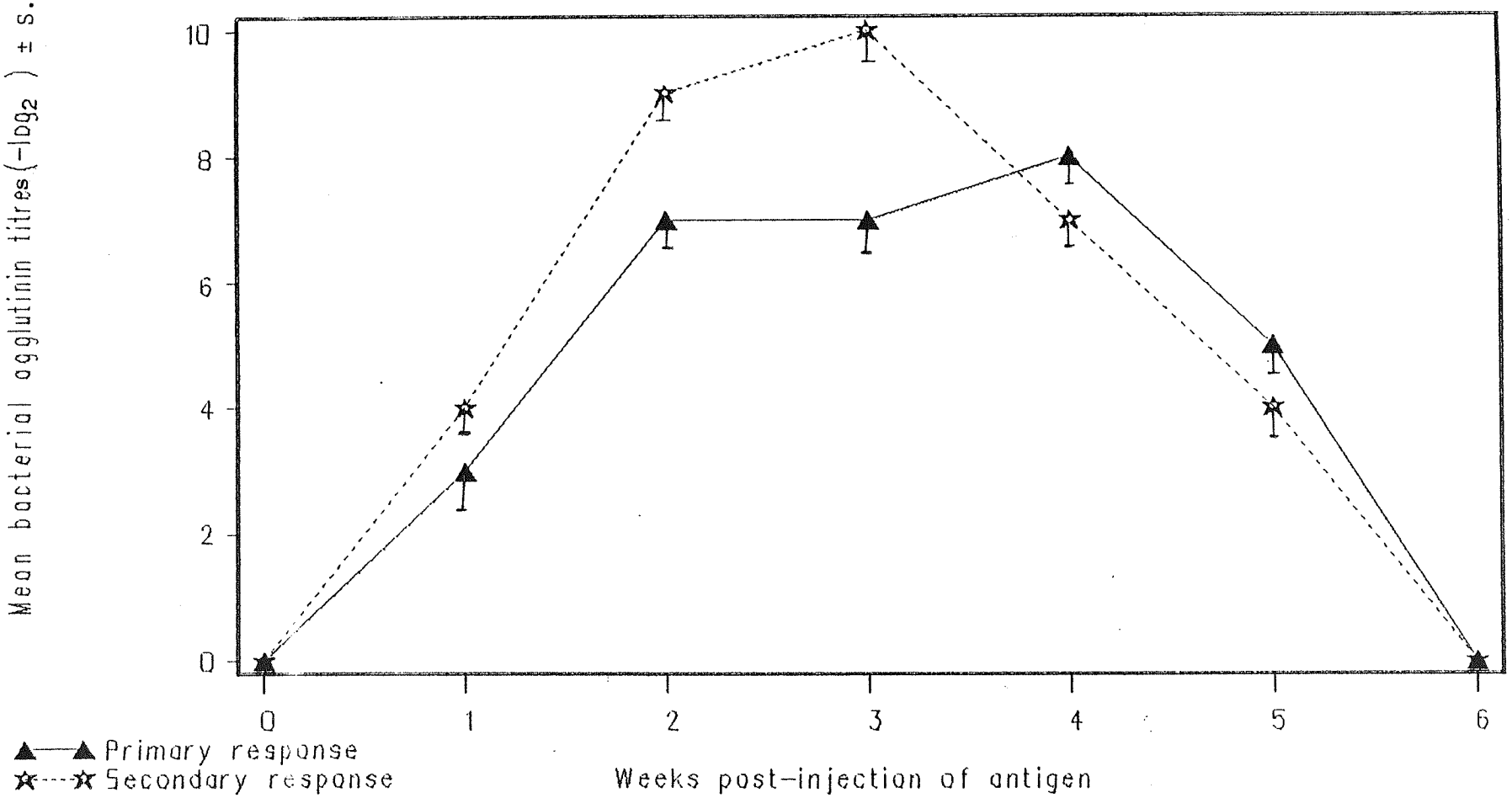
Escherichia coli administered by hyperosmotic infiltration produced only a poor serum antibody response. Titres did not exceed 3.2 ($-\log_2$) at any time, and were certainly less than the titres seen following immunisation via the intraperitoneal route (Table 4.1).

Table 4.1

Antibody Titres of *Tilapia mossambica* Immunised with *Escherichia coli* by Hyperosmotic Infiltration

Day Tested	7	14	21	28	35	42	49	56
Mean of Ab titres expressed as $-\log_2$	1.5	2.3	3.2	3.2	2 .8	1.7	1.0	0.8

Fig 4.4



Primary and secondary humoral immune response of adult Tilapia mossambica to Escherichia coli.

4.3.6 Antibody Response to Human Gamma Globulin (HGG)

The antibody response to HGG as measured by passive agglutination of latex particles is seen in Fig. 4.5. During the primary response maximal agglutinin titres were seen 1 - 2 weeks post injection and declined slowly to zero by the fourth week post challenge. Following secondary challenge with HGG, maximal antibody titres were double those seen during the primary response, but again declined to zero by the fourth week post immunisation. Antibody responses for both *E. coli* and HGG were slower to develop and more persistent than those recorded for SRBC.

4.3.7 Ontogenesis of Humoral Immunity in Fry

The response to SRBC

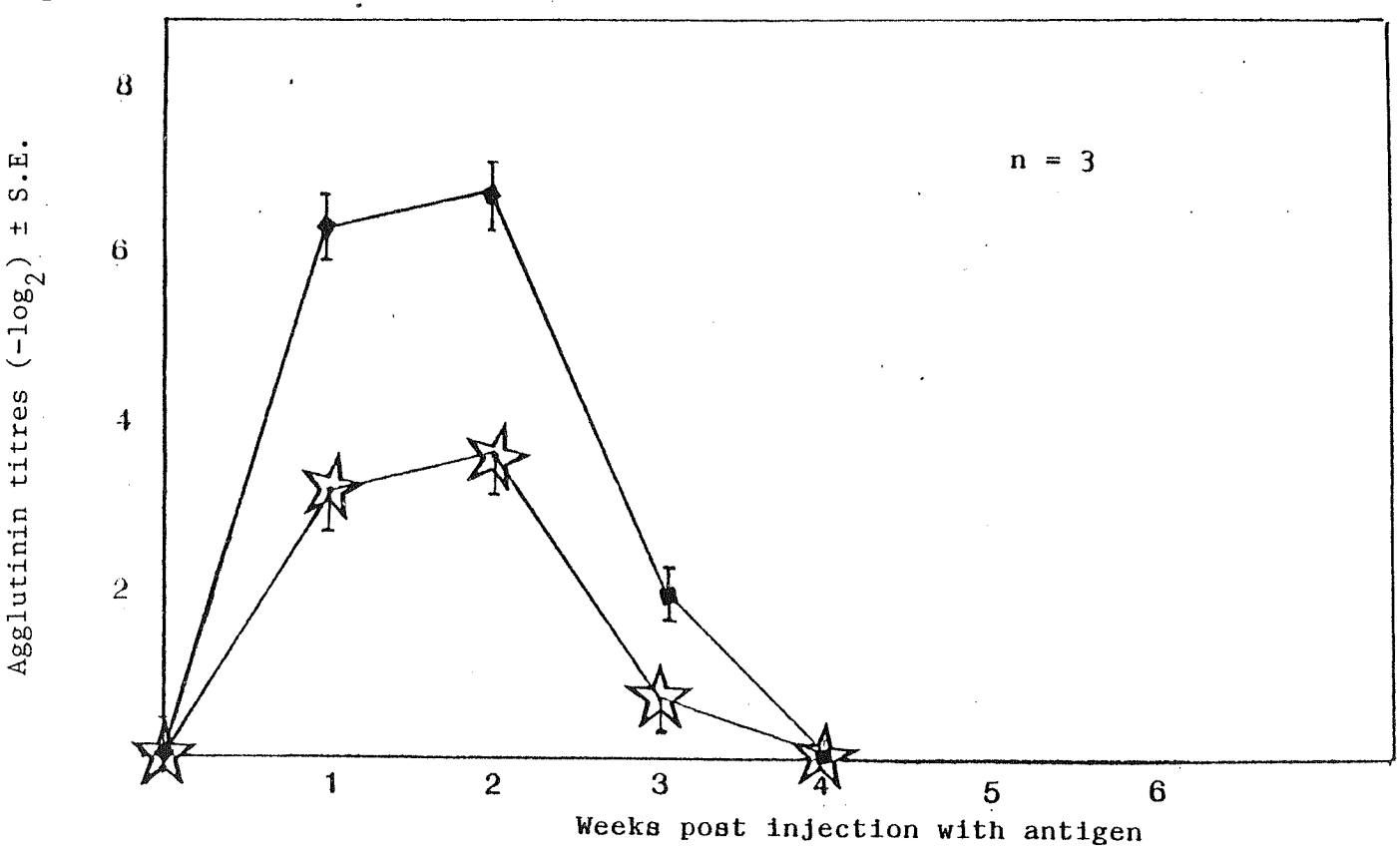
The humoral immune response to SRBC following antigenic challenge at different times during ontogeny is summarised in Table 4.2. A single injection of SRBC at 10 weeks of age failed to elicit detectable haemagglutinin titres in the sera of fry examined over a period of six weeks post injection. Similarly, antigen injections at 10 weeks and 7 days later also failed to promote an anti-sheep red blood cell response. Only fry receiving SRBC injections at 10 weeks and 12 days later were able to produce antibody against this antigen. The response was slower to develop and more persistent than in adults. Specific haemagglutinin titres were first detected in this group from the first week post challenge and after achieving a peak titre of (6.98) at two weeks post-injection, slowly declined to background levels over the next three to four weeks (Fig 4.6).

Antibody specificity was tested against rat red blood cells, mouse red blood cells and chicken red blood cells but no cross-reactivity was detected. Saline injected controls gave no detectable antibodies against SRBC.

PFC in Fry Immunised at 10 weeks of age with SRBC

Because of the difficulties experienced in obtaining adequate numbers of lymphoid cells from fry, the PFC response was only studied at 14 days post SRBC injection. This time was chosen to coincide with the maximal observed titre of haemagglutinating antibodies in the serum (Fig. 4.6). Table 4.3 shows the results of experiments on four

Fig 4.5



Primary and secondary humoral immune response of adult Tilapia mossambica to human gamma globulin

Table 4.2

Ontogenetic Emergence of the Antibody Response of Fry to SRBC

Number of Fish	Time of injections [1] in days			Antibody Response [2]					
	0	7	12	1	2	3	4	5	6 weeks [3]
30	√			-	-	-	-	-	-
30	√	√		-	-	-	-	-	-
30	√		√	[4] +	+	+	+	+	+

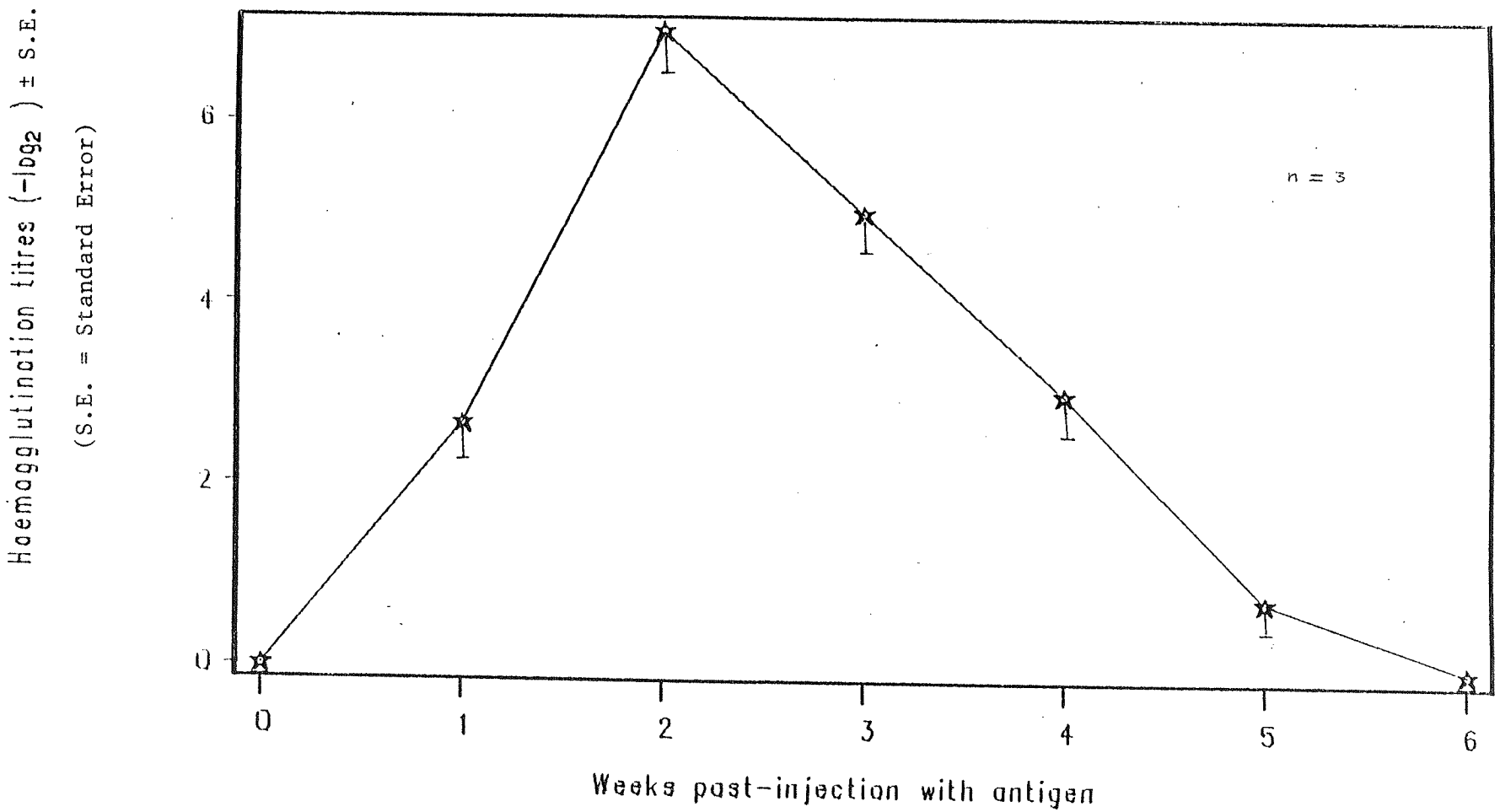
1 Time 0 = 10 weeks of age

2 As assessed by haemagglutination test

3 Weeks after final injection of antigen

4 Individual results are shown in Fig.4. 6

Fig 4.6



Primary immune response of Tilapia mossambica fry (10 weeks old) to sheep red blood cells.

Table 4.3

Plaque Forming Cell Response in Spleen and Head Kidney of Fry 14 Days after Immunisation with SRBC

Antigen used for Testing PFC	Mean PFC/10^6 white cells of treated spleen ± S.D.	Mean PFC/10^6 white cells of treated head kidney ± S.D.	Mean PFC/10^6 white cells of control spleen ± S.D.	Mean PFC/10^6 white cells of control head kidney ± S.D
SRBC	220 ± 30	178 ± 35	0.73 ± 0.33	1.07 ± 0.45
SRBC	260 ± 48	233 ± 43	0.93 ± 0.33	1.27 ± 0.42
SRBC	241 ± 54	162 ± 36	1.62 ± 0.24	0.34 ± 0.09
SRBC	251 ± 60	182 ± 38	1.36 ± 0.53	2.91 ± 0.92
RRBC	62 ± 14	73 ± 16	0.07 ± 0.07	0.20 ± 0.11
MRBC	-	-	-	-
CRBC	-	-	-	-
Total Mean for SRBC only	243 ± 17	188.75 ± 31	1.16 ± 0.4	1.3 ± 0.4

groups of fry. The mean number of PFC/10^6 white blood cells in the spleen was 243 ± 17 PFC/10^6 white blood cells and in the head kidney the mean number was 188 ± 31 PFC/10^6. No PFC were observed against mouse and chicken erythrocytes, but small numbers of PFC were seen when spleen and head kidney cells from SRBC immunised and control fry were incubated with rat red blood cells.

RFC in Fry Immunised at 10 weeks of age with SRBC

RFC in fry were measured at the same time as PFC assays were carried out. As shown in Table 4.4, the mean number of RFC/10^6 white blood cells in the spleen was 29 ± 17 RFC/10^6 white blood cells and in the head kidney the mean number was 23 ± 10 RFC/10^6 white blood cells. No RFC were observed against rat, mouse and chicken erythrocytes.

The response of *Escherichia coli*

Table 4.5 shows that, as with SRBC, a single injection of bacteria at 10 weeks of age did not stimulate antibody production. However fry challenged with *Escherichia coli* at 10 weeks and 12 days later did produce detectable bacterial agglutinins as detailed in Fig. 4.7. These antibodies were specific for *Escherichia coli* as judged by their lack of cross-reactivity with another bacterial species (*Klebsiella aerogenes*).

The effects of FCA on the anti-bacterial response are seen in Table 4.5 and Fig 4.7. Antibody titres were higher in the group of fry injected with *E. coli* in FCA emulsion than in the group receiving bacteria in saline alone. The highest agglutination titre in this group was 11 compared to a maximal titre of 8 in the group injected with bacteria in saline. Non-immunised control fry produced no humoral reaction against *Klebsiella aerogenes* but did exhibit low background titres of agglutinin to *E. coli* (Fig. 4.7).

The response to Human gamma globulin (HGG)

The data presented in Table 4.6 show that whilst 5 week old fry failed to respond to HGG challenge, fry challenged at 10 weeks and 12 days later with the same antigen produced detectable antibody titres as assessed by passive haemagglutination of

Table 4.4 Rosette Forming Cell Response in Spleen and Head Kidney of Fry (14 days) after Immunisation with SRBC

Antigen used for Testing RFC	Mean RFC/10^6 white cells of treated spleen ± S.D.	Mean RFC/10^6 white cells of treated head kidney ± S.D.	Mean RFC/10^6 white cells of control spleen ± S.D.	Mean RFC/10^6 white cells of control head kidney ± S.D
SRBC	32 ± 4	23 ± 4	0.33 ± 0.16	0.07 ± 0.07
SRBC	50 ± 7	36 ± 6	0.28 ± 0.08	0.33 ± 0.16
SRBC	26 ± 5	19 ± 3	0	0
SRBC	9 ± 2	12 ± 2	0	0
RRBC	-	-	-	-
MRBC	-	-	-	-
CRBC	-	-	-	-
Total Mean for SRBC only	29.25 ± 16.9	22.5 ± 10	0.152 ± 0.17	0.1 ± 0.14

Table 4.5

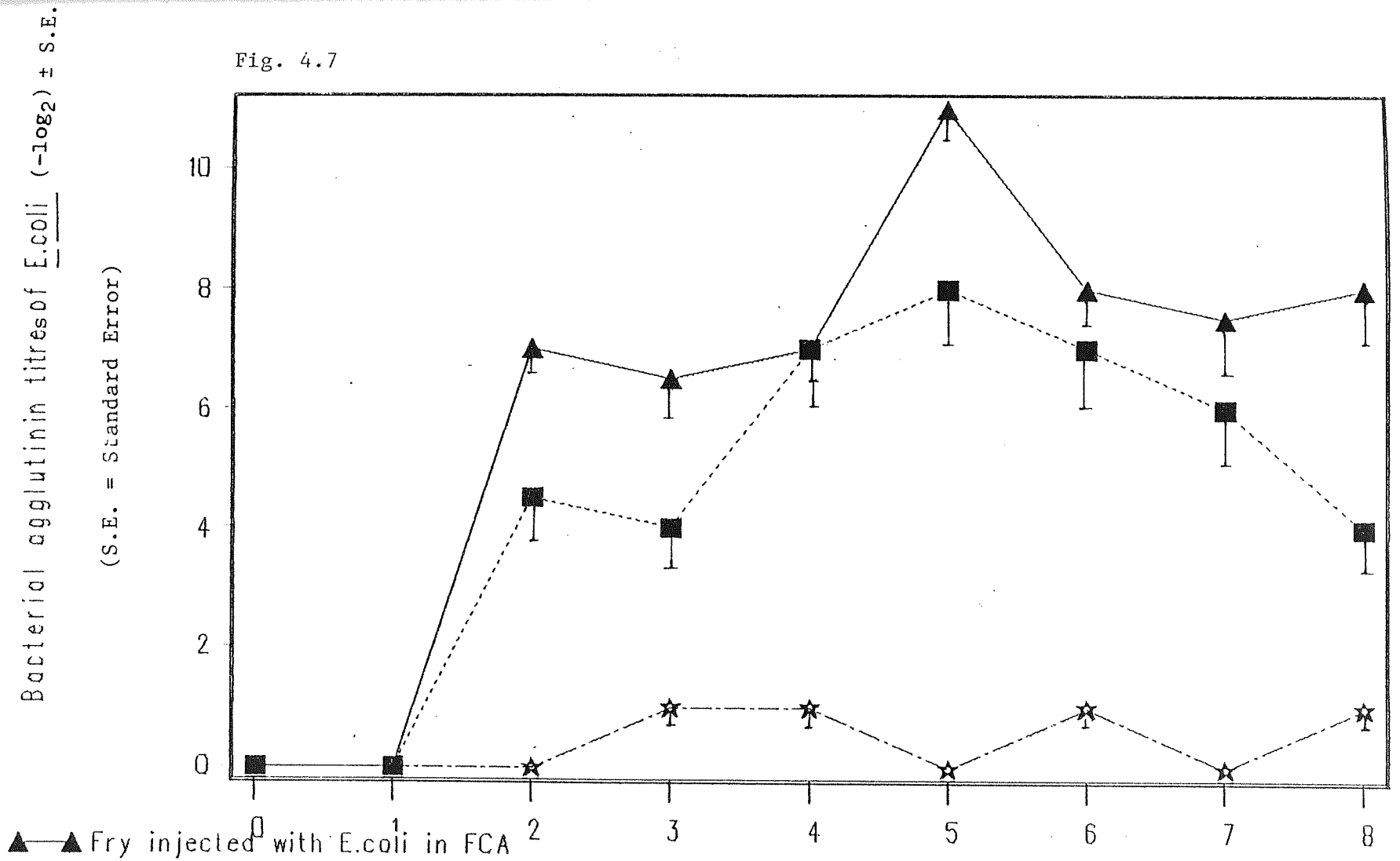
Ontogenetic Emergence of the Antibody Response of Fry to *Escherichia coli*

Number of Fish	Time of injections[1] in days	Antibody Response [2]							
	0 7 12	1	2	3	4	5	6	7	8 [3]
30	✓	-	-	-	-	-	-	-	-
30	✓ ✓	+ [5]	+	+	+	+	+	+	+
30	✓+ FCA [4] ✓+FCA	++ [5]	++	++	++	++	++	++	++

1 Time 0 = 10 weeks of age
2 As assessed by agglutinating antibody
3 Weeks after final injection of antigen
4 +FCA = with Freund's complete adjuvant
5 Individual results are shown in Fig. 4.7

- = No response
+ = Response
++ = Higher response

Fig. 4.7



Effect of adjuvant on immune response of Tilapia mossambica fry to Escherichia coli.

Table 4.6

Ontogenetic Emergence of the Antibody Response of Fry to Human Gamma Globulin

Number of Fish	Time of injections[1] in days			Antibody Response [2]							
	0	7	12	7	14	21	28	35	42	49	56[3]
15 *	✓		✓	-	-	-	-	-	-	-	-
15	✓		✓ 4	+	+	+	+	+	+	+	+[6]
15	✓+ FCA		✓ +FCA[5]	++	++	++	++	++	++	++	++[7]

1 Time 0 = 10 weeks of age
N.B. * For this group time 0 = 5 weeks of age
2 As assessed by passive agglutination of latex particles
3 Days after final injection
4 Saline injected antigen
5 +FCA = with Freund's complete adjuvant
6 Individual results are given in Fig.4.9
7 Individual results are given in Fig. 4.8
8 Antibody response of non-immunised control fish were negative

HGG-coated latex particles. Administration of HGG in FCA emulsion produced higher agglutinin titres than injections of saline dissolved antigen. The highest titre obtained against HGG administered in FCA was 13.7 (day 28) as shown in Fig 4.8 compared with a maximal titre of 7.2 (day 28) against HGG dissolved in saline (Fig. 4.9). Non-immunised control fish produced no antibodies against HGG.

Long Term Effects of Early Exposure to Antigen

SRBC

Table 4.7 shows that groups of fry given either a single injection of SRBC at 10 weeks or two injections of the same antigen at 10 weeks and 12 days later, failed to respond to a further challenge with SRBC 56 days after the first injection. These findings contrast with those for two groups of fry which were challenged with SRBC for the first time at 126 days of age (10 weeks + 56 days) which both produced heightened levels of haemagglutinins (Fig. 4.10) represents one of these two last groups.

Escherichia coli

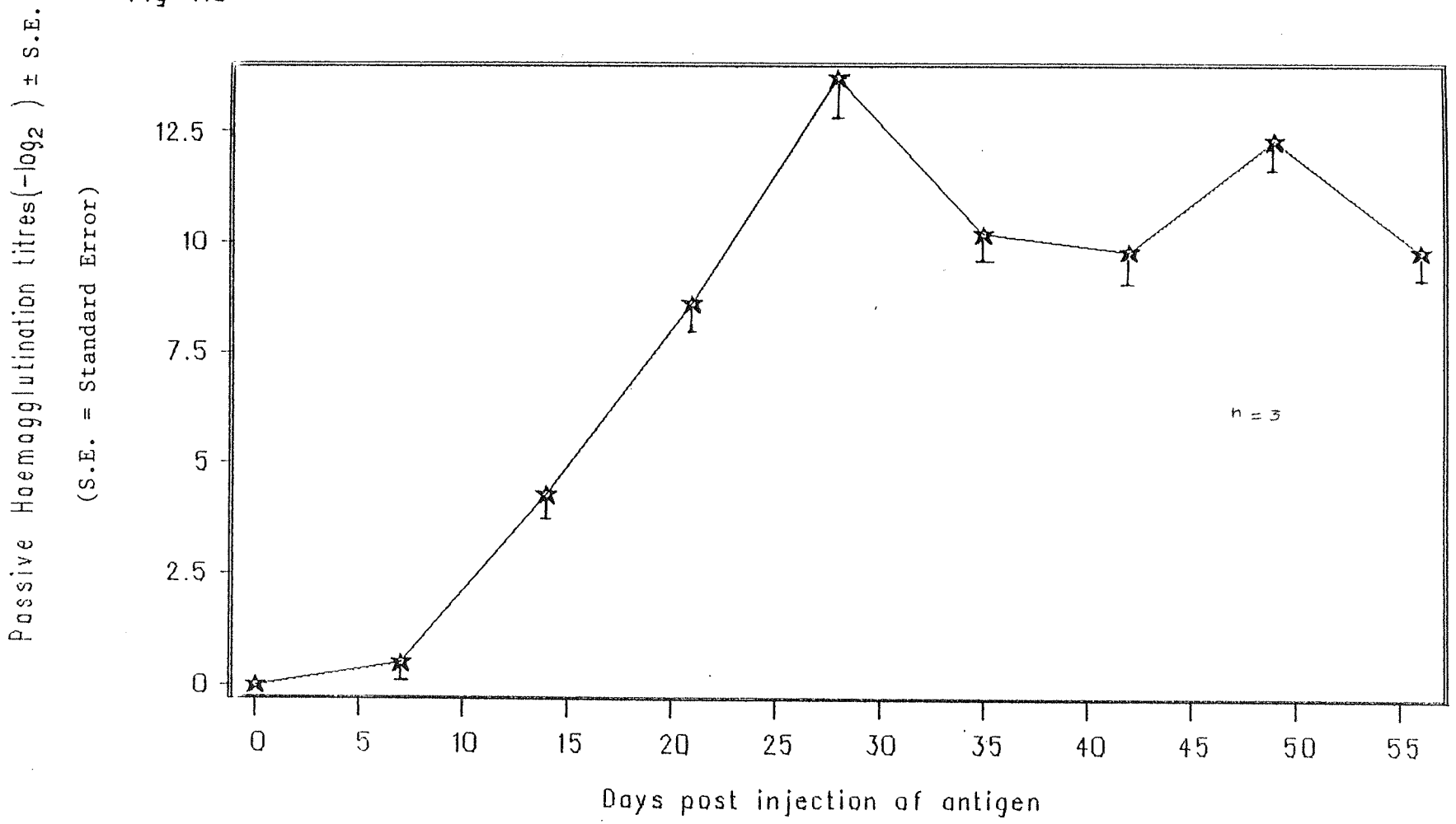
The effects of early exposure to *Escherichia coli* on responses to the same antigen in later life are summarised in Table 4.8. An injection of bacteria (*E. coli*) at 10 weeks followed by two more injections 12 days and 56 days later resulted in a prolonged antibody response which was first detectable one week after the final injection and persisted for about 8 weeks post injection when observations ceased as outlined in Fig. 4.11. A similar pattern of antibody response was seen when the first and final injections of bacteria were administered in FCA/saline emulsion. In this case, the maximal agglutination titres were nearly the same as those observed for fish injected with bacteria in saline as shown in the same figure.

4.3.8 Histological and Proliferative Changes following Exposure to Antigen

Imprints

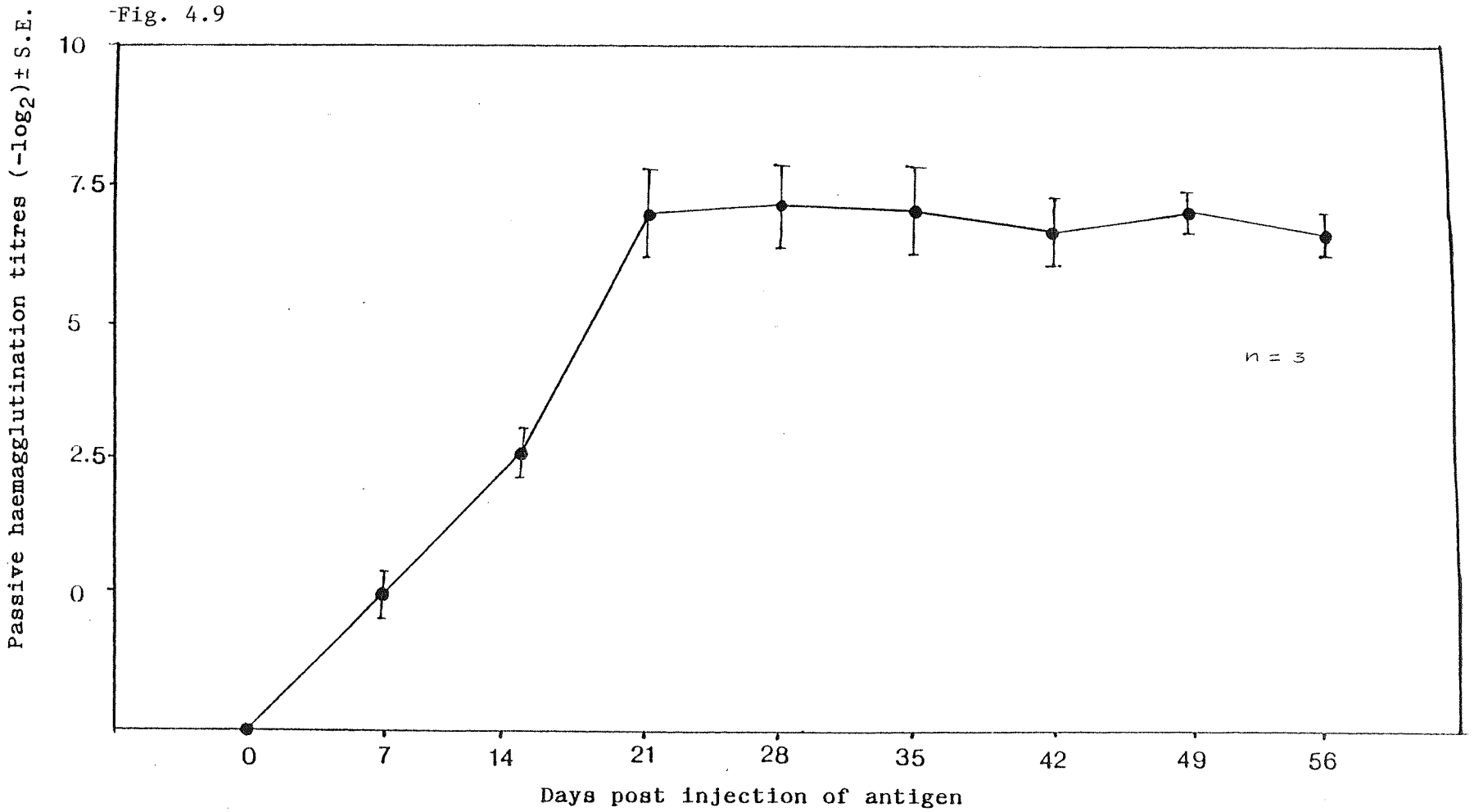
Cellular imprints taken from spleen, head kidney and thymus of SRBC injected fish were examined at intervals following tritiated thymidine injection. Studies over a four day

Fig 4.8



Antibody response of fry injected at 10 weeks of age and 12 days later with HGG in FCA.

Fig. 4.9



Antibody response of fry injected at 10 weeks of age and 12 days later with HGG in saline.

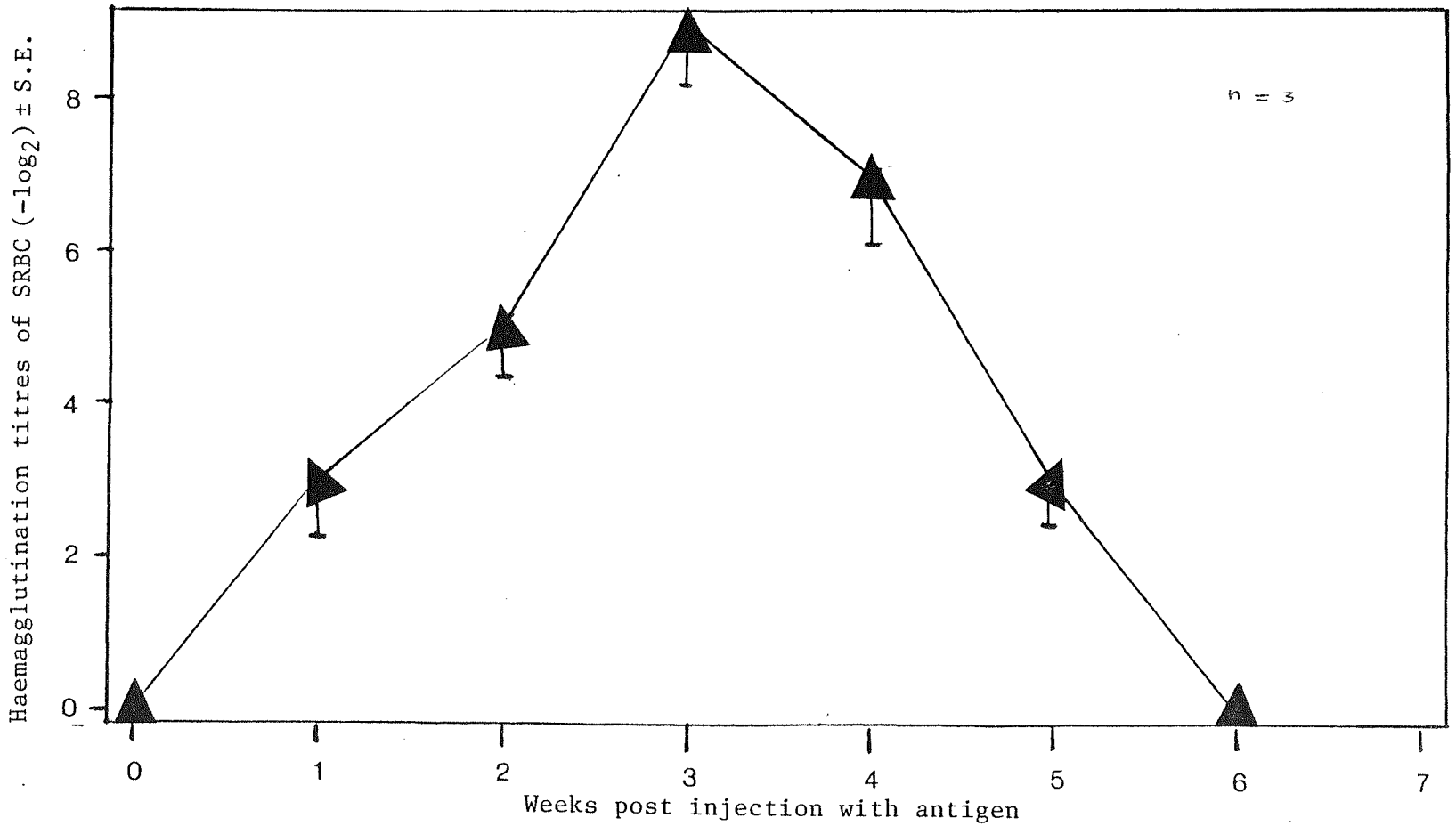
Table 4.7

Tolerogenic Effects of Early Exposure to SRBC

Number of Fish	Time of injections [1] in days			Antibody Response [2]				
	0	12	56	1	2	3	4	5 weeks [3]
30	✓		✓	-	-	-	-	-
30			✓	+	+	+	+	+
30	✓	✓	✓	-	-	-	-	-
30			✓	+	+	+	+	+ [4]

1 Time 0 = 10 weeks of age
2 As assessed by haemagglutination test
3 Weeks after final injection of antigen
4 Individual results are shown in Fig.4. 10
N.B. - = no response
+ = response

Fig. 4.10



Antibody response of Tilapia mossambica fry immunised at 126 days of age to SRBC.

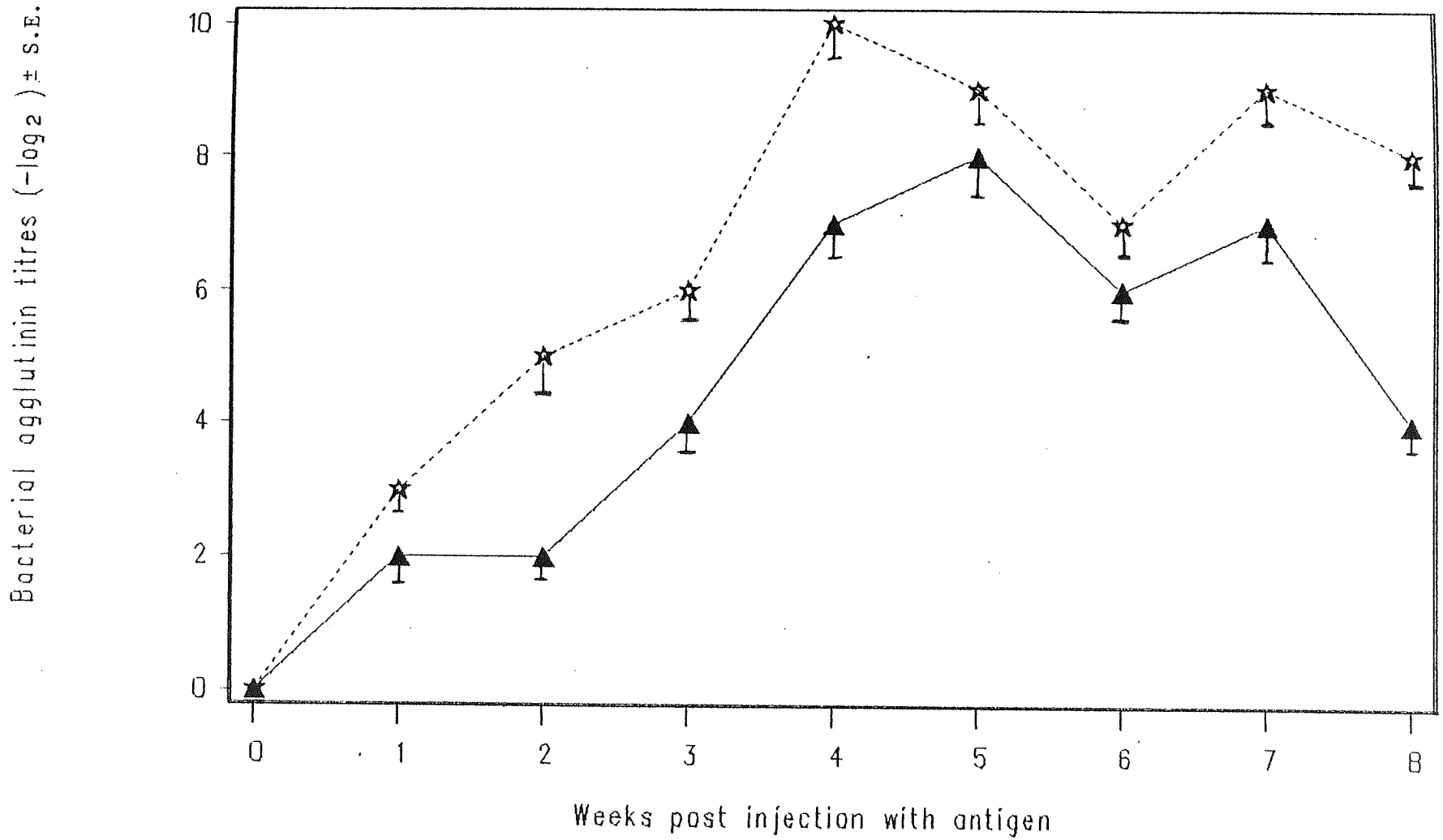
Table 4.8

Long Term Effects of Early Exposure to *E. coli*

Number of Fish		Time of injections[1] in days			Antibody Response [2]							
		0	12	56	1	2	3	4	5	6	7	8 [3]
30	4	✓	✓	✓	-	-	-	-	-	-	-	-
30	5	✓	✓	✓	+	+	+	+	+	+	+	+ [7]
30	6	✓+FCA		✓ +FCA	++	++	++	++	++	++	++	++ [7]

1 Time 0 = 10 weeks of age
2 As assessed by slide agglutination test
3 Weeks after final injection
4 Control fish injected with saline
5 Fry injected with *E. coli* in saline on day 0, 12 & 56
6 Fry injected with *E. coli* in FCA on day 0 & 56
7 Individual results are shown in Fig 4. 11

Fig. 4.11



The effect of adjuvant on the immune response of fry to E.coli

▲—▲ Without adjuvant
☆···☆ Using Freund's complete adjuvant
S.E.= Standard Error
n = 3

period after the injection of radioisotope revealed heavy labelling of cells. Imprints of head kidney cells taken at 1 - 2 days post thymidine administration were so heavily labelled that quantitative counts were not possible. Indeed it was often difficult to precisely quantify the percentage of labelled cells in imprints treated for autoradiography.

Figure 4.12 shows an imprint taken from the head kidney three days after tritiated thymidine injection and ten days after injection with SRBC. Large numbers of labelled cells are present but it is difficult to discern any histological detail of the cells involved. They are presumed to be lymphocytes in the process of cell division. Both spleen and head kidney imprints showed increased levels of labelling, when compared with imprints taken from non-immunised saline injected animals.

Autoradiographical Studies

Figure 4.13 illustrates a section of the spleen from a fish injected with SRBC 7 days prior to administration of tritiated thymidine. Four days after injection of the radioisotope, heavy concentrations of label are evident throughout the spleen., The label occurs in localised areas throughout the spleen and may reflect accumulations of dividing cells. A similar pattern of labelling after tritiated thymidine was seen in sections of head kidney. Particularly high levels of label were seen on the 3rd day after tritiated thymidine injection. Labelling was low or absent in spleen and head kidney of saline injected control fish.

Labelling in the thymus of both control and SRBC injected fish was generally restricted to zones of cells just below the thymic capsule. There was no apparent increase in label in the thymus of the immunised fish.

In general the heavy concentrations of label observed made it difficult to examine histological details of labelled cells.

Histology of Lymphoid Organs in Immunised Fish

Histological sections of spleen and head kidney from HGG and *Escherichia coli* injected fish were examined following staining with methyl green pyronin. The results obtained with this stain were variable, but in well stained sections it was possible to discern clusters of pyroninophilic cells in both spleen and head kidney of immunised fish.

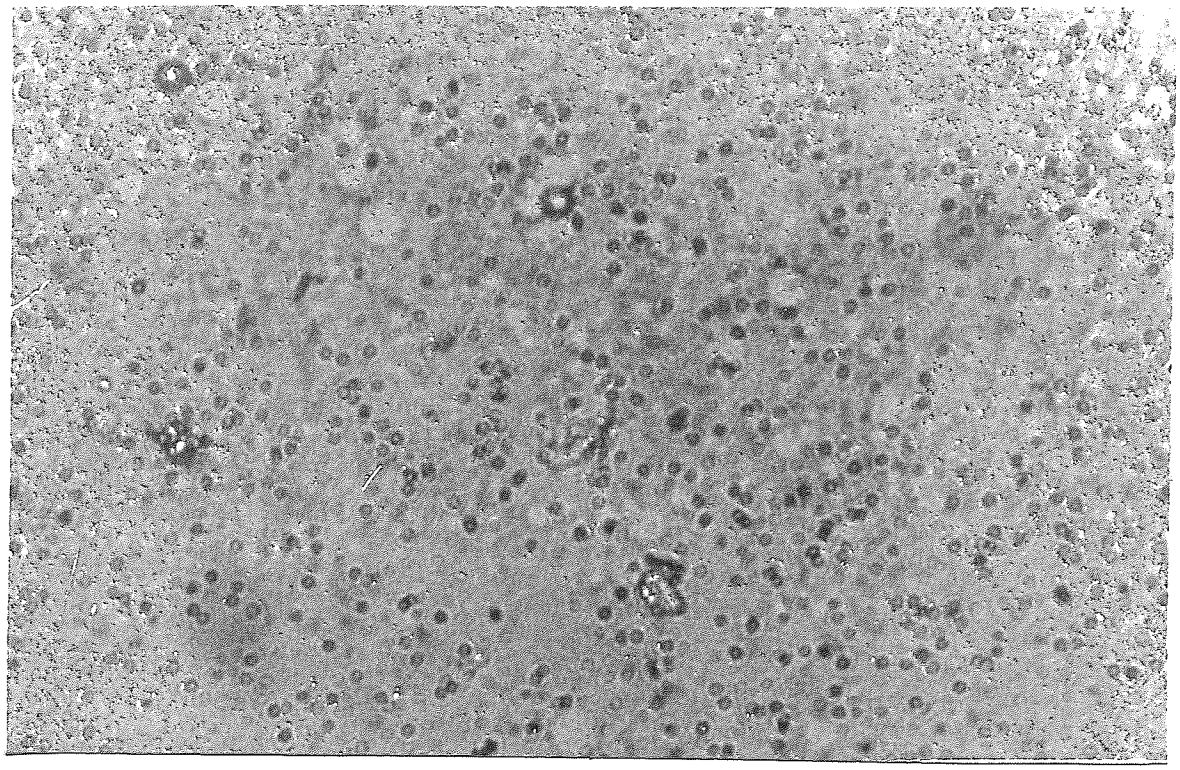


Fig. 4.12 Autoradiograph of Kidney imprints from fish immunised with 20% SRBC and killed 3 days after $3^{+}H$ thymidine injection. Note, the silver grains labelling the division of cells appearing as dark spots. Magnification 512. Stain - Methyl green pyronin
Exposure time is 4 weeks (as in Appendix 7).

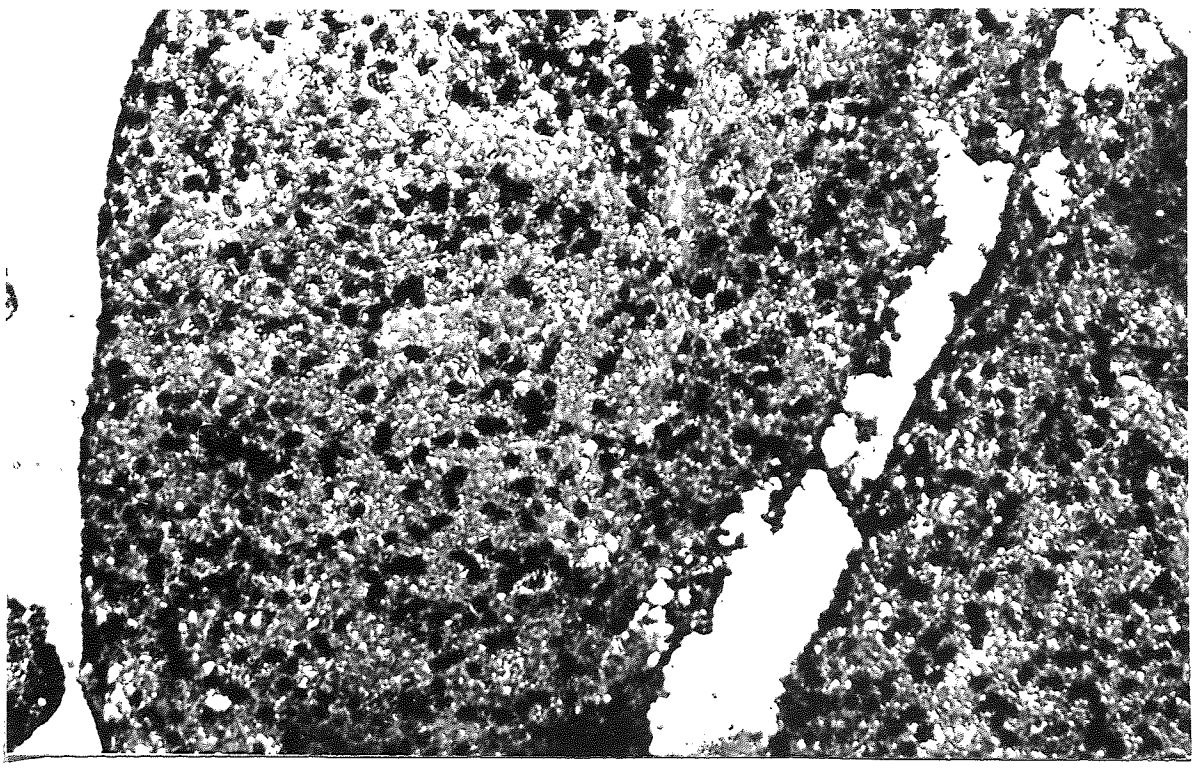


Fig. 4.13 Autoradiograph of spleen section from fish, immunised with 20% SRBC and killed 4 days after $3^{+}H$ thymidine injection. Note the presence of labelled cells.
Magnification 400. Stain - Methyl green pyronin.
Exposure time is 4 weeks.

A comparison of spleen and head kidney sections taken from immunised fish over a period of 5 weeks revealed that pyroninophilia was more pronounced in the cells of the head kidney. Pyroninophilic cells were detected one week after immunisation and persisted in the head kidney for up to five weeks.

The level of pyroninophilia in the spleen was more variable and in some immunised fish, no pyroninophilic cells were detected at all. Studies on fish at 4 and 5 weeks post antigen challenge revealed little or no pyroninophilic staining in the spleen. Levels of pyroninophilia in the spleen and head kidney of control fish were low or absent. The thymus of both control and immunised fish showed little or no evidence of pyroninophilic staining (Table 4.13 - Appendix 12).

4.3.9 Proliferation in Spleen of Adult *Tilapia* 7 days after Injection of SRBC

The following (Table 4.9) shows the results of experiments on ^{3}H-thymidine incorporation *in vitro* by spleen cells of three SRBC injected fish and three saline injected control fish as assessed by scintillation counting.

Statistical analysis using Student's t-test confirmed that the stimulation indices observed in experiments 2 and 3 were significant ($p = < 0.05$).

Table 4.9

Proliferation in Spleen of Adult *Tilapia*

Experiment	Mean counts per minute in spleen cells from SRBC injected *Tilapia mossambica*	Mean counts per minute in spleen cells from control uninjected *Tilapia mossambica*	S.I.
1	717 ± (269)	444 ± (100)	1.6
2	291 ± (20)	124 ± (01)	2.3
3	228 ± (13)	141 ± (6)	1.6

- Each count represents three suspension aliquots from one individual
- S.I. is the stimulation index
- S.E. standard error given in parentheses.

4.3.10 Mixed Leucocyte Culture

The results of this reaction as shown below in Table 4.10. The stimulation indices observed for the three experiments ranged from 1.4 to 4.2.

Table 4.10

Mixed Leucocyte Reactivity in Spleen and Pronephros of *Tilapia*

Exp	Type of cell suspension	Stimulation Index S.I.
1	$pro_1 + pro_2$	1.4
	$sp_1 + sp_2$	1.5
2	$pro_1 + pro_2$	1.75
	$sp_1 + sp_2$	4.2
3	$pro_1 + pro_2$	1.6
	$sp_1 + sp_2$	1.4

pro = pronephros
sp = spleen
All S.I. were significant ($P = < 0.05$) as assessed by student's t-test

4.3.11 Scale Transplantation

Table 4.11 (in Appendix 10) shows the results of allograft rejection studies on adult *Tilapia*. The mean survival time for first set grafts was (6.6 ± 1.2) days. Second set grafts were always rejected in acclerated fashion and gave a mean survival time of (4.8 ± 0.6) days.

4.4 DISCUSSION

This study describes the kinetics of primary and secondary immune responses to sheep red blood cells, *Escherichia coli* and HGG in *Tilapia mossambica* adults and fry of 10

weeks old. The humoral immune response to SRBC was studied by injecting the animals with SRBC and the peak response was determined by counting the numbers of plaque forming cells (PFC/10^6 WBC) and measuring haemagglutinating antibodies in serum. For adult *Tilapia*, anti-sheep red blood cell antibodies appeared within two days after immunisation at 32°C and in the primary response, the peak response for haemagglutination occurred on day 8 post-injection. A second injection of the same antigen always elicited a higher level of antibody response, and the peak titre was reached on the eleventh day. The secondary response was also more prolonged than the primary response. The individual variability of antibody titres reported here is typical of immunological responses in poikilothermic vertebrates. The titres were also much lower than those recorded for mammalian species (Manning and Turner, 1976). Mohan (1977) reported that the humoral immune response to SRBC in *Tilapia* was dose dependent, however the present study did not examine a range of antigen doses.

In 1975 Corbel, concluded that immunocompetent cells in teleosts in general are localised not only in spleen but also in the thymus and head kidney. This observation is in accordance with the findings of the present study. Although plaque forming cells were not studied in the thymus, they were detected in the spleen and head kidney of *Tilapia* within 48 hours of immunisation. The appearance of a maximum number of plaque forming cells in the spleen and head kidney of immunised adult animals coincided with the time of the peak serum antibody response. The primary response in adult *Tilapia mossambica* showed a maximum of (407 ± 0.5 PFC per 10^6 WBC) on day eleven using spleen cell suspensions and (3,352 ± 1 PFC) per 10^6 WBC on day 8 using cell suspensions from the head kidney. This figure is comparable with those reported for other teleost fish such as *Salmo gairdneri* (165 PFC per 10^6 WBC) (Smith et al. 1967) and *Perca fluviatilis* (848 PFC per 10^6 WBC) (Pontius and Ambrosius, 1972). A second injection with the same antigen elicited a higher PFC response (5-fold increase) in the spleen at day 11 and a similar (4 fold increase) in the head kidney. This heightened secondary reaction illustrates the immunological nature of the PFC response. The occurrence of plaque forming cells in spleen and head kidney following immunisation with foreign erythrocytes has also been reported for blue gill (Smith and et al. 1967) rainbow trout (Chiller and et al., 1969) and

perch (Pontius and Ambrosius, 1972). In these species, plaque forming cells appeared four to six days after immunisation and peaked by about the fourteenth day at 17°C or by the tenth day at 20°C. In the present study, the peak response of plaque forming cells in *Tilapia* at 32°C was found to be more rapid, possibly because the fish were maintained at a higher temperature. In the present study the number of antibody secreting cells represented by PFC/10^6 WBC was higher in the spleen than in the anterior kidney and this result is similar to those obtained by others (Ambrosius & et al., 1971; Chiller et al., 1969; Pontius and Ambrosius, 1972). One possible explanation for this difference is that the anterior kidney contains both lymphoid and excretory cells in some teleosts. However, in *Tilapia* there is little or no excretory tissue in the adult head kidney (see Chapter 3 and Sailendri, 1975).

The measurements of rosette forming cells (RFC) gives a more complete picture of cellular reactivity in lymphoid organs than the plaque forming cell assay, since the rosette forming cell assay detects any immune cells which have the appropriate surface receptors for antigen, and these will presumably include T-equivalent lymphocytes as well as B-cells which have not yet begun to secrete antibody. Evidence for an increase in antigen reactive lymphocytes in both spleen and head kidney is provided by the immunocytoadherence studies carried out here. Thus, the SRBC immunised animals showed a dramatic increase in rosette forming cells when compared with control animals. As expected, the numbers of rosette forming cells observed were far in excess of the numbers of plaque forming cells recorded, and provided further evidence that both spleen and head kidney are major sites of immunological reactivity.

The thymic dependence of PFC and RFC has not been widely studied in fish. However, thymectomy throughout the first three weeks of larval life in the amphibian, *Xenopus laevis* affected the heterologous red cell responses of young adults. No plaque forming cells were observed in thymectomized toadlets. Moreover, use of the immune cytoadherence assay, indicated a complete absence of induced responsiveness to SRBC in the spleens of thymectomized toadlets, even in those animals thymectomized as late as 22 days of age. (Horton, Rimmer and Horton, 1976).

In mammals, the hapten carrier effect has been used to demonstrate interactions between T-helper cells and B-cells in antibody production (Raff, 1970). In teleosts, hapten-carrier studies have also been performed and have suggested the presence of two functional populations of lymphocytes : one of which responds to the carrier, the other producing antibodies to the hapten (Stolen and Makela, 1975; Yocum, Cuchens and Clem, 1975). Whether the helper population is thymus dependent in fish has not been clearly established. Experiments by Warr and et al,. (1977) investigated this tissue in the goldfish (*Carassius auratus*) and demonstrated that the hapten primed cells were found only in pronephros and not in thymus. There have been few thymectomy studies in fish. Sailendri, (1975) was unable to demonstrate any impairment of humoral immunity to SRBC following adult thymectomy in *Sarotherodon (Tilapia) mossambica.* However unpublished observations by Jayaraman (cited in Jayaraman et al., 1979) claim to have demonstrated impairment of thymus dependent (SRBC) antibody production in adult thymectomized *Tilapia* 2 - 3 months later. Responses to a thymus- independent antigen (polyvinyl pyrrolidine) were unaffected. Further studies - particularly on fish thymectomized early in life - are required to fully elucidate the role of the thymus in antibody responses.

Two methods of administration were employed in the present study to investigate the response of *Tilapia mossambica* to the bacterial antigen *Escherichia coli.* Intraperitoneal injection was used for both adult and younger fish and the second method, hyperosmotic infiltration, was used for adults only - the latter gave a very weak response in comparison to the first route of injection. The effect of hyperosmotic infiltration, especially in young fish, needs to be carefully examined to determine the risks of inducing tolerance by early exposure of the lymphoid system to antigen. Also, the possible effects of stress in young fry subjected to hyperosmotic infiltration needs to be investigated as it is known in mammals that corticosteroid hormones can cause involution and destruction of the lymphoid tissues (Burnet, 1962).

A number of workers have used pyroninophilic staining in lymphoid organs as an indication of immune reactivity in lower vertebrates (Secombes, 1981). The presence of pyroninophilia is thought to be related to the presence of large amounts of cytoplasmic

RNA, and therefore an indication of active protein synthesis. It is a characteristic of protein secreting cells such as plasma cells. Immunisation of *Tilapia mossambica* with *Escherichia coli* in saline, elicited pyroninophilia in pronephros and spleen during the subsequent immune response. In this experiment the number of pyroninophilic cells in pronephros was higher than that in the spleen. The increase in pyroninophilic cells in the pronephros and mesonephros roughly concides with the first appearance of circulating antibody, plaque forming cells and rosette forming cells during the first week after antigenic challenge. In other poikilotherms, such as the frog, *Xenopus laevis* (Turner and Manning, 1973) a large increase in pyroninophilic cells, but not plasma cells, was noted after immunisation. At the same time the numbers of pyroninophilic cells increased for about 4 weeks, during which time some loose clusters of cells were formed. Secombes (1981) suggested that the large pyroninophilic cells seen in carp during an immune response were just a proliferative phase. Horton and et al., (1977) demonstrated that the spleen is a major site of alloimmune reactivity in *Xenopus*, and reported that examination of well stained autoradiographs revealed that large pyroninophilic cells were invariably labelled, suggesting that these lymphoid cells are a proliferating population. Turner and Manning (1973) suggested that the pyroninophilic cells appearing after immunisation may be involved in antibody secretion. Fish, like all poikilotherms, lack germinal centres however, the pyroninophilic cell clusters seen in the mesonephros following HGG injection in saline, and those seen in the pronephros after injection with HGG and with *Escherichia coli*, appeared to be sites of cellular reactivity. These cell clusters do not occur until after the time when antibody can first be detected in the serum and in some cases they do not appear at all, even though antibody production is certainly taking place. So, they are not the sole producers of antibody and may not be involved in antibody production at all. In the present study on *Tilapia mossambica* and in studies on *Cyprinus carpio* (Secombes, 1981) large numbers of pyroninophilic cells were seen by the third week after immunisation. Pyroninophilia was accompanied by other marked cellular changes including an increase in the number of lymphocytes in the splenic red pulp this increase in cell numbers occurred following injection of both SRBC and HGG.

The increased pyroninophilia reported here was accompanied by evidence of cellular proliferation as revealed by autoradiographic and scintillation counting studies. These confirm that the spleen and head kidney are major sites of immune reactivity in *Tilapia mossambica*. High levels of ^{3}H-TDR labelling were apparent in imprints of thymus, spleen and head kidney of fry following antigenic challenge with SRBC. In general the highest levels of label were seen in head kidney (pronephros) followed by spleen cells and thymus in descending order of labelling. In many slides it was difficult to accurately assess the numbers of labelled cells and indeed some slides were so heavily labelled that it was impossible to perform labelled cell counts. In the autoradiographic experiments, increased levels of label were seen also in the lymphoid organs of fry (head kidney and spleen) following challenge with SRBC. In the spleen these studies revealed labelling of cells in both white pulp and amongst the lymphoid accumulations of the red pulp. Labelling in head kidney of *Tilapia* was observed in lymphocytes throughout the kidney tissue, however, labelling in the thymus of immunised fry was restricted to the peripheral zone and was not noticeably higher than that seen in control fish except in a few slides. Scintillation counting experiments were only carried out on adults, since it was difficult to obtain enough cells for *in vitro* labelling from fry. These studies revealed increased levels of proliferation in the spleen of antigen injected fish when compared with those of saline injected controls. The proliferative changes reported here for the spleen are similar to those reported elsewhere in other poikilotherms (Turner and Manning, 1973; Horton and et al., 1977).

The effects of administration of antigen using FCA was also studied in *Tilapia,* and it was found that the antibody titres obtained following injection of *Escherichia coli* in FCA were higher and persisted for longer than titres obtained by injection of the same antigen without FCA. The higher titres and longer duration of the antibody response using FCA are probably the result of a number of factors. In mammals, adjuvants are known to enhance the immune response in a number of ways. Antigen in emulsion is more resistant to dispersal and can therefore be released over a prolonged period of time to stimulate antibody production. FCA also contains extracts of *Mycobacterium tuberculosis* which are known to stimulate the immune response, possibly via the induction of soluble,

non-specific, macrophage products such as Interleukin-I, (Roitt, Brostoff and Male, 1985). Krantz et al., (1963) reported that high antibody titres to *A. liquifaciens* in brown trout (*Salmo trutta*) persisted for two years after administration of vaccine emulsified in an oil adjuvant. Antibody titres in fry of the same species, (to the same antigen suspended in FCA) persisted for more than eight months, whereas the antibody titre obtained after the injection of the same antigen with saline solution was lower and began to decline after only three months (Khalifa and Post, 1976). In 1974, Paterson and Fryer reported similar results using *A. Salmonicida* and juvenile coho salmon (*Oncorhynchus Kisutch*). Secombes,(1984) employed a different approach to antigen presentation and concluded that priming with immune complexes can under certain circumstances be as effective as priming with adjuvant. However, it was also stated that the use of immune complexes in place of adjuvants may be limited by the type of antigen used for immunisation. Results obtained from studies on antigen presentation may help in understanding factors which promote long term immunity in fish and may therefore be of considerable importance in establishing successful vaccination procedures.

In addition to studying the immune responses of adult fish, the experiments presented here extend our knowledge of the ontogenesis of immunological competence in *Tilapia mossambica*. A single antigenic challenge at 10 weeks of age or earlier failed to provoke a humoral immune response to SRBC and *E. coli*. Fry challenged with two injections of HGG at 5 weeks of age and 12 days later also failed to respond positively. However a first antigenic challenge at 10 weeks of age followed by a second injection twelve days later resulted in the production of PFC in spleen and head kidney, together with detectable serum agglutinin titres. Taken together these results would seem to indicate that the ability to respond immunologically to a range of antigens appears sometime between 10 weeks of age and twelve days later. For SRBC at least, the emergence of immunocompetence may be pinpointed to a stage later than 11 weeks of age since injections at 10 weeks and 7 days later also failed to elicit a humoral immune response.

By 10 weeks of age in the present study, *Tilapia* fry possessed a spleen with small lymphocytes and well differentiated red and white pulp, together with structurally mature thymus and head kidney which, despite being smaller, showed most of the features of the

adult lymphoid organs (Chapter 2). The lack of immune responsiveness following a single antigenic challenge at 10 weeks may have been the result of too low an antigen dose. However, it may indicate that even though small lymphocytes are present in all the major lymphoid organs, this in itself does not signify the aquisition of full immunological competence in *Tilapia*. In this respect, it would be important to determine when surface immunoglobulins first became apparent on *Tilapia* lymphocytes during ontogeny. As in mammals, these molecules may be important in antigen recognition.

It is clear that there is a good deal of variability between fish species with regard to the onset of detectable immunological competence. This is also true of higher vertebrates. The developmental stage at which immunocompetence to different antigens appears varies from species to species. Solomon,(1981) has proposed the concept of an "immunological clock", whereby each species acquires the ability to respond to particular antigens at a certain stage during ontogeny. For mice, the point at which the ability to respond to a given antigen appears to vary between different inbred strains and appears to be related to the possession of different MHC antigens (Rowlands, Collins, Raab and Bonner, 1979). For amphibians, Horton,(1969) correlated the appearance of the allograft response with the maturation of the thymus gland, and in sequential thymectomy studies (Horton et al., 1977) was able to demonstrate that thymectomy at different stages of development resulted in differential impairment of cell mediated and humoral immune responses.

Further studies are required in order to establish whether such a strict developmental heirarchy of antigen responsiveness exists in *Tilapia* and other teleosts.

When compared with adult *Tilapia* , the immune responses seen in younger fish were slower to develop. Studies of PFC in fry revealed a longer latent period prior to the detection of antibody producing cells in spleen and head kidney. Furthermore, total numbers of PFC observed in immunised fry were less than those seen in adults. One unusual feature of the humoral immune response of fry when compared with that of older fish, was that the response in fish immunised as fry was often more persistent than that observed in the adult. A similar phenomenon was reported by Khalifa and Post,(1963) for brown trout fry. Although the most persistent titres resulted from the injection of antigen suspended in FCA, fry injected with antigen in saline only, gave responses which only began to decline after three months.

In studies on the effects of early exposure to antigen on long term responsiveness of *Tilapia,* there was a marked contrast between the data obtained for SRBC and *E. Coli*.. Fry immunised with SRBC at 10 weeks of age and twelve days later gave no detectable antibody response following secondary challenge 56 days after the first injection. Earlier studies presented here have shown that the period during which the first injections were carried out corresponds to a time when the ability to respond to different antigens, including SRBC, is first emerging. The possibility must be considered that exposure to antigen during this period may give rise to a form of immunological tolerance in later life. In mammals it is well established that exposure of the immature lymphon to antigen can result in specific persistent immunological tolerance (Humphrey & Frank, 1976); Howard & Mitcheson, 1975; Howard, 1979). This apparent induction of tolerance in young *Tilapia* exposed to SRBC at 10 weeks and 12 days later is seemingly at variance with our earlier findings that SRBC challenge at these times elicited both PFC and humoral antibody production when observed immediately after the second injection. It may be that the ability to respond to SRBC emerges at slightly different ages in different batches of fry. Further studies are required to clarify this issue.

Tolerance induction following early exposure to SRBC has also been described by Van Loon et al.,(1981) in carp. They were able to demonstrate tolerance induction to SRBC in carp injected at 4 weeks of age and challenged with a second injection some 3 months later. However, the induced unresponsiveness may not be very long-lasting since by 5 or 13 months after the first injection a secondary challenge induced a normal primary reaction (Van Muiswinkel). In adult carp a stage specific and long-lasting tolerance can be induced at low temperature in fish previously injected with a high dose of soluble antigen (Serero and Avtalion, 1978; Avtalion et al., 1980; Avtalion, 1981). The factors which might possibly lead to tolerance induction in young fish therefore require further investigation.

In marked contrast to the findings for SRBC, early exposure at 10 weeks and 12 days later to *E. coli* as an antigen gave rise to prolonged increased titres of antibody following secondary challenge in later life (56 days after 1st injection). The results obtained here for *E. coli* may be compared with a study carried out by Khalifa and Post,(1976) on the

immune response of brown trout to *Aeromonas liquifaciens*. They too were able to demonstrate that exposure to bacterial antigens in early life resulted in the production of persistent antibody titres against the eliciting antigen. In their study, high titres of antibody against *A. liquifaciens* persisted for eight months. In the present study observations ceased at 4 months through lack of animals but high titres of antibody against *E. coli* were still being recorded.

The studies carried out here represent preliminary findings on the long term effects of early antigenic challenge to the fish immune system. However, even from the limited range of immunogens employed here, it is apparent that different antigens can produce quite different effects on adult immunity when introduced during early life. The implications of such findings for studies on fish vaccines are far-reaching , since challenge with a vaccine at an inappropriate stage during ontogeny could result in tolerance rather than immunity.

Cell mediated immunity in adult *Tilapia* was studied here by examining the rejection of scale transplants and by examining the *in vitro* proliferative activity of spleen and head kidney cells in the mixed leucocyte culture (MLC). Time did not permit more extensive studies on adults nor was it possible to study these reactions in fry, but it is hoped that the results reported here may prove useful in establishing techniques for further examination of CMI in *Tilapia* . Adult *Tilapia* were able to reject first set allografts in acute fashion (MST 6.6 ± 1.2 days) and demonstrated accelerated second set rejection times (MST 4.8 ± 0.6 days) when re-challenged with scales grafted from the original donor. Rejection times for third party grafts were not examined and the specificity of the immunological memory demonstrated here has therefore not been confirmed. The rejection times reported here correspond closely to those described for *Tilipia* by Sailendri,(1973). Rejection times in fry have not been examined, but Sailendri has shown that 1.5 month old *Tilapia* rejected grafts more slowly than adults. By 4.5 months of age, however, rejection times were similar to those seen in adult fish. Six month old carp display a fully developed allograft response (Rijkers and Van Muiswinkel, 1977) and Botham et al.,(1980) have shown that allograft responsiveness is present in carp fry at only 16 days of age. These results suggest that fish develop allograft responsiveness early in life.

The development of the allograft response in the amphibian *Xenopus laevis* has been correlated with the appearance of small lymphocytes in the thymus gland (Horton, 1969), and it is now well established that graft rejection is abrogated or severely impaired in this species by early thymectomy (Horton and Manning, 1972; Tochinai and Katagiri, 1975; Rimmer and Horton, 1977). Few thymectomy studies have been carried out on fish, but Sailendri,(1973) reported that adult thymectomy of *Tilapia mossambica* had no effect on allograft and antibody responses, although the removal of the thymus in young adult *Tilapia* at 2 months of age completely abolishes the response to SRBC. Prolonged allograft survival has been reported in *Tilapia* thymectomized at four months of age (Muthukkaruppan and Sailendri in preparation). Grace,(1981) found that rainbow trout thymectomized at 2 months of age and allografted one month later, showed impaired rejection times in comparison to non-thymectomized controls.

In mammals the mixed leucocyte culture (MLC) test has become a major technique for the *in vitro* analysis of the major histocompatibility complex and its gene products. The magnitude of the mixed lymphocyte response (MLR) or stimulation is determined by the degree of MHC disparity between responder and stimulator cells. The use of MLC in fish has met with varying degrees of success. McKinney et al.,(1976) consistently failed to obtain a positive MLR in cell cultures from snappers, gars and sharks. Cells from Salmonid fish, however, do seem to be capable of responding in MLC. Etlinger et al., (1977) obtained a positive MLR with peripheral blood leucocyte cultures from brown trout. Ellis,(1977b) reported a positive MLR in salmon fry and described the ontogeny of the reaction. The maximum stimulation index (~2) was relatively small, but nevertheless considered to be positive. In the present study, pronephric and splenic lymphocytes of *Tilapia* responded positively in two way MLC. The stimulation indices obtained were low, but similar to those reported in other teleost fish. Although the number of fish tested were small, this is believed to be the first demonstration of MLC reactivity in *Tilapia mossambica*. The MLC test may provide a suitable method for detecting the onset of cell mediated immune reactions in small fry. This approach has been successfully employed by Ellis,(1977b) in salmon fry.

CHAPTER 5

CHAPTER 5

GENERAL DISCUSSION

A study of the histogenesis of the lymphoid organs presented in this thesis, showed that in *Tilapia* the thymus was the first organ to become lymphoid at 5 days post-hatching. Lymphocytes were first seen in the head kidney at 14 days of age but were not seen in the spleen until about 5 weeks of age. The spleen was slow to mature and at 5 weeks of age was still predominantly erythroid in nature. By ten weeks the major lymphoid organs had increased in size, and there was some demarcation of the thymus into cortical and medullary zones and the spleen had begun to exhibit distinct areas of red and white pulp. These findings are broadly similar to those described for *Tilapia* by Sailendri,(1973).

The present study was unable to shed any light upon the origin of stem cells in Tilapia, but as in most other teleosts the thymus is the first organ to develop (Manning and et al., 1982). Sailendri,(1973) suggested that thymocytes might migrate from the thymus to the head kidney along a lymphoid "bridge" between the two organs. No physical bridge was observed in the present study, but lymphocytes were occasionally seen in the area between the thymus and head kidney. Ellis,(1977) suggested that in salmon, where the head kidney becomes lymphoid before the thymus, stem cells may migrate from the head kidney to the thymus during ontogeny. In the rainbow trout it has been suggested that perhaps both thymus and head kidney develop their own stem cell populations (Grace and Manning, 1980; Grace, 1981). Further approaches to determining the origins of stem cells in *Tilapia* might include an extension of the ultrastructural observations commenced here to include very early development of major lymphoid organs. This approach has been used to identify the early appearance of stem cells in amphibians (Rimmer, 1977; Tochinai & Katagiri, 1975). In order to observe the possible early migration of stem cells and immature lymphocytes cell markers may be of value (Secombes et al.,1983a,b). Early thymectomy of *Tilapia* fry might also prove useful in establishing the presence of thymus dependent and thymus independent pathways of lymphoid cell development.

Adult *Tilapia* were able to respond humorally to a range of antigens, to reject allografts and produce MLR's *in vitro*. Although the latter studies were only included as pilot experiments in adults, both techniques could be usefully applied to younger stages in order to provide further information about the onset of alloimmunity in *Tilapia*. Previous studies have revealed that alloimmune reactions to grafts could be detected at approximately six weeks of age, but that young *Tilapia* did not reject grafts as quickly as adults until they were four and a half months old (Sailendri, 1973; Sailendri and Muthukkaruppan, 1975). Ellis,(1977) has employed the MLR technique to study the onset of cell-mediated immunity in salmon. Similar studies may therefore be possible with younger stages of *Tilapia*.

The humoral response of *Tilapia* was assessed using a number of techniques and, following appropriate antigenic challenge, both fry and adult fish were found to give good primary and secondary responses to SRBC, *Escherichia coli* and HGG. Studies on PFC and RFC were correlated with the appearance of serum antibodies, and revealed that both spleen and head kidney were major sites of specific cellular reactivity and antibody production. Studies of PFC in adult thymus were not performed, but Sailendri and Muthukkaruppan,(1975) have previously demonstrated the presence of PFC in the adult thymus. (Unpublished studies in this laboratory have also shown the presence of PFC in the thymus of fry injected at 10 weeks of age and 12 days later). Cellular reactivity in spleen and head kidney was confirmed by histological observations and assessment of cellular proliferation using autoradiography and scintillation counting, and these observations serve to confirm that head kidney and spleen are important secondary lymphoid organs in teleosts.

Despite the fact that the differentiation of the major lymphoid organs is well advanced by 10 weeks of age, studies on the ontogeny of humoral immunity presented in Chapter 4 suggest that, for *Tilapia* the period between 10 and approximately 12 weeks of age represents a critical stage in the acquisition of antibody responsiveness to a number of different antigens. Thus, the ability to respond to foreign erythrocytes, bacteria and soluble protein antigen appears to emerge following two injections during this time period. Single injections at ten weeks of age failed to elicit positive antibody titres under the conditions described in this thesis.

Previous research on *Tilapia* (Sailendri, 1973; Sailendri and Muthukkaruppan, 1975) revealed that immunological reactivity to SRBC occurred at two and a half months after hatching, which is in line with the present observations. A survey of the onset of immunological competence in other teleosts reveals a good deal of individual variation. The humoral immune response to bacterial antigens appears to mature fairly quickly in a number of different fish species. In addition to the present studies on *Tilapia mossambica* fry, which showed persistent antibody titres following early exposure to *E. coli*; Tatner and Horne,(1983) showed that, in rainbow trout, it was possible to raise protective immunity to *Vibrio* bacteria following bath immersion as early as two weeks post-hatch. The same species was found to produce antibodies against *Aeromonas salmonicida* vaccine when immunised at three weeks post-hatch (Secombes, 1981) and also responded to *Aeromonas liquifaciens* approximately 23 days after the start of feeding - about 40 days of age (Khalifa and Post, 1976). Juvenile coho salmon were able to produce antibody against *Aeromonas salmonicida* when they achieved a body weight of 1.2 grams (Paterson and Fryer, 1974). Four week old carp, challenged by immersion in formalin killed *Aeromanas salmonicida* were able to give an improved secondary response when subsequently re-challenged (Mughal and Manning, 1985).

In constrast, rainbow trout failed to produce antibodies when challenged with HGG at three weeks of age (Secombes, 1981); although in another study they were able to respond to DNP-KLH (Dinitrophenol attached to keyhold limpet haemocyanin) by four weeks post hatch (Dorson, 1974). In carp, van Muiswinkel et al.,(1985) found that intramuscular injection of SRBC at 4 to 5 weeks of age did not result in PFC formation. They also observed that there was a lack of responsiveness if the fish were given a second injection 2 - 3 months later. However, this unresponsiveness or tolerance appeared transitory in nature, since a second injection of antigen five or thirteen months later, elicited antibody production.Van Loon et al.,(1981) also found that SRBC challenge gave no antibody reponse in four week old carp. Studies on the response to HGG in carp of the same age revealed that the antigen was tolerogenic if injected, but not if introduced by immersion. Injection of HGG at 9 - 10 weeks of age did not result in tolerance or immunosuppression (Mughal and Manning, 1985).

The results summarised above have some parallels with the studies presented in this thesis which showed that, in *Tilapia,* single injections of HGG or SRBC at 10 weeks of age were unable to elicit antibody production. Furthermore, in one experiment, injections of SRBC at this time and even two injections at 10 weeks and twelve days later, resulted in a lack of responsiveness when the fish were challenged with the same antigen 56 days after the first injection - a time when they would normally respond in a positive manner. By way of constrast, early exposure to bacterial antigen in the form of *Escherichia coli* , did not induce tolerance but gave rise to prolonged heightened titres of bacterial agglutinins. The reasons for the differences in responsiveness of young fish to bacterial antigens and other antigens such as HGG and SRBC is not known. However, Etlinger et al.,(1979) observed that juvenile rainbow trout gave poor responses to T-dependent antigens at a stage of development when they were already capable of producing antibodies against antigens which are T-independent in mammals. Many T-independent antigens are polyclonal activators with mitogenic properties and some, eg. L.P.S. (Lipopolysasaccharide) and POL (Polymerized flagellin), are derived from bacterial components. It is possible, therefore,that bacteria are able to trigger antibody production in fish by virtue of inherent mitogenic qualities not possessed by other antigens. Thymus independent antigens in mammals trigger IgM responses in B-cells without the need for T-cell "help", and it is tempting to speculate that this represents an ancient and efficient mechanism which first arose in primitive vertebrates, in order to promote a rapid agglutinating response against common determinants of bacterial cell walls and flagellae.

In amphibians, sequential thymectomy studies suggest that T-helper cells may emerge from the thymus after the appearance of alloreactive T-cells (Horton et al., 1977). Thymectomized amphibians are also capable of producing a humoral immune response against thymus - independent antigens (Collie, et al., 1975). Whether the same is generally true of fish is not known, but one study in *Tilapia* suggests that adult thymectomy does not affect the humoral immune response to PVP (polyvinyl pyrrolidone), a T-independent antigen (Jayaraman et al., 1979). Further studies are required to establish whether *Tilapia* (and teleosts in general) possess T-dependent and T-independent lymphocyte subsets.

The onset of humoral immunocompetence and alloimmune reactivity in *Tilapia* appears to occur later than that observed in a number of other teleost species, although even 6 - 7 month old grey mullet (*Chelon labrosus)* gave no detectable antibody responses following a single injection of HGG in adjuvant (Mughal and Manning, 1985). Grace, (1981) has suggested that the different times at which each species attains immunological competence probably depends upon their individual life styles. Thus, young stages of Tilapia are protected inside the mouth of the parent fish until about three weeks of age. Whether the young fry receive any passive immune protection during this time is not known, but if hatchings are to survive away from the parent, they require constant sterilization. According to Hildemann,(1962) at least one case of passive transfer of immunity has been recorded in the Amazonian discus fish (*Symphysodon discus*). The newly hatched fry feed upon parental skin mucous which is known to contain antibody and appears to confer resistance against bacterial infection. Whether similar mechanisms operate in mouth brooders such as *Tilapia* is not known.

Studies on the emergence of immunocompetence are important for the development of vaccination programmes involving immature stages of fish, since premature exposure to antigen has been shown to result in tolerance rather than the desired state of immunity. Of equal importance in optimising the immune response to antigens and vaccines are a number of other factors including the dose of antigen; its route of presentation; its persistence inside the body; and its physical state and manner and presentation. In mammals, variations in one or more of these factors can result in tolerance rather than immunity (Roitt et al., 1985). In recent years, fish immunologists have begun to study the importance of such variables in fish vaccination and, to the list above, may be added the effects of environmental temperature (Serero and Avtalion, 1978; Wishkovsky and Avtalion, 1982). The present work has made some preliminary studies on the effects of adjuvant on the immune response of *Tilapia* to HGG and *E. coli* and it is envisaged that future work will extend these observations. It is apparent from the present study that the administration of antigen in Freund's complete adjuvant leads to some enhancement of antibody production. However, the use of adjuvant in fish culture may not be viable since secondary effects of adjuvant may produce peritoneal lesions such as fibrosis and scarring which may affect the marketability of treated fish (Horne et al., 1983).

Pilot studies were also made of the efficacy of hyperosmotic infiltration of antigen compared with intraperitoneal injection. These studies were only applied to adult *Tilapia*, but it is proposed to extend the comparison to younger stages, particularly since injection of small fry is difficult and time consuming, and produces some mortality. A number of reports have shown that direct immersion in an antigen bath is an effective method for fish vaccination (Egidius and Anderson, 1979; Gould et al., 1979; Anderson and Dixon, 1980; Tatner and Horne, 1983). This method is particularly effective for small fish and promotes good protective immunity against bacteria in young salmonids at 1 gram body weight and greater (Johnson et al., 1982). However, Mughal and Manning,(1985) report that this method is unsuitable for soluble antigens in young carp, although good secondary responses were obtained following immersion of four week old carp in a bacterial vaccine.

A number of other methods of immunisation might also be investigated in *Tilapia* including oral administration (e.g. Ward et al., 1985; Mughal and Manning, 1985). Ward and coworkers found that antigen applied to food was able to produce immunity in rainbow trout, but required a large number of antigen administrations over a period of 30 days in order to produce acceptable levels of immunity to *Vibrio anguillarum*. Another means of immunising fish commonly used in the aquaculture industry is spray vaccination (Gould et al., 1978). Here large numbers of fish may be vaccinated by spraying bacteria over the body surface (Itami and Kusada, 1978; Rosenkrist-Jensen, 1982; Hockney, 1985). Together with bath immunisation, this method is unskilled and involves minimal handling and stress of fish (Pickering and Willoughby, 1982); it is therefore widely recommended for commercial vaccines.

The importance of temperature in the generation of immunity or tolerance could also be investigated in *Tilapia*. In addition to the effects of temperature on vaccine administration, such studies might prove useful in functionally dissecting out the different sub-populations of cells involved in "helper" and "suppressor" functions in *Tilapia*. (Although preliminary experiments by Avtalion et al., (1980) failed to produce tolerance in *Tilapia*). Such studies, together with an extension of the thymectomy studies carried out on *Tilapia* by Sailendri,(1973) and Jayaraman et al.,(1979) could be combined with attempts to raise monoclonal antibodies specific for different lymphocyte subsets

(Secombes et al., 1983a; 1983b). A combination of such approaches, together with studies on the effects of T-dependent and T-independent mitogens and antigens on *Tilapia* lymphocytes, and the application of *in vitro* MLR studies developed in chapter 4, should provide a clearer insight into the functioning of the immune system in teleosts.

APPENDICES

APPENDIX 1

Preparation of *Escherichia coli* Suspension

Using a sterile, pasteur pipette and teat, a small volme of 0.9% saline was added to plates inoculated with *Escherichia coli*. A loop was flamed to red heat, allowed to cool, and then gently rubbed over the surface of the plates to loosen the growth. Using a sterile pasteur pipette and teat, the bacteria were washed off and collected. About four plates of *Escherichia coli* washed off into one litre of saline gave a suspension of approximately 10^9cells/ml^{-1} . The number of bacteria was counted and adjusted using a haemocytometer chamber.

APPENDIX 2

Preparation of Hank's Solution

Stock solutions of the salt mixtures are convenient and are made up with ten times the concentration of all the components except for the bicarbonate solution, which is made up separately.

Phenol Red Indicator (0.4%)

This is made by dissolving 1.0g phenol red in the minimum volume of 0.05M Na OH and then bringing the volume to 250 ml by the addition of distilled water.

Stock Solution A

(1)	Nacl	160.0g
	KCl	8.0g
	$MgSO_4.7H_2O$	2.0g
	$MgCl_2.6H_2O$	2.0g
	H_2O	800.0ml
(2)	$CaCl_2$	2.8g ml
	H_2O	100.0 ml

The two solutions are mixed slowly and the volume adjusted to 1000 ml with water. 2.0 ml chloroform is added and the solution is stored in a polythene bottle at 4^oC until used.

Stock Solution B

$Na_2 H PO_4.12 H_2O$	3.04g
$KH_2 PO_4$	1.2g
Glucose	20.0g
Water	800.0 ml. When dissolved, add:-
Phenol red solution	100.0 ml
Water	1000.0 ml
Chloroform	2.0 ml and store as with solution A

Sodium Bicarbonate Solution

$Na HCO_3$	1.4 g
Water	100.0 ml

Sterilize by autoclaving in a half filled container with a tightly closed screw cap for 10 minutes at 115°C (9 lb pressure). Hank's solution is made by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of distilled water. It is sterilized by steaming for 1 1/2 hour. Immediately before use 0.5 ml of sterile 1.4% $NaHCO_3$ solution is added to each 20 ml of Hank's solution.

Glycine saline buffer, pH 8.2, 0.5M (for latex agglutination)

Glycine	14.00g
Sodium Hydroxide, Solid	0.7 g
Sodium Chloride	17g
Sodium Azide (preservative)	1g

Method

Dissolve the constituents listed above in 500 ml of distilled water and adjust pH to 8.2. Make up to 1000 ml.

Phosphate Buffered Saline (PBS), pH 7.2, 0.20 M (for tanning erythrocytes)

Materials

Potassium di-hydrogen phosphate ($KH_2 PO_4$) 0.02 M (12.2g) Di-Sodium hydrogen phosphate ($Na_2 HPO_4$) 0.06 M (40.4g) Sodium Chloride 0.12 M (36.0g)

Method

Dissolve in 5 litres of distilled water.

Phosphate Buffered Saline (PBS), pH 7.2 (0.15 M)

Materials

Sodium Chloride 8.00 gram per litre

Potassium Chloride 0.20 gram per litre

Di-sodium hydrogen phosphate ($Na_2 HPO_4$) (0.008M) 1.15 gram per litre

Potassium di-hydrogen phosphate, 0.20 gram per litre

Method

Dissolve in 1000 ml of distilled water.

It is convenient to make up a 10 x solution for storage and dilute as required.

Physiological Saline or 'Normal' Saline

0.14 M sodium chloride 8.5 gram per litre.

Store at a 10 x concentrated solution and dilute as required.

Cacodylate buffer, pH(6.9), 0.28M (E.M)

Materials

Sodium cacodylate

Hydrochloric acid 3M

Method

1. Dissolve sodium cacodylate (60 gram per litre) in distilled water.
2. Titrate to pH 6.9 with 3M HCl.
3. Adjust to 1L with distilled water.

APPENDIX 3

Preparation of Fixatives for Histology

Carnoy's Fluid

Absolute alcohol	60 ml
Chloroform	30 ml
Acetic acid	10 ml

$3mm^3$ tissue blocks are fixed from 30 - 90 mins.

Bouin's Fluid

Saturated Aqueous Picric Acid	75 mls
Formaldehyde	25 mls
Acetic Acid (Glacial)	5 mls

Fixation time is from 6 - 24 hours

Formal Saline 10% [not suitable for H.E. staining]

Formaldehyde solution	100 mls
0.9% saline	900 mls

Fixation time is about 10 days.

APPENDIX 4

Slide Agglutination Test Using Latex Particles

Latex Agglutination

Latex provides a convenient carrier for antigens in agglutination tests.

Latex coating

Materials

Latex suspension 10% w/v

Antigen, HGG (human gamma globulin) 0.27M and 0.054 M, Glycine-saline buffer pH 8.2 (Appendix 2).

Method

1. Wash 800 µl of latex suspension twice by adding 40 µl of 0.054 M glycine-saline; mix and centrifuge at 12,500 g for 15 mins.
2. Resuspend the latex in 20 ml 0.054 M glycine saline and add 300 µl of a 10 mg ml^{-1} solution of antigen.
3. Mix the suspension for 30 minutes at room temperature.
4. Wash the latex twice by adding 40 µl of 0.054 M glycine-saline; mix and centrifuge at 12,500g for 15 minutes.
5. Resuspend the latex in 20 µl of 0.27 M glycine - saline containing 0.1% of an irrelevant protein (BSA) to block any remaining latex protein-binding sites and store at $4^{o}C$.

The Slide Agglutination Test

Materials

Coated latex as above

0.27 M Glycine Saline buffer, pH 8.2 (Appendix 2).

Method

1. Prepare doubling dilutions of test antiserum
2. Add 25 µl of antiserum dilution to 25 µl coated latex on a glass slide.
3. Rock gently for 2 min and read agglutination visually, illuminating the slide from the side, against a dark background.

APPENDIX 5

Tissue Processing for Histological Techniques

1. Fix in Bouins or formalin for 24 hours.
2. Store in 70% alcohol
3. Change the 70% alcohol to wash out picric acid colouring.
4. If stored in 70% alcohol, go directly to 90% alcohol for 2 hours, otherwise in 70% first for 2 hours.
   (a) From 70% alcohol to 80% alc. for one hour
   (b) 90% alc. for 3/4 hour
   (c) 96% alc. for 1/2 hour
   (d) 100% alc for 1/2 hour
5. Introduce to (100% alc + xylol) for 2 hours till the margins become transparent.
6. Transport to xylol 1 for 30 minutes
   xylol 2 for 30 minutes
7. Transport to xylol + wax 1 at the percentage of 3/4 xylol & 1/4 wax in oven at 40°C.
8. Transport to xylol + wax 2 at the percentage of 1/2 xylol & 1/2 wax in oven at 40°C.
9. Transfer to wax 1 only for one hour
10. Transfer to wax 2 only for one hour
11. Transfer to wax 3 only for one hour

    N.B. (in 9, 10 and 11 samples can be left outside the oven in wax overnight).
12. Embed in trays and float on cold water for 1/2 hour to speed up setting.

APPENDIX 6

Methyl green pyronin

This is a quick and effective stain for plasma cells in tissues.

Staining Solution

Methyl Green	1.0 gram
Pyronin Y	0.15 gram

Put the dry dyes into a flask and add 100 ml of hot distilled water. Agitate to mix and let stand 2 days at room temperature. Filter. The staining solution is ready for use and keeps at least 6 months.

Staining Technique

Tissue is fixed in 4 changes of absolute ethyl alcohol for 2 - 4 days and embedded in paraffin wax. Sections are cut at (4 - 6 microns), and mounted on slides coated with Mayer's albumen-glycerol adhesive.

1. Remove paraffin and bring slides to distilled water as usual
2. Stain each slide separately (on a rack) by flooding with the staining solution and allowing it to remain for 5 - 6 minutes.
3. Rinse in distilled water and lightly blot off excess water but do not allow section to dry.
4. Immediately dip in and out 3 times, into each of 2 jars containing dioxane, total time 5 to 10 sec. Clear in 4 changes of xylene and mount in resin dissolved in xylene.

Results

Plasma cells show dark blue nuclei and brilliant red cytoplasm. The other nuclei stain light blue and the cytoplasm faint pink to lavender.

APPENDIX 7

Autoradiographic Techniques

About sixty fry of 10 week old *Tilapia mossambica* average weight two grams were immunised intraperitoneally with 20% sheep red blood cells (0.01 ml/gram fish for each fry). The fry were left for a week before injecting with tritiated thymidine (3HT).

A stock solution of tritiated thymidine (3HT) containing specific activity of 5Ci/mmol (1microcurie/ ml) was stored at 4°C. This stock was diluted before use to 200 microcurie per ml 0.85% saline and was injected intraperitoneally into the fish at a rate of 10 µl per gram body weight [using a glass micrometer syringe fitted with a sterile 30 gauge needle (S.G.E)] 18 hours prior to sacrifice. A group of three fish were sacrificed on days 1, 2, 3 and 4 after 3HT injection. Spleen, kidney and thymus were dissected out, and imprints were made on glass microscopic slides [organs were cleared of the connective tissues].

Slides had been prepared previously by washing them for 24 hours in concentrated hydrochloric acid, followed by 24 hours rinse in running tap water. They were then left in several changes of distilled water for a further day, and then dipped in gelatin (5 grams of gelatin and 0.5 gram chrome alum in one litre distilled water), before being left to dry.

The organs from which imprints were to be taken were gently pressed against the slide and this process was repeated several times along the length of the slide. Slides were left to air dry and then fixed with absolute methanol, stained with Leishman's stain and dried ready for autoradiography.

Spleen, kidney and thymus area were removed from another group of fish and fixed in Carnoy's fluid as in Appendix 3, these organs were processed and embedded in paraffin wax, sectioned and mounted on gelatin coated slides. All slides were dewaxed in xylene and hydrated (passed down the alcohol to distilled water).

The autoradiography of both the organs and their imprints was performed using Ilford K_5 Emulsion which was melted in a 43°C water bath under safelight conditions and then diluted 1:1 with distilled water and 1% v/v glycerine was added in a coplin jar.

Dewaxed slides were dipped individually into emulsion for a few seconds, withdrawn slowly and the backs wiped. Then they were placed on stainless steel trays over ice baths, in a stream of cold air blowing horizontally for the emulsion to solidify. Slides were then placed in light proof slide boxes, securely wrapped in black polythene, stored in a cold room ($4^{o}C$) for a period of 4 weeks with some silica gel or calcium chloride to ensure a regulated, low humidity.

After exposure, again in the dark room, slides were placed into distilled water to moisten them. Under safelight conditions, they were developed in Kodak D19 developer for 4 minutes, rinsed quickly in distilled water or 1% acetic acid and then fixed in 1:6 Amfix high speed fixer for 5 minutes. Then slides were washed thoroughly in running tap water for at least 15 minutes before staining in methyl green pyronin (Appendix 8) as for normal slides and then mounted in D.P.X. For scoring each organ, the labelled cells were counted from ten fields of view under a X 40 objective. Photographs were taken using a Carl Zeiss photo microscope with 3 5 mm camera attached.

APPENDIX 8

Methods to Stain Autoradiographs using Methyl Green Pyronin

The developed sections which are fixed in suitable fixer and washed in running tap water are stained as follows:-

1. Rinse in acetate buffer pH 4.8 for 30 minutes or in distilled water and blot dry.
2. Stain in staining solution (M.G.P) for 25 minutes.
3. Sections are rinsed again in acetate buffer (pH 4.8) or in distilled water for a few seconds.
4. Blot dry carefully (because if any moisture is left - the methyl green will be lost from the sections at the next step) some pyronin is removed during this washing and the correct time should be determined by trial and error.
5. Dehydrate in acetone for not more than one minute.
6. Rinse in acetone-xylene (1:1) or in n-butyl alcohol for two minutes.
7. Rinse in xylene and mount in D.P.X. or suitable synthetic resin medium.

* Sections on slides need red filter to be photographed.

APPENDIX 9

Preparation of L-15 [Liebovitz] - Liquid Medium

1. Measure out 90% of final required volume of deionized-distilled water (e.g. 900 ml for a final volume of 1000 Microlitres). Select an appropriate container as close in size to the final volume as possible. Water temperature should be 15 - 20^{o} C.
2. While gently stirring the water, add the powdered L-15 Medium (Sigma) (Cat No. L-4386) stir until dissolved. Do not heat water.
3. Rinse original package with a small amount of deionized distilled water (less than 5% of final volume) to remove all traces of power. Add to solution in step 2.
4. While stirring, adjust the pH of medium from 7.2 - 7.6. The use of 1 N HCL is recommended.
5. Add additional deionized distilled water to bring medium to final volume (1L).
6. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns or less. (It is recommended that positive pressure be used for filtering.)
7. Aseptically dispense medium into sterile container (TCM bottles). Store liquid medium refrigerated at 2 - 6^{o}C in darkness.

   Addition of 10% new born calf serum optional.

APPENDIX 10

Scale Transplantation Allograft Rejection Studies on Adult*Tilapia*

Table 4.11

Species	Recipient animals per set	Mean Survival Times ± S.E.		Interval between first and second set grafting days	Temperature and other comments
		First set Mean ± S.D. (S.E.)	Second set Mean ± S.D. (S.E.)		
Adult*Tilapia mossambica*	3 grafts on each fish			27 days (23)	28°C (26)
	(1)	8.6 ± 0.5 (2.1) *	6.0 ± 0.3 (1.1)		
	(2)	7.5 ± 0.3 (1.5)	4.1 ± 0.2 (1.0)		
	(3)	7.2 ± 0.3 (1.0)	4.7 ± 0.2 (0.7)		
	(4)	7.2 ± 0.4 (1.8)	4.5 ± 0.2 (1.0)		
	(5)	7 ± 0.0 (0)	5 ± 1.1 (0.1)		
	(6)	6.0 ± 1.2 (0)	5 ± 1.1 (0.1)		
	(7)	5.3 ± 0.8 (0)	N.D.		(7) Died in 2nd day after grafting
	(8)	5.2 ± 0.2 (1.0)	4.4 ± 0 (0)		
	(9)	5.1 ± 1.1 (0.1)	4.1 ± 1.1 (0.1)		
	(10)	-	6 ± 0 (0)		No rejection till 21 days
Total mean ± S.D.		6.6 ± 1.2 (0.8)	4.8 ± 0.6 (0.4)		

* n = 3 One exceptional fish (allografted) was still viable 21 days after grafting, however the comparable set graft was rejected after 6 days (matching histocompatibility).

* N.D. = Not Done

APPENDIX 11

Table 4.12 Immune response of adult *Tilapia mossambica* to SRBC at (22 - 27°C)

	Days Post injection of SRBC	Haemagglutination		Plaque Forming Cells/10^6 White Cells				Rosette Forming Cells/10^6 White Cells			
		HA		Spleen		Head Kidney		Spleen		Head Kidney	
		Mean $\log_2$ ± S.D.	S.E.	Mean PFC/10^6 ±S.D.	S.E.	Mean PFC/10^6 ± S.D	S.E.	Mean RFC/10^6 ± S.D.	S.E.	Mean RFC/10^6 ± S.D.	S.E.
1ry immune response	2nd day	0.66 ± 1.1	0.4	40 ± 0	0	50 ± 0	0	222 ±0	0	1420 ± 0	0
	5th day	2 ±1	0.45	100 ± 0	0	120.22 ±0.33	0.19	568.2 ±15.8	9.12	3381 ± 0.36	0.21
	8th day	3.66 ± 2	0.47	163.5 ±0.45	0.26	3352.3 ±1.84	1.06	8249.6 ± 0.52	0.30	5961.4 ± 1.21	0.70
	9th day	2.36 ± 1.12	0.34	273.5 ± 0.39	0.22	1227.5 ±0.45	0.26	1330 ±0	0	1166 ± 57.7	33.3
	11th day	2.5 ± 1.29	0.65	407.33 ±0.52	0.30	464.4 ±0.46	0.27	5926.5 ±0.46	0.26	5435.4 ± 30.6	17.6
	14th day	0		0		0		0		0	
2 ry immune response	2nd day	2 ± 1	0.45	100 ± 0	0	118.22 ± 0.33	0.19	N.D		N.D.	
	5th day	3.1 ± 1.66	0.52	628.26 ± 1.85	1.07	1145.4 ± 0.47	0.27	1916 ± 12.2	7.04	4140 ± 37.4	21.6
	8th day	3.45 ± 1.96	0.59	711.3 ± 9.01	5.20	1199.34 ± 0.33	0.19	3878.7 ± 105.0	60.6	4887.3 ± 56.7	327.8
	11th day	4.36 ± 2.83	0.85	2240.41 ± 22.6	13.1	1765.27 ± 0.8	0.32	8406.7 ± 5.83	3.36	5336.8 ± 141.5	81.7
	14th day	2.6 ± 1.41	0.49	0	0	0	0	0	0	0	0
	16th day	0	0	N.D.		N.D		N.D.		N.D.	

n = 3
N.D. - Not Done
S.D. - Standard Deviation
S.E. - Standard Error

The 9th day for 2°C immune response not done in all tests as SRBC was not available on that day

APPENDIX 12

Table 4.13

Histology of Lymphoid Organs in Immunised Fish (by detection of pyroninophilic cells)

	Testing Weeks	1st week	2nd week	3rd week	4th week	5th week
Organs from fish immunised with HGG	Thymus	+	+	-	-	-
	Head Kidney (Pronephros)	+ + +	+ + +	+ + +	++ *	+ +
	Spleen	+	*	*	+	-
Organs from fish immunised with *E. coli*	Thymus	+	+	-	-	+
	Head kidney (Pronephros)	+++	+++	++	+	+
	Spleen	*	+	-	-	-

* = Cell Clusters
+++ = High numbers of pyroninophilic cells
++ = Medium numbers of pyroninophilic cells
\+ = Low numbers of pyroninophilic cells
\- = No pyroninophilic cells
All non-injected control organs were negative

n = 3
HGG = Human Gamma Globulin
E. coli = *Escherichia coli*

REFERENCES

REFERENCES

Ahlsted, S., Holmgren, J., and Hanson, L.A. (1973)
The primary and secondary antibody response to *Escherichia coli* lipopolysaccharide, analysed at the humoral and cellular level - amount and avidity of the antibodies in relation to protective capacity.
Immunology 24, 191

Agius, C. (1980)
Aspects of the melano-macrophage centres in fish.
Ph.D Thesis, Stirling University.

Agius, C. (1985)
The melano-macrophage centre of fish ' A Review'.
'Fish Immunology', edt. by Manning, M.J. & Tatner, M.F.,
Academic Press, London, New York.

Albert, C. (1977)
Reactions of fish red blood cells with mucus and sera from other fish(es).
Calif. Fish and Game, 63(1) : 52 - 57.

Alexander, J.B. (1980),
Precipitins in the serum of the Atlantic Salmon.
Dev. & Comp. Imm. Vol. 4 pp 641 - 651.

Alexander, J.B. (1982),
The antibody minetic precipitins of fish.
Dev. and Comp. Imm. Supplement 2 pp 133 - 138.

Alexander, J.B., Ingram A.G.(1983),
Antigen Clearance in Trout.
Dev. and Comparative Immunology. Vol. 7. pp 707 - 708.

Alexander, J.B., Bowers, A., and Shamshoom, S.M. (1981)
Hyperosmotic infiltration of bacteria into trout:
Route of entry and the fate of the infiltrated bacteria.
International Symposium on Fish Biologics : Developmental Biol. Standard 49 p. 441 - 445.

Alexander, J.B., Bowers A., and Ingram, A.G. (1982),
The portal of entry of bacteria into fish during hyperosmotic infiltration and the fate of antigens.
Dev. and Comp. Immunology. Supplement 2, pp 41 - 46.

Amend, F.D., and Fender, C.D.(1976),
Uptake of Bovine Serum Albumin by Rainbow Trout from hyperosmotic solutions. A model for vaccinating fish.
Science Vol. 192., pp 793 - 794

Anderson, D.P. (1974),
Fish Immunology,
T.F.H. Publications, Inc. Ltd.,
4th Edition in books of fish diseases.

Anderson, D.P., and Dixon, O.W. (1980,
Immunology memory in rainbow trout to a fish disease bacteria adminstrated by flush exposure. In "Phylogeny of immunological memory", Ed. Manning, M.J, Elsevier/North Holland, Amsterdam, pp 103 - 111.

Anderson , D.P., and Merchant, B. (1983),
Flush exposure and injection immunisation of Rainbow Trout to selected (DNP) Conjugates.
Dev. and Comp. Imm. Vol 7 pp 261 - 268.

Anderson D.P., Dixon, O.W., and Roberson, B. S. (1979),
Kinetics of the primary immune response in Rainbow Trout after flush exposure to *Yersinia Ruckeri* O- Antigen.
Dev. and Comp. Imm. Vol 3 pp 739 - 744.

Anderson, D.P., Roberson, B.S., and Dixon, O.W. (1979),
Plaque - Forming cells and Humoral Antibody in Rainbow Trout (*Salmo gairdneri*). Induced by Immersion in a *Yersinia ruckeri* O-Antigen Preparation.
Journal of Fish Res. Board Can. Vol. 36; pp 636 - 639.

Andrew, W.R. (1979),
Techniques of Autoradiography.
3rd Edition.
Elsevier/North - Holland Biomedical Press., Amsterdam, New York, Oxford.

Antipa, R. (1976),
Field testing of injected *Vibrio anguillarum* bacterins in Pen-Reared Pacific salmon.J. Fish, Res. Board Can. Vol. 33: pp 1291 - 1296

Antipa, R., and Amend, F.D., (1977),
Immunisation of Pacific Salmon. Comparison of Intraperitoneal Injection and Hyperosmotic Infiltration of *Vibrio anguillarum* and *Aeromonas salmonicida* Bacterins.
Journal of Fish Res. Board Can. Vol. 34, pp 203 - 208

Argyris, B.F., Delustro, F., and Horowitz, M. (1976),
Cell interaction during mitogenesis and mixed lymphocyte reaction. Phylogeny of thymus and bone marrow. Ed. by Wright R.K. and Cooper E.L. Elsevier/North - Holland Biomedical Press, Amsterdam, The Netherlands.

Arnall, J.C. and Horton, J.O. (1986),
Impaired Rejection of Minor-Histocompatibility-Antigen-Disparate Skin Grafts and Acquisition of Tolerance to Thymus Donor Antigens. In Allothymus-Implanted, Thymectomised Xenopus.
Transplantation, Vol. 41. No.6 PP 770 - 775

Ashley, L.M., and Smith, E.C. (1963),
Advantages of tissue imprints over tissue sections in studies of blood cell formation.
Progressive Fish Culturist, Vol. 25 pp 93 - 96.

Ashman, R.B., Holmes, R.M., and Keast, D. (1977),
The effect of neonatal thymectomy on the ontogeny of mitogen responses in the Quokka *(Setonix Brachyurus)*.
Development and Comparative Immunology,Vol. 1 pp 47 - 58.

Avtalion, R.R., Wojdani, A., Malik, Z, Shahrabani, R.
and Duczminer, M.(1973)
Influence of environmental temperature on the immune response in fish. Current Topics in Microbology and Immunology. Vol 61, Part 2., pp 1 - 35.

Axelson, N.H., Krolly, J., and Weeke, B. (1973)
A manual of quantitative immuno-electrophoresis methods and applications.
Scandinavian Journal of Immunology. Vol. 2(1)

Azzolina, L.S. (1978),
Antigen recognition and immune response in cold fish *Carassius auratus* at different temperatures.
Developmental and Comparative Immunology. Vol. 2, pp 77 - 86.

Bach, F.H., and Bach, M.L. (1971),
Mixed leukocyte cultures in transplantation immunology
Transplantation proceedings. Vol. 3(1), pp 942 - 948.

Bach, R., Chen, P.K., and Chapman G.B. (1978),
Changes in the spleen of the channel catfish *Ictalurus punctatus*. Rafinesque induced by infection with *Aeromonas hydrophila*.
Journal of Fish Diseases. Vol. 1, pp 205 - 217.

Baldwin III, W.M. (1983),
Antigen trapping cells in *Xenopus laevis*: Tissue distribution.
Developmental and Comparative Immunology. Vol. 7, pp 709 - 710.

Balfour, F.M. (1882),
On the nature of the organ in Adult Teleosteans and Ganoids, which is usually regarded as the Head Kidney or Pronephros.
Quarterly journal of Microscopical Sciences. Vol XXII, pp 12 - 16.

Balinsky, B.I. (1948),
On the development of specific characters in Cyprinid fishes.
Proc. Zool. Soc. London. Vol. 118, pp 335 - 344.

Bancroft, J.D., and Stevens, A. (1982),
Theory and Practice of Histological Techniques, 2nd Edition.
Churchill Livingstone.
Edinburgh, London, Melbourne & New York.

Bang, B.F. (1975),
Comparison of immune mechanisms in Vertebrates and Invertebrates.
Invertebrate Immunity by Karl Maramorosch & Robert E. Shope.
Academic Press, INC. (London) Ltd.

Bartos, J.M., and Cynthia, V.S. (1981),
In vivo cell mediated immune response to M. Tuberculosis and M. Salmoniphilum in Rainbow Trout (*Salmo gairdneri*),
Development and Comparative Immunology Vol. 5, pp 75 - 83.

Batch, F.H., and Hirschhorn, K.(1964),
Lymphocyte Interactions: A potential histocompatibility Science,
Vol. 143, pp 813 - 814.

Battle, H.I. (1944),
The embryology of the Atlantic Salmon (*Salmo solar* Linnaeus),
Can. J. Res., Vol. 22 (5), pp 105 - 125.

Beard, J. (1894),
The development and probable function of the thymus.
Anatomischer Anzeiger. Vol. 9, pp 476 - 486.

Bearman, R.M., Bensch, K.G., and Levine, G.D. (1975),
The normal human thymic vasculature: An ultrastructural study,
Anat. Rec., 183, 485.

Bell, R.G. (1964),
A guide to the properties, characteristics and uses of some general anaesthetics for fish.
Fish Res. Board. Canada. Bull., Vol. 148.

Bejerano, I., and Sarig, S. (1980),
Stress Induced Infection of *Sarotheradon aureus* under laboratory conditions. Special Publication European Maricult.
Society No.6

Bezkorovainy, A., Springer, G.F. and Desai, P.R. (1971),
Physicochemical properties of the cell anti-human blood group H(0) antibody Biochemistry. Vol. 10, pp 3761 - 4.

Billingham, R.E., Brent, L., and Medawar (1953),
Actively 'acquired tolerance' of foreign cells.
Nature, Vol. 172 (3) pp 603.

Bisset, K.A., (1948),
The effect of temperature upon antibody production in cold blooded vertebrates. J. Path. Bact., Vol. 60, pp 87 - 92

Blaxhall, P.C. (1972),
The haematological assessment of the health of freshwater fish,
A review of selected literature,
Journal of Fish Biology. Vol 4, pp 593 - 604.

Blazer, V.S., and Wolke, R.E. (1984),
The effects of α - Tocopherol on the immune response and non-specific resistance factors of Rainbow Trout (*Salmo gairdneri* Richardson). Aquaculture, (37), pp 1 - 9.

Bloom, W., and Fawcett, D.W. (1970),
'A text book of histology' (10th edition),
W.B. Saunders Co., Philadelphia, London. Toronto.

Bly, J. (1984),
The ontogeny of immunity in teleost fishes with particular reference to foeto-maternal relationships.
Ph.D. Thesis, University of Wales.

Bockman, D.E. (1970),
The thymus. In: Biology of the reptilia.,
C. Gans (Ed.), Academic Press, New York, Vol.3, pp 111.

Bogner, K.H. 1975,
Aspects of Mitogen Responsiveness in Teleost Fish, MSc Thesis,
Unit of Aquatic Pathobiology, University of Stirling, Scotland.

Bogner, K.H., and Ellis, A.E. (1977),
Properties and functions of lymphocytes and lymphoid tissues in Teleost fish, Fish and Environmental, Vol. 4, pp 59 - 72.

Boomker, J. (1979) & (1981),
The haemocytology and histology of the haemopoietic organs of South African fresh water fish.
I. The haemopoietic organs on *Clarias gariepinus* and *Sarotheradon mossambicus*.

II Erythrocytes and Thrombocytes of *Clarias gariepinus* and *Sarotheradon mossambicus*.

III The leucocytes, plasma cells and macrophages of *Clarias gariepinus* and *Sarotheradon mossambicus*.
Onderstepoort J. Vet. Res., 48, pp 185 - 193.

Borysenko, M. (1976),
Phylogeny of Immunity: An Overview Immunogenetics. Vol.3, pp 305 - 326.

Botham, J.W. (1982),
Studies on the ontogeny of the lymphoreticular system of the carp *Cyprinus carpio linnaeus*, Ph. D, Thesis, University of Hull.

Botham, J.W., Manning, M.J. (1981),
The histogenesis of the lymphoid organs in the carp *Cyprinus carpio* L, and the ontogenetic development of allograft reactivity,.
Journal of Fish. Biol., 19, pp 403 - 414.

Botham, J.W., Grace, M.F., and Manning, M.J. (1980)
Ontogeny of first and second set alloimmune reactivity in fishes.
Phylogeny of Immunological Memory edited by Margaret J. Manning Vol. 10. pp 83. Elsevier/North-Holland Biomedical Press.
Amsterdam, New York, Oxford.

Bourne, P.K. (1984),
The use of MS222 (Tricaine Methane-sulphonate) as an Anaesthetic for routine blood sampling in three species of Marine Teleosts.
Aquaculture, 36, pp 313 - 321.

Bowen, S.H. (1984),
Differential habitat utilization by sexes of *Sarotheradon mossambicus* in Lake Valencia, Venezuela.
Significance for fitness. Journal of Fish Biology. Vol. 24, pp 115 - 121.

Bowers, A. (1979),
Hyperosmotic immunisation of teleosts: The portal of entry of the bacterium. M.Sc. Thesis, University of Salford, Salford, U.K.

Bowers, A. and Alexander, J.B. (1981),
Hyperosmotic infiltration : immunological demonstration of infiltrating bacteria in brown trout, *Salmo trutta* L.
Journal Fish. Biol., Vol 18, pp 9 - 13.

Bradshaw, C.M., Clem, L.W., and Michael, S.M. (1969),
I-Immune responses and characterisation of Antibody,
Journal of Immunology, Vol. 103, No.3.

Bromage, N.R., and Fuchs, A. (1976),
A histological study of the response of the interrenal cells of the goldfish.*(Carrassius auratus)* to treatment with sodium lauryl sulphate.
Journal of Fish Biol., Vol. 9, 529 - 535.

Bullock, W.L. (1963),
Intestinal histology of some salmonid fishes.
Journal of Morphology, Vol. 112, pp 23 - 43.

Bullock, M.A., and Roberts, R.J. (1974),
The Dermatology of Marine Teleost Fish.
I. The normal integument.
Oceanogr. Mar. Biol. Ann. Rev., Vol. 13, pp 383 - 411.

Burne, R.N. (1927),
A contribution to the anatomy of the ductless glands and lymphatic system of the angler fish, *Lophius piscatorius*.
Phil. Trans. Roy. Soc. London B, Vol. 215, pp 1 - 56.

Burnet, M. (1962),
Role of the thymus and related organs in immunity.
British Medical Journal, Vol. 2, pp 5308.

Caspi, R.R., and Avtalion, R.R. (1984),
The mixed leukocyte reaction (MLR) in carp:
Bidirectional and unidirectional MLR responses.
Dev. and Comp. Immunology,
Vol. 8 (3), pp 631 - 637.

Caspi, R.R., Shahrabani, R., Avtalion, R.R. (1979),
The cells involved in the immune response of fish I, separation and study of lymphocyte sub-populations in carp *Cyprinus-carpio*. A new approach. Manning, M.J., (Ed.), Developments in Immunology, Vol. 10. Phylogeny of Immunological Memory; Proceedings of the international symposium. Elsevier/North-Holland, Biomedical Press: Amsterdam. Netherlands, New York, N.Y. USA, pp 131 - 142.

Caspi, R.R., Shahrabani, R., Kekati-Dan, T. &
Avtalion, R.R. (1984),
Heterogeneity of mitogen - responsive lymphocytes in carp (*Cyprinus carpio*). Dev. and Comp. Immunol., Vol. 8(1), pp 61 - 70.

Catton, W.T. (1951),
Blood cell formation in certain teleost fishes,
Blood 1951, Vol. 6, pp 39 - 60.

Charlemagne, J. (1974),
Larval thymectomy and transplantation immunity in the urodele, *Pleurodeles waltlii* Michach (Salamandridae).
Eur. J. Immunol., Vol. 4 , pp 390 - 392.

Chiller, J.M., Hodgins, H.O., Chambers, V.C., and Weiser, R.S. (1969),
Antibody response in Rainbow Trout. (*Salmo gairdneri*).
I - Immunocompetent cells in the spleen and anterior kidney.,
Journal of Immunology, Vol. 102, No. 5, pp 1201 -

Chilmonczyk, S. (1983),
The thymus of the rainbow trout (*Salmo gairdneri*) light and electron microscope study. Dev. and Comp. Immunol., Vol. 7, pp 59 - 68.

Clark, S.L. Jr. (1968)
Incorporation of sulfate by the mouse thymus : its relation to secretion by medullary epithelial cells and to thymic lymphop oie s.
J. Exp. Med., Vol. 128, pp 927.

Clem, L.W. (1971),
Phylogeny of immunoglobulin structure and function - IV
Immunoglobulins of the giant grouper, Epinephelus itaira.
J. Biol. Chem., Vol. 246, pp 9 - 15.

Clem, W., and Michael, S.M. (1966),
Immunological & immunochemical studies on holostean and marine teleost fishes immunised with bovine serum albumin.
Phylogeny of Immunity by Robert Good.
Gainseville University of Florida press.

Clem, W.L., McLean, E.W., Shankey, T., Vincet and Cuchens, M.A. (1977),
Phylogeny of lymphocyte heterogeneity,
I - Membrane immunoglobulins of teleost lymphocytes.
Dev. and Comp. Imm., Vol. 1, pp 105 - 118.

Cole, M.B., (1982),
Glycol methacrylate embedding of bone and cartilage for light microscopic staining.
Journal of Microscopy, Vol. 127, Part (2), pp 139 - 148.

Cole, M.B., Sykes, S.M. (1974),
Glycol Methacrylate in light microscopy. A routine method for embedding and sectioning animal tissues.
Stain Technology, Vol. 49, No.6.

Collie, M.H., Turner, R.J. and Manning, M.J. (1975),
Antibody production to lipopolysaccharide in thymectomised *Xenopus*.
Europ. J. Immunol., Vol.5, pp 426.

Colorni, A., and Paperna, I. (1983),
Evaluation of Nitrofurazone Baths in the treatment of Bacterial Infections of Sparus Aurata and *Oreochromius mossambicus*.
Aquaculture, Vol. 35, pp 181 - 186.

Cooper, E.L. (1973),
The thymus and lymphomyeloid system in poikilothermic vertebrates.
Vol. 2, Thymus dependency by Davies, A.J.S., and Carter, R.L.
New York, London.

Cooper, E.L. (1982),
"General Immunology", Pergamon Press, Oxford, New York.

Corbel, M.J. (1975),
The immune response in fish : A review
Journal of Fish Biol., Vol. 7, pp 539 - 563.

Cruickshank, R. (1968),
Medical Microbiology,
(A guide to the laboratory diagnosis and control of infection). 11th edition,
E & S. Livingstone Ltd., Edinburgh & London.

Cunningham, A.J., and Szenberg, A. (1968),
Further improvements in the plaque technique for detecting single antibody - forming cells.
Immunology, 1968, Vol. 14, pp 599.

Curtis, S.K., Volpe, E.P., and Cowden, R.R. (1972),
Ultrastructure of the developing thymus of the leopard frog. *Rana pipiens*.
Z. Zellforsch. 127, 323.

Cushing, J.E., (1970),
Immunology of Fish. In Fish Physiology. Vol. IV.
The nervous system, circulation and respiration. (Ed.) Hoar, W.S., and Randall, D.J. Academic Press, London, pp 465 - 500.

Cushing, J.E., and Campbell, D.H., (1957),
"Principles of Immunology", McGraw-Hill Book Comp. Ing. New York, Toronto, London.
"Chapter 12 - Comparative Immunology.

Davies, D.H., and Lawson, R. (1985),
Serum precipitins of the Atlantic Salmon, *Salmo salar* , and their possible role in Immunological Defence in Fish Immunology 1985.
Edt. by Manning, M.J., and Tatner, M.F.,
Academic Press, London, New York.

Davina, J. H.M., and Rijkers, G., Rombout, J.W.M.,
Timmermans, L.P.M. and Van Muiswinkel, W.B. (1980),
Lymphoid and non-lymphoid cells in the intestine of Cyprinid Fish, Development and Differentiation of Vertebrate Lymphocytes edited by J.D. Horton. Elsevier.North Holland Biomedical Press.

Deanesly, R.B.A., (1927),
The structure and development of the thymus in fish, with special reference to *Salmo fario*. Quarterly journal of Microscopical Science. Vol. 71, pp 113 - 145, No. 281.

Dickerson, J. B., and Dawe, D.L.(1984),
Tetrahymena pyriformis as a protective antigen against Ichthyophthirius multifilus infection. Comparisons between isolates and ciliary preparations.
Journal of Fish. Biol.,Vol. 24, pp 523 - 528.

DiConza, J.J., Halliday, W.J. (1971),
Relationship of Catfish Serum, Antibodies to Immunoglobulin in Mucus Secretions.
Aust. J. Exp. Biol. Med. Sci. ,Vol. 49, pp 517 - 519.

Donald W.L, and Evans, E.E., (1967),
Comparative Immunology: Serum complement of Fresh Water fishes.
Transactions of the American Fisheries Society, Vol. 96(3).

Dorson, M.M. (1974),
Immunologie : Production d'anticorps precipitants antidinitrophenol chez les alevins detruits Arc-en-ciel (*Salmo gairdneri*) immunises a l'age d'un mois. C.R. Acad. Sc. Paris. Vol. 278, pp 3151 - 3152.

Dorson, M. and De Kinkelin, F. (1975),
Mortality and interferon production in rainbow trout experimentally infected with Egtved virus : Role of temperature.
Annls. Roch. Vert.,Vol. 59, pp 365 - 372.

Dresser, D.W., (1962),
Specific Inhibition of Antibody Production
I - Protein - Overloading Paralysis Immunology,Vol. 5, pp 161.

Drury R.A.B., and Wallington, E.A. (1980),
Carleton's Histological, 5th Edition,
Oxford Medical Publication.

Duff, D.C.B. (1939),
Some serological relationships of the S.R., and G. Phases of *Bacillus salmonicida.*
Journal of Bacteriology, Vol. 38, pp 91.

Dumonde, D.C., Wolstencroft, R.A., Panaji, G.S., Matthew, M., Morley, J., and Howson, W.T. (1969),
"Lymphokines" : Non-antibody Mediators of Cellular Immunity generated by Lymphocyte Activation. Nature, Vol. 224, pp 38 - 42.

Du Pasquier, L. (1970),
Ontogeny of immune response in Animals having less than one million lymphocytes: The larvae of the toad *Alytes obstetricans.*
Immunology, 1970,Vol. 19, pp 353 - 361.

Du Pasquier, L. (1973),
Ontogeny of the Immune Response in Cold-Blooded Vertebrates.
Current topics in Microbiol Immunol., Vol. 61, pp 37 - 88.

Du Pasquier, L. (1982)
Ontogeny of immunological functions in amphibians in : Cohen N, Sigel M.M. eds. The reticulo endothelial system : Phylogeny and Ontogeny, New York, Plenum, 1982, 633.

Du Pasquier,L., and Horton J.D. (1976),
The effect of Thymectomy on the MLR and phytohemagglutination responsiveness in the clawed toad *Xenopus laevis* .
Immunogenetics, Vol. 3,pp 105 - 112, 1976.

Egberts, E., Secombes, J.C., and et al. (1983),
Analysis of lymphocytes heterogeneity in carp, *Cyprinus carpio* L, using monoclonal antibodies.
Dev. and Comp. Imm.,Vol. 7, pp 749 - 754.

Egidius, E.C. and Anderson, K. (1979),
Both immunisation : a practical and non-stressing method of vaccinating formed rainbow trout (*Salmo gairdneri*) against vibrosius.
J. Fish. Dis.,Vol. 2, pp 405 - 410.

Elizabeth, F.M. (1962),
Histophysiological studies of the adrenal tissues of the goldfish.
Can. Journal of Zoology, Vol. 40, page 451.

Elliot, J.A.K. (1982),
Neuroendocrine mechanisms involved in the control of ovarian development and vitellogenesis in the rainbow trout.
Ph.D. Thesis, Chapters 2 & 6, pp 14 - 43 & pp 327 - 372.

Ellis, A.E. (1974),
Aspects of the lymphoid and reticulo-endothelial systems in the plaice *Pleuronectes platessa*, L.
Ph.D. Thesis, University of Aberdeen.

Ellis,A.E. (1977a),
The leucocytes of fish: A review Journal of Fish Biol.,Vol. 11, pp 453 - 491.

Ellis, A.E. (1977b),
Ontogeny of the immune response in *Salmo salar* : Histogenesis of the lymphoid organs and appearance of membrance immuno-globulin and mixed leucocyte reactivity.
Dev. Immunobiology by Solomon J.B. and Horton J.D. (1977), Elsevier/North - Holland Biomedical Press, Amsterdam.

Ellis, A.E. (1978),
The immunology of Teleosts,
Fish Pathology edit. by Ronald J. Roberts.
Bailliere Tindall, London.

Ellis, A.E. (1982),
Differences between the immune mechanisms of fish and higher vertebrates Microbial Diseases of Fish by Roberts R.J., pp 1 - 29, Academic Press, London.

Ellis, A.E. (1985),
Fish serum precipitins to *Aeromonas salmonicida* Exotoxins and Protease Lipoprotein Reactions : A critical appraisal.
In "Fish Immunology (1985), Edt. by Manning, M.J., and Tatner, M.F., Academic Press, London, New York.

Ellis, A.E., and De Sousa, M. (1974),
Phylogeny of the lymphoid system. 1 - A study of the fate of circulating lymphocytes in plaice.
Eur. Journal of Immunology, Vol. 4, pp 338 - 343.

Emmrich, F., Richter, R.F., and Ambrosius, H. (1975),
Immunoglobulin determinants on the surface of lymphoid cells of carp, European Journal of Immunology, Vol. 5, pp 76 - 78.

Emmrich, F., Richter, R.F., and Ambrosius, H. (1980),
Anti-Idiotypic IgM antibodies Produced in carps with BAL B/C Mouse Myeloma Protein S117,
Dev. and Comp. Imm., Vol.4 pp 245 - 254.

Etlinger, H.M., Hodgins, H.O., and Chiller, J.M. (1976a),
Rainbow trout leucocyte culture : A simplified method.
In vitro; Vol. 12, pp 599 - 601.

Etlinger, H.M., Hodgins, H.O. and Chiller, J.M. (1976b),
Characterisation of lymphocytes in a primitive teleost, *Salmo gairdneri.*
In: Phylogeny of Thymus and Bone Marrow Bursa Cells.
Ed. Wright, R.K. and Cooper E.L., Elsevier/North Holland Biomedical press. pp 83 - 91.

Etlinger, H.M., Hodgins, H.O., and Chiller, J.M. (1976c),
Evolution of the lymphoid system
I - Evidence for lymphocytes Heterogeneity in Rainbow Trout Revealed by the Organ Distribution of Mitogenic Responses.
The Journal of Immunology, Vol. 116(6).

Etlinger, H.M., Hodgins, H.O., and Chiller, J.M. (1977),
Evolution of the lymphoid system.
II Evidence for immunoglobulin determinants on all rainbow trout lymphocytes and demonstration of mixed leukocyte reaction.
Eur. J. Immunology, Vol. 7, 881.

Etlinger, H.M., Hodgins, H.O. and Chiller, J.M. (1978),
Evolution of the lymphoid system. III. Morphological and functional consequences of mitogenic stimulation of rainbow trout lymphocytes.
Dev. and Comp. Immunol., Vol. 21, pp 263 - 276.

Etlinger, H.M., Hodgins, H.O., and Chiller, J.M. (1979),
Evolution of the lymphoid system.
IV Murine T-dependent but not T-independent antigens are very immunogenic in rainbow trout (*Salmo gairdneri*).
Cell Immunol ., Vol. 47, pp 400.

Ezzat, A.A., Shabana, M.B., and Farghaly, A.M. (1974),
Studies in the blood characteristics of *Tilapia zilli* (Gervais),
I- Blood cells.
J. Fish Biol., Vol. 6 , pp 1 - 12.

Fänge, R. (1966),
Comparative aspects of excretory and lymphoid tissue.
Phylogeny of Immunity by R.T. Smith & et al.
University of Florida Press - Gainseville.

Fänge, R. (1968),
White blood cells and lymphomyeloid Tissues in fish.
Bull. of int. Epiz., Vol. 69, pp 9 - 10.

Fänge, R., and Pulsford, A. (1985),
The thymus of the angler fish, *Lophius piscatorius* (Pisces : Teleostei).
A light and electron microscopic study.
In Fish Immunology, Ed. by Manning, M.J. and Tatner, M.F.
Academic Press, London, New York.

Fänge, R., Lundblad, G., and Lind, J. (1976),
Lysozyme and chitinase in Blood and Lymphomyeloid tissues of marine fish. Mar. Biol., Vol. 36, pp 277 - 282

Ferguson, H.W. (1976a),
The reticulo-endothelial system of teleost fish with special reference to the plaice (*Pleuronectes platessa*).
Ph.D. Thesis, University of Stirling.

Ferguson, H.W. (1976b),
The Ultrastructure of plaice (*Pleuronectes platessa*) leucocytes
J. Fish Biol., (Vol.8) pp 139 - 142.

Ferren, F.A. (1967),
Role of the spleen in the immune response of teleosts and elasmobranchs.
J. of the Florida Medical Association, Vol. 54, No. 5

Fichtelius, K.E., Finstad, J., and Good, R.A. (1969),
The phylogenetic occurence of lymphocytes within the Gut epithelium.
Int. . Arch. Allergy, 35 : pp 119 - 133.

Finstad, J.F., Good, R.A. (1966),
Phylogenetic studies of adapative immune responses in the lower vertebrates Phylogeny of Immunity.
Edit. by Smith R.T.

Finstad, J., Papermaster, B.W., and Good, R.A. (1964),
Evolution of the Immune response,
II. Morphologic studies on the origin of the thymus and organized lymphoid tissue.
Lab. Invest., 13 : 490→512.

Flajnik, M.F., DuPasquier, L., and Cohen, N. (1985),
Immune responses of thymus lymphocyte embryonic chimeras: Studies on tolerance and MHC restriction in *Xenopus*.
Eur. J. Immunol.,Vol. 15, pp 540 - 547.

Fletcher, T.C. (1982),
Non-specific defence mechanisms of fish.
Dev. Comp. Immunol.,Suppl. 2, pp 123 - 132.

Fletcher, T.C. and White, A. (1973),
Antibody production in the plaice Pleuronectes platessa L. after oral and parental immunisation with *Vibrio anguillarum*.
Aquaculture, Vol. 1, pp 417 - 428.

Flitney, E. (1982),
Autoradiography,
Theory and Practice of Histological technique by John D. Bancroft and Alan Stevens forward by I.M.P. Dawson - 2nd Edition, Churchill Livingstone, Edinburgh, London.

Gahan, P.B., (1972),
Autoradiography for Biologists.
Academic Press, London and New York.

Galili, U., and Schlesinger, M. (1974),
The formation of stable E. Rosettes after Neurominidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells.
Journal of Immunology, Vol. 112, No.5

Gomori, G.M.D. (1937),
Silver impregnation of reticulum in paraffin sections,
American Journal of Pathology, Vol. 13, pp 993 - 1002

Gomori, G., M.D. (1942),
Distribution of acid phosphatase in the tissues under normal and under pathological conditions.
Archives of Pathology, Vol. 32, pp 189 - 199.

Good, R.A., and Finstad, J. (1967),
The phylogenetic development of immune responses and the germinal center system. In : Germinal Centers in Immune Responses (Ed. by Cottier, H., Odartchenko, N., Schindler, R., and Congdon, C.C.), pp 4 - 27. Springer - Verlag, Berlin.

Good, R.A., Finstad, J., Pollara, B. and Gabrielsen, A.E.(1966),
II Morphologic studies on the evolution of the lymphoid tissues among the lower vertebrates.
Phylogeny of Immunity by Smith R.T. Miescher, P.A., and Good, R.A. Gainesville University of Florida Press.

Good, R.A., Dalmasso, A.P., Martinez, C., Archer, O.K., Pierce, J.C. and Papermaster, B.W.(1962),
The role of the thymus in Development of Immunologic capacity in Rabbits and Mice. Journal of Experimental Medicine, Vol. 116 Page 773 - 803.

Gould, R.W. Antipa, R. and Amend, D.F. (1979),
Immersion vaccination of sock-eye salmon (*Onchorhynchus nerka* with two pathogenic stains of *Vibrio anguillarum*.
J. Fish Res. Bd. Can., Vol. 36, pp 222 - 225.

Goven, B.A., Dauve, O.L., and Gratze, K.J.B. (1980),
In vivo and *In vitro* Anaphylactic type reactions in fish.
Dev. and Comp. Imm., Vol. 4 pp 55 - 64.

Grace, M.F. (1981),
The functional histogenesis of the immune system in rainbow trout, *Salmo gairdneri.*
PhD. Thesis, Richardson, 1836, University of Hull.

Grace, M.F., and Manning, M.J. (1980),
Histogenesis of the lymphoid organs in Rainbow Trout *Salmo gairdneri* . Rich, Dev. and Comp. Imm., Vol. 4 pp 255 - 264.

Grace, M.F., Botham, J.W., and Manning, M.J. (1981),
Ontogeny of Lymphoid Organ Function in Fish.
Aspects of Dev. and Comp. Immunology I., by J.B. Solomon
pp 467 - 468. Pergamon Press, Oxford and New York

Gräf, R., and Schlüns, J. (1979),
Ultrastructural and Histochemical Investigation of the terminal capillaries in the spleen of the carp (*Cyprinus carpio* L.)
Cell and Tissue Research, Vol. 196, pp 289 - 306.

Griffin, B.R. (1983),
Opsonic effect of rainbow trout (*Salmo gairdneri*) Antibody on phagocytosis of *Yersinia ruckeri* by Trout Leukocytes.
Dev. and Comp., Imm. Vol. pp 253 - 259.

Grodzmskim, C. (M.Z.) (1948),
The influence of temperature upon the rate of the heart in the embryos of teleost fishes. Bulletin international of L'Academie Polonaisse des Sciences et des lettres. 1948 - 1949, Ser B.N.7 10B 11.

Grondel, J.L. and Boesten, J.A.M. (1982),
The influence of Antibiotics on the immune system
I. inhibition of the mitogenic leukocyte response in vitro by oxytetra cycline, Dev. and Comp. Imm., Vol. (2), pp 211 - 216.

Grondel, J.L., and Harmsen, E.G.M. (1985),
Do fish have interleukin? "Fish Immunology", Edt. by Manning, M.J
Academic Press, London.

Hafter, E. (1952),
Histological age changes in the thymus of the teleost, Astyanax.
Journal of Morphology, Vol. 90, pp 555 - 579.

Hall, R.G. Jr. (1970),
Basic fuchsin and picro-indigo Carmine:
A selective stain for cells in mitosis and for autoradiography
Stain technology, Vol. 45, No.2.

Hammar, J.A. (1909),
Funfzig jahre thymusforschung kritische ubersicht der normalen morphologie.
Eng. Anat. Ent wickle, 19, pp 1 - 74.

Harboe, M. (1963),
A note on the absence of immune reactions in Myxinoids. In the biology of Myxine. A. Brodal and R. Fängé (Eds).
Oslo: Universitet for laget, page 456.

Hayat, M.A. (1970),
Principles and techniques of E.M. Biological applications Vol. 1.
Van Nostrand, Reinhold, Comp.

Henderson, A.A., and Stichney R. R. (1980),
Immune hypersensitivity in Intensively cultured Tilapia species.
Trans. Amer. Fish Society, 109: pp 244 - 247.

Henrickson, R.C. and Matolsty, A.G. (1967),
The fine structure of teleost epidermis.
Journal of Ultrastructure Res.,Vol. 21, pp 194 - 232.

Hildemann, W.H. (1957),
Scale homotransplantation in goldfish (*Carassius auratus)*
Annals, New York Academy of Science, Vol. 64, pp 775 - 791.

Hildemann, W.H. (1970),
Transplantation immunity in fishes : Agnatha, Chondrichthyes and,
Osteichthyes., Transplant. Proc.,Vol. 2, pp 253 - 259.

Hildemann, W.H. (1972),
Transplantation reactions of two species of Osteichthyes (Teleostei),
from South Pacific Coral reefs, Transplantation, Vol. 2, pp 261 - 269

Hildemann, W.H. and Haas, R. (1960),
Comparative studies of homotransplantation in fishes.
Journal of cell and comp. Phys.,Vo. 55, pp 227.

Hinuma Shuji, Abo Toru, Kumagai Katsuo and Hata Mitsuo (1980),
The potent activity of fresh water fish kidney cells in cell killing.
1- characterization and species distribution of cytotoxicity.
Dev. & Comp. Imm.,Vol 4, pp 653 - 666.

Hill, B.H. (1935),
The early development of the thymus glands in *Amia calva.*
Journal of Morphology, Vol. 57, pp 61 - 89.

Hines, R., and Yashouv, A. (1970),
Differential leucocyte counts and total leucocyte and erythrocyte counts
for some normal Israeli, Mirror Carp,
Bamidgeh, Vol. 22, pp 106 - 116.

Hisaaka, K.K., and Battle, H.I.(1958),
The normal developmental stages of the Zebrafish, Brachydanio rerio
(Hamilton - Buchanan). Journal of Morphology, Vol. 102,
January, March, May.

Hightower, J.A., McCumber, L.J. (1984),
Blood cells of *Fundulus heterochlitus* (L),
Journal Fish Biol., Vol. 24, pp 587 - 598

Hockney, M.J. (1985),
An investigation of the skin of rainbow trout, *Salmo gairdneri*
Richardson, for antigen uptake mechanisms following spray
vaccination. "Fish Immunology", Edt. by Manning, M.J., &
Tatner, M.F., 1985, Academic Press, London, New York.

Hodkinson, M., and Hunter, A. (1970),
Immune response of U.D.N-infected Salmon to Saprolegnia.
Journal Fish Biol.,(1970), Vol. 2, pp 305 - 311.

Holmgren, N. (1950),
On the pronephros and the blood in *Myxine glutinosa*.
Acta Zoologica ,tockholm, Vol. 31, pp 233 - 348.

Hood, L.E., Weissman, I.L., Wood, W.B. (1978),
"Immunology". The Bengamin/Cummings Publishing Company Inc.
London, Amsterdam.

Horne, M.T. and ~~et al.~~, Roberts, R.J., Tatner, M.F.
and Ward, P.D.(1983),
The effects of the use of potassium alum adjuvant in vaccines against
vibriosius in rainbow trout, *Salmo gairdneri*, Richardson.
J. Fish Dis. In press.

Horton, J.D., and Manning, M.J. (1972),
Response to skin allografts in *Xenopus laevis* following thymectomy
at early stages of lymphoid organ maturation.
Transplantation, Vol. 14, pp 141.

Horton, J.D., and Manning, M.J. (1980),
Effect of early thymectomy on the cellular changes occuring in the spleen.
Journal of Fish Diseases, Vol. 3, pp 399 - 412.

Horton, J.D., Rimmer, J.J, and Horton, L.T. (1976)
The effect of Thymectomy at different stages of larval development
on the Immune Response of the Clawed Toad to Sheep Erythrocytes.
Journal of Exp. Zool., Vol. 196, No.2

Horton, J.D., Rimmer, J.J., and Horton, L.T. (1977),
Critical role of the thymus in establishing humoral immunity
in Amphibians: Studies in Xenopus thymectomized in larval
and adult life. Dev. & Comp. Immunology, Vol. 1, pp 119 - 130

Howard, J.G. (1975),
Immunological tolerance. In Defence and Recognition, Vol. 22,
Cellular aspects E.S. Lennos (Ed.) University Park Press,
Baltimore, U.S.A.

Howard J.G. and Mitchison, N.A. (1975),
Immunological tolerance. In progress in Allergy, 18, 43.
Waksman B.H. (ed.) Karger, Basel.

Hudson, L. and Hay, F.C. (1980),
"Practical Immunology", 2nd edition.
Oxford : Blackwell Scientific Publications.,

Humphrey, J.H. and Frank, M.M. (1976),
Immunological Tolerance.
British Medical Bulletin, 32, 99.

Ichiro Iuchi (1982),
Characterization of Rabbit Antisera against rainbow trout (*Salmo
gairdneri*, irideus) Adult Hemoglobins and preparation of their
specific antibodies.
Journal of Faculty of Science , Vol. 15(2).

Ichiro Iuchi (1985),
Cellular and Molecular Bases of the larval - Adult shift of hemoglobins
in Fish. Zoological Science, 2 : pp 11 - 23.

Ichiro Iuchi and Masamichi Yamamoto (1983),
Erythropoiesis in the developing rainbow trout, *Salmo gairdneri* , irideus: Histochemical and Immunochemical Detection of Erythropoietic Organs.
Journal of Experimental Zoology, pp 226 - 409 - 417.

Ingebrigtsen K.&Bergsjo T.,
Methodological problems in whole body auto-radiography of rainbow trout. Acta vet ScAND, 20(4), 1979, pp 604 - 606

Ingram, G.A. (1978),
The immune response of the brown-trout (*Salmo trutta* L) to injected antigens. PhD Thesis, University of Salford.

Ingram G.A. (1980),
Substances involved in the natural resistance of fish to infection - A review. J. Fish Biol.,(1980) 16, pp 23 - 60.

Ingram, G.A. and Alexander, J.B. (1977),
The primary immune response of brown trout (*Salmo trutta)* to injection with cellular antigens.
Journal of Fish Biology, Vol. 10, pp 63 - 72.

Ingram, G.A., and Alexander, J.B. (1979),
The immunoglobulin of the brown trout; *Salmo trutta* and its concentration in the serum of antigen - stimulated and non-stimulated fish. J. Fish Biol., (1979), 14, pp 249 - 260.

Ingram, G.A. and Alexander J.B. (1980)
The immune response of the brown trout *Salmo trutta* to lipopolysaccharide.
J. Fish Biol.,(1980), pp 16, 181 - 197.

Ingram, G.A., and Alexander J.B. (1981a),
The primary immune response of brown trout (*Salmo trutta*) to cellular and soluble antigens.
Enumeration of antibody - secreting and antigen - binding cells, and the production of antibody.
Acta. Biol. meol. germ. Band 40, Siete, pp 317 - 330.

Ingram, G.A. and Alexander, J.B (1981b)
A comparison of the methods used to detect the cellular and humoral immune response of Brown Trout .
Dev. Biol. Standard, 49, pp 295 - 299.

Ingram, G.A., Alexander, J.B. (1981c),
A comparison of the immune response of the brown trout (*Salmo trutta*) to a protein and a carbohydrate antigen.
Aspects of Dev. and Comp. Immunology I by J.B. Solomon.
Perganon Press, New York. 1981.

Itami, T., and Kusuda, R. (1978),
Efficacy of a vaccination by spray administration against vibrosis in culture ayu. Bull. Japan Soc. Sci. Fish, Vol. 44, pp 1413

Ito, T., and Hoshino, T. (1966),
Fine structure of the epithelial reticular cells of the medulla of the thymus in the golden hamster, *Z. Zellforsch, mikros. Anat.*, 69, 311.

Jaraslow, B.N., and Beverly, A.S. (1972),
Differential Sensitivity to Hibernation of Early and Late Events in Development of the Immune Response.
Journal of Exp. Zool.,181, pp 111

Jayaraman, S., Muthukkaruppan, VR., (1977),
In vitro correlate of transplantation Immunity: Spleen Cell Migration Inhibition in the lizard, *Calotes versicolor.*
Dev. & Comp. Immunology, Vol., 1, pp 133 - 144.

Jayaraman, S., Mohan, R. and Muthukkaruppan, VR. (1979),
Relationship between Migration Inhibition and Plaque - Forming Cell Responses to Sheep Erythrocytes in the teleost, *Tilapia mossambica,*
Dev. & Comp. Imm.,Vol 3, pp 67 - 75.

Jerne, N.K.C., Henry, Nodin, A.A., Fuji, H. , Koros, A.M.C., and Lefkovits, I. (1974),
Plaque forming cells : Methodology & Theory.
Transplant. Rev.,18, pp 130 - 191.

Johnson, K.A., Flynn, J.K.S., & Amend, D.F. (1982),
Onset of Immunity in Salmonid fry vaccinated by direct immersion in *Vibrio anguillarum* and *Yersinia ruckeri* Bacterins.
Journal of Fish Diseases, (5), pp 197 - 205.

Jones, A.J. (1972),
The early development of Substrate Brooding Cichlids (Teleostei : Cichlidae) with a discussion of a New System of staging. Journal of Morphology,Vol. 136, January, April. 1972

Jurd, R.D. (1985),
Specilization in the teleost and Anuran Immune Response. A Comparative Critique. In Fish Immunology, Edited by Manning, M.J., and Tatner, M.F.
Academic Press, London, New York.

Kaastrup, P., and Claus, K. (1983)
Complement in the Carp fish.
Dev. Comp. Immunology, Vol. 7, pp 781 - 782.

Kallman , K.D. (1970),
Genetics of Tissue Transplantation in Teleostei.
Transplantation proceedings, Vol. II, No.2.

Kanakambika, P. and Muthukkaruppan, VR. (1972),
Immunological competence in the Newly Hatched Lizard, *Calotes versicolar.*
Proc. Soc. Exp. Biol. Med.,Vol. 140, pp 21 - 23.

Kawakami, Masaya, Ihara Izumi, Ihara Setsunosuke, Suzuki, Tsushi, A., and Fukui Kazuhiro (1984),
A group of bactericidal factors conserved by vertebrates for more than 300 million years. Journal of Immunology, Vol. 132, No.5, May 1984.

Khalifa, A.K., and Post, G. (1976),
Immune response of advanced rainbow trout fry to *Aeromonas liquefaciens.* Prog. Fish. Culturist, Vol.5, 38, pp 66

Klapper, D., and William, C.L. (1977),
Phylogeny of Immunoglobulin structure and function; characterisation of the Cysteine - containing peptide involved in the Penta merisation of Shark IgM.
Dev. & Comp. Imm.,Vol. 1, pp 81 - 92.

Klein, J. (1977),
Evolution and function of the major histocompatibility system : facts and speculation. In "The Major Histocompatibility System in Man and Animal". (Ed. Gotze, D.), Springer-Verlag, Berlin, pp 339 - 378.

Knight, A.E., (1963),
The embryonic and larval development of the rainbow trout.
Trans. Am. Fish. Soc.,92(4), pp 344 - 355.

Koo, G.C., Goldberg, C.L. (1979),
A simplified technique for H.Y.
Typing differentiation 14, pp 189 - 192.

Krantz, G.E., Reddecliff, J.M., and Heist, C.E. (1963),
Development of antibodies against *Aeromonas salmonicida* in trout.
Journal of Immunology, Vol. 91, pp 757 - 760.

Laale, H.W. (1984),
Poly embryony in teleostean fishes : double monstrosities and triplets.
J. Fish Biol.,Vol. 24, pp 711 - 719.

Lafferty, K.J., and Cunningham, A.J. (1975),
A new analysis of allogeneic interactions.
Aust, J. for Exp. Biol. Med. Science, Vol. 53 : 27.

Lavid, L.M., and Oswald, R.L. (1975),
A note on the use of benzocaine (ethyl-P-amino-benzoate) as a fish anaesthetic.
J. Inst. Fish Mgnt.,Vol. 6, pp 92 - 93.

Lamers, C.H.J., and De Haas, M.J.H. (1983),
The development of immunological memory in carp *(Cyprinus carpio L.)* to a bacterial antigen.
Dev. & Comp. Immunology, Vol. 7, pp 713 - 714.

Lele, S.H. (1933a),
On the phasical history of the thymus gland in plaice of various ages with note on the involution...
Journal of the University, Bombay, Vol.1, pp 37 - 53.

Lewis, D.N., Eurell, T., Cannon, N.S., Crumbles, L.C., (1979),
T & B Cell analogues from peripheral blood of immune channel catfish, *Ictalurus punctatus.*
J. Fish Biol.,14 : pp 31 - 37.

Liewes, E.W., Van Daby R.H. (1982),
Optimisation & Kinetics of in vitro stimulation of carp, *Cyprinus carpio* - leucocytes.
Vet. Immunol. Immunopathol.,Vol.3(3), pp 325 - 344.

Lobb, G.J., and William, C.L. (1982),
Fish lymphocytes differ in the expression of surface immunoglobulin.
Dev. & Comp. Imm.,Vol. 6, pp 473 - 479.

Lopez, D.M., Sigel, M.M. and Esquerazi, V. (1973),
Differential response of shark lymphocytes to Mitogens.
Fed. Proc.,Vol. 32, pp 1012.

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 concanavalin A.
Cell Immunol.,10, pp 287 - 293.

Lucas, A.M. and Casimir, J. (1961),
Arneth counts on granulocytes of chickens,
Atlas of Avian Hematology. Agriculture Monograph 25,
U.S. Department of Agriculture, Washington.

Luna , L.G. (1960, 1968),
Manual of Histological Staining Methods of the Armed Forces Institute of Pathology, 3rd edition.
McGraw Hill Book Company, New York, Toronto, London, Sydney.

Mac Arthur, J.I., and Fletcher, T.C. (1985)
Phagocytosis in Fish "Fish Immunology". Edited by Margaret Manning and Tatner, M.F. Academic Press London, New York.

Madison, B. C. Jr. (1968),
A simple apparatus for Ultraviolet polymerization of water soluble Embedding Media Employed in Electron Microscopy.
Journal of microscopy, Vol. 7, pp 441 - 444.

Mahajan, C.L., and Dheer, J.M.S (1979),
Autoradiography and different hemoglobin staining as aids to the study of fish hematology.
Experienta (BASEL), 35(6), 1979, pp 834 - 835.

Majoor, G.B., Van't Veen, M.B., Zaalberg, G.B. (1975),
Quick and easy production of monolayer plaque assay slides.
Journal of Immunological Methods, Vol. 7, pp 301 - 304.

Mandel, T. (1968),
Ultrastructure of epithelial cells in medulla of guinea pig thymus.
Aust, J. Exp. Biol. Med. Sci.,46, pp 755.

Manning, M.J. (1979),
Phylogeny of Immunological Memory,
Elsevier/North Holland Biomedical Press,
Amsterdam, New York, Oxford.

Manning, M.J. (1980),
The Evolution of vertebrate lymphoid organs.
Proceedings of 1st congress of Dev. Journal of Comp. Immunology,
27th July - 1st August, 1980, Aberdeen.

Manning, M.J. (1981),
A comparative view of the thymus in vertebrates.
In " The thymus gland" (ed. Kendall Marion, D.)
Academic Press, London, pp 7 - 17.

Manning , M.J., and Turner, R.J. (1976),
"Comparative Immunobiology" Blackie, Glasgow and London.

Manning, M.J., Grace, M.F. and Secombes, C.J. (1982a),
Ontogenic Aspects of Tolerance and Immunity in carp and rainbow trout: Studies on the role of the thymus.
Dev. & Comp. Imm. Suppl. 2, pp 75 - 82.

Manning, M.J., Grace, M.F. and Secombes, C.J. (1982b),
Developmental aspects of immunity and Tolerance in fish.
In "Microbial Diseases of Fish", (Ed. R.J. Roberts) pp 31 - 46.
Acad. Press, London.

Marchalonis, J.J. (1969),
Isolation and characterization of Immunoglobulin like Proteins
of the Australian Lungfish (*Neoceratodus forsteri*).
Austral. J. Exp. Niol. Med. Sci., 47, pp 405 - 419.

Marchalonis, J.J. (1971),
Isolation and partial characterisation of immunoglobulins of Gold fish
(*Carassius auratus* and *Cyprinus carpio*).
Immunology, Vol. 20, pp 161 - 173.

Marshall, H.E., and White R.G. (1961),
The immunological reactivity of the thymus.
Brit. Journal of Experimental Pathology, 42 : pp 379 - 384.

Masamichi Yamamoto and Ichiro Iuchi (1975),
Electron Microscopic Study of Erythrocytes in Developing Rainbow
Trout. Salmo gairdneri irrideus, with particular reference to changes in the
cell line.
Journal of Experimental Zoology, Vol. 191, No.3.

Meyer, R.K. (1980),
Mapping the normal and Regenerating Retinotectal Projection of
Goldfish, with Autoradiographic Methods. Journal of Comparative
Neurology 189, pp 273 - 289.

Miller, J.F.A.P., and Osoba, N. (1967),
Current concepts of the immunological function of the thymus.
Physiol. Rev., Vol. 47, pp 437 - 520.

Mishell, R.I. and Dutton, R.W. (1967)
Immunisation of Dissociated spleen cell cultures from Normal Mice.
Journal of Experimental Medicine, Vol. 126, page 423.

Mohsin, A.K.M., ONG, S.L., AMBAK, M.A. (1984).
Effect of Malathion on sepat-Siam Trichogaster- pectoralis and Tilapia
Sarotheradon mossambicus
Pertanika, Vol. 7(1), pp 57 to 60.

Moore, M.A.S. and Owen J.M.T. (1967)
Experimental studies on the development of the thymus.
Journal of Experimental Medicine , Vol.126, pp. 715 to 725.

Mori, Y. (1931),
On the transformation of ordinary scales into lateral line scales
in the goldfish. J. Fac. Sci. Imp., University of Tokyo.
Vol. 2, pp 185 - 194.

Morrow, W.J.W. (1978)
The immune response of the dogfish.....
PhD Thesis, Plymouth Polytechnic, U.K.

Mount, D.I. (1961).
Additional Information on a system for controlling the dissolved
Oxygen content of water.
Trans . Am.fish Soc. 90(3) pp. 100 to 103.

Mughal M.S. and Manning, M.J. (1985),
Antibody Responses of Young Carp, *Cyprinus carpio* and Gray Mullet, *Chelon labrosus*, Immunized with Soluble Antigen Fish Immunology , Edt. by Manning, M.J. , Academic Press, London.

McClure (1915)
The development of the lymphatic system in Fishes [with special reference to its development in Trout] Memoirs of the Wistar - Institute of Anatomy & Biology, Vol .4.

Mckinney, E.C., Smith, S.B, Sigel, M.M. (1974).
Studies on the function of fish Leucocytes and thrombocytes. In vitro (Rochville), Vol. 9(5) , pp 367 - 368.

Mckinney, E.C., Mcleod, T.F. and Sigel, M.M. (1981)
Allograft Rejection in a Holostean fish, *Lepisosteus platyrhincus*. Dev. & Comp. Imm., Vol.5, pp. 65 to 74.

Mckinney, E.C., Ortiz, G. , Lee, J.C., Sigel, M.M., Lopez, D.M., Epstein, R.S. and McLeod, T.F. (1976).
Lymphocytes of Fish: In "Phylogeny of thymus & Bone Marrow-Bursa cells" . Edt. by Wright, R.K., and Cooper, E.L. Elsevier/North - Holland Biomedical Press, Amsterdam The Netherlands.

Nagata, S. (1976),
An electron microscopic study on the thymus of larval and metamorphosed toads, *Xenopus laevis* Daudin.
J. Fac. Sci., Hokkaido University, Ser. VI, Zool., 20: 263.

Nandi, J. (1962),
The structure of the Interrenal Gland In Teleost Fishes.
University of California Publications in Zoology, Vol. 65, No. 2, pp 129 - 212.

Nossal, G.J.V. and Shellam, G.R. (1967),
Recent advances in immunological tolerance to a potent - Non-Living antigen In: "Radiation & Control of Immune Response",
Report of a panel on Radiation and the control of immune response organized by the International Atomic Energy Agency and held in Paris, pp 22 - 24, June 1967.

Novikoff, P. M., Alex, B., Novikoff, N.Q and Hana J.J. (1971),
Golgi Apparatus and Lysosomes of neurons in rat dorsal root ganglia - studied by thick section and thin section.
Cytochemistry Journal of Cell Biology, Vol. 50. pp 859 - 886.

Omura, Y., and Mikio, O. (1969)
Histological studies on the pineal organ of 15 species of Teleosts.
Bulletin of Japanese Society of Scientific Fisheries, Vol. 35, No.10.

O'Neill, J.G. (1978),
The immune response in Teleosts:
The effects of temperature & heavy metals
Ph.D. Thesis, Department of Life Sciences,
Trent Polytechnic, Nottingham, England.

O'Neill, J.G. (1980a),
Antegenic Competition in Teleosts
Aspects of Dev. & Comp. Immunology, I by J.B. Solomon.

O'Neill, J.G. (1980b),
Blood clearance of MS 2 bacteriophage in *Salmo trutta*: a paradoxon
Experientia, 36.

Opstad, A.M. (1959),
A methyl green - pyronin stain for plasma cells in tissues.
Stain tech.,Vol. 34, pp 293.

Ortiz-Muniz, G., and Sigel, J.J. (1971),
Antibody synthesis in lymphoid organs of two marine teleosts.
Journal of Reticuloendothelial Society, 9, pp 42 - 52.

Ourth, D. D. (1980),
Secretory IgM, Lysozyme & lymphocytes in the skin, Mucus of the channel catfish, *Ictalurus punctatus* ,
Dev. & Comp. Imm.,Vol. 4, pp 65 - 74 (1980).

Ourth, D.D., Wilson, E.A. (1982),
Alternative pathway of complement and bactericidal response of the channel catfish to *Salmonella paratyphi.*
Dev. Comp. Immunol., Vol. 6, pp 75 - 86.

Owen, J.J.T., Andrew, T., Jordan, R.K., McElroy, P., Robinson, J.H., Singh, U., and Willcox, H.N.A. (1977),
Factors influencing the primary differentiation of lymphocyte population.
Dev. Immunobiology, Edited by Solomon, J.B. and Horton J.D.
Elsevier/North - Holland Biomedical Press, Amsterdam.

Owen, R. D. (1945),
Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins.
Science, Vol. 102, No. 2651.

Padmanabham, K.G., (1955),
Breeding habits and early embryology of *Macropodus cupanus*.
Bulletin of the Central Research Institute,
University of Travancore Ser. C. 4(1), pp 1 - 46.

Palmer, C.J., and Culley M.B. (1984),
The egg and early life stages of the Sandmelt, Atherina presbyter Cuvier.
J. Fish Biol.,(24), pp 537 - 544.

Paterson, W.D. and Fryer, J.L. (1974),
Immune response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* cells administered in Freund's Complete Adjuvant.
J. Fish. Res. Bd. Can. 31, 1751,

Payne, A.I., Collinson, R.I. (1983),
A comparison of the biological characteristics of *Sarotherodon - niloticus* with those of *Sarotherodon hureus* and other *Tilapia* of the Delta and Lower Nile, Egypt.
Aquaculture, 30(1 - 4), (335 - 352).

Pearse, A.G.E. (1961),
Histochemistry, theoretical and applied Histology, 2nd Ed.
Pub. J. & A. Churchill, London.

Pechan, P., Wachtel, S.S. & Reinboth, R. (1979),
H - Y antigen in the Teleost.
Differentiation, Vol. 14, part 3, pp 189 - 192.

Petty, C.L., and McKinney , C. E. (1981),
Mitogen Induced Cytotoxicity in the Nurse Shark.
Dev. & Comp. Imm.,Vol. 5, pp 53 - 64.

Pickering, A.D., and Willoughby, L.G. (1982),
Saprolegnia Infections of Salmonid Fish.
Microbial Diseases of Fish, Edt. by Roberts, R.J.
Academic Press, London, New York.

Pillai, P.S., and Muthukkaruppan, VR. (1982),
The relationship between Immune Rosette Forming cells & Plaque Forming cells in the lizards, *Calotes versicolor*.
Dev. & Comp. Immunology, Vol. 6, pp 321 - 328.

Pontius, H. and Ambrosius, H. (1972)
Contribution to the immune biology of poikilothermic vertebrates.
Acta Biologica Medica. Germanica., Vol. 39, pp 319

Popovic, M., Galic, M., Dujin, T. (1980),
Effect of parenteral injection of therapeutic doses of penicillin and oxytetracycline on the titre and persistance of brucella agglutinins in the blood of immunized pigs.
Aquaculture, 19 (pp 172 - 189).

Post, G. (1962),
Immunisation as a method of disease control in fish.
American Fishes and U.S. Trout new. Vol. 7, pp 14 - 17.

Rabinowitch, M. (1968),
Phagocytosis " The engulfment stage".
Seminars Haematology, Vol. 5, pp 134

Raff, M.C. (1970),
Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence.
Immunology, Vol 19, pp 637 - 650.

Reith, E.J., and Ross, M.H. (1972),
"Atlas of Descriptive Histology",
New York, Evanston, London, 2nd Edition.

Rich, R.R. and Pierce, W.C. (1974),
Biological Expressions of Lymphocyte Activation,
III Suppression of Plaque Forming cell Responses in Vitro by Supernatant Fluids from Concanavalin A- activated Spleen Cell cultures. Journal of Immunology, Vol. 112, No.4

Richardson, R.C., Jarett, L., and Finke, E.H. (1960),
Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology, Vol. 35, pp 313 - 323.

Richter, R., (1971),
Specificity of Immune Reactions in Bony fishes from Antigen - Antibody Reactions - Contributions to the IVth Symposion on Immunology of the Gesells chaft fur allergie und Immunitats forschung der DDR "
Edited by Prof. Dr. H. Ambrosius & et al.
V.E.B. Gustav - Fischer Verlag Jena.

Ridgway G.J., Hodgins, H.O., and Klontz, G.W. (1966),
The immune response in teleosts.
Phylogeny of Immunity by R.T. Smith
University of Florida, Gainesville.

Rijkers, G.T. (1980a),
The immune system of Cyprinid fish,
Landbow Hoge School, Wageningen (Netherlands). Ph.D Thesis

Rijkers, G.T. (1981),
Introduction to Fish Immunology,
Dev. & Comp. Immunology, 5(4), pp 527 - 534.

Rijkers, G.T. (1982),
Kinetics of cellular and humoral immune reactions in fish.
Dev. Comp. Immunol., Suppl. 2, pp 93 - 100.

Rijkers, G.T., Frederix - Wolters, E.M.H. (1980b),
The haemolytic plaque assay in carp (*Cyprinus carpio*).
Journal of Immunological Methods, Vol. 33, pp 79 - 86.

Rijkers, G.T., and Van Muiswinkel, W.B. (1977),
The immune system of Cyprinid Fish,
The development of Cellular and Humoral responsiveness
in the Rosy barb (*Barbus conchonius*)
Developmental Immunobiology eds. by J.B. Solomon & J.D. Horton
Elsevier/North Holland Biomedical Press, pp 233 - 240.

Rijkers, G.T., Frederix-Wolters, E.M.H., Van Muisurnkel, W.B. (1980c)
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 edited by M.J. Manning.
Elsevier/North - Holland. Biomedical Press, Amsterdam, pp 93 - 102.

Rijkers, G.T., Van Oosterom, R. and van Muiswinkel, W.B. (1981),
The immune system of cyprinid fish.
Oxytetracycline and the regulation of humoral immunity in carp
(Cyprinus carpio).
Veterinary Immunology & Immunopathology, Vol. 2, pp 281 - 290.

Rijkers, G.T., Wiegerinck, J.A.M., Van Oosterom, R., and
Van Muiswinkel W.B. (1980c),
Temperature Dependence of Humoral Immunity in carp (*Cyprinus carpio*).
Aspects of Dev. & Comp. Immunology -1

Rijkers, G.T., Teunissen, A.G. , Van Oosterom, R. and
Van Muiswinkel, W.B. (1980e),
The immune system of Cyprinid Fish. The immuno-suppressive
effect of the Antibiotic oxytetracycline in carp (*Cyprinus carpio*)
Aquaculture, Vol. 19, pp 177 - 189.

Rimmer J.J. , (1977),
Electron microscopic studies of the Developing Amphibian Thymus
Developmental and Comparative Immunology,Vol.1, pp 321 - 332.

Rimmer, J.J. and Horton, J.D. (1977),
Allograft rejection in larval and adult *Xenopus* following early
thymectomy.
Transplantation, Vol. 23, pp 142.

Rizkalla, W. (1969),
Studies on the thymus of the Nile teleost fish, *Clarias lazella*.
Acta Veterinaria - Academic Scientiarum Hungarica, Vol. 19, pp 331.

Robinson, J. H. (1980),
A review of thymus Ontogeny in Organ Culture.
Development and Differentiation of Vertebrate Lymphocytes
(Edited by J.D. Horton) Elsevier/North - Holland Biomedical Press.

Roch, Ph; Valenbois, P., and Du Pasquier, L. (1975),
Response of Earthworm Leucocytes to Concanavalin A and Transplantation Antigens.
Advances Experimental medicine & biology, Vol. 64, pp 45 - 54.

Rogers, A.W. (1979),
Techniques of Autoradiography 3rd edition,
Elsevier/North - Holland Biomedical Press,
Amsterdam, New York, Oxford.

Roitt, M.I., Brostoff, J., Male, K.D. (1985),
"Immunology",
Churchill Livingstone, Edinburgh, London,
Melbourne, New York.

Rosenkrist, Jensen, L. (1982),
Results from vaccination of Danish rainbow trout against vibriosus.
Dev. Comp. Immunol. Suppl. 2, pp 187 - 191.

Ruben, L.N., Warr, G.W., Decker, J.M and Marchalonis, J.J. (1977),
Phylogenetic origins of immune recognition : Lymphoid heterogeneity and the hapten carrier effect in the goldfish. (*Carassius auratus*).
Cell. Immunol. 31, pp 266 - 283.

Ruglys, M.P.(1985),
Studies on Immunosuppresion in Teleost Fish.
Ph.D. January 1985, University of Hull.

Sailendri, K. (1973),
Studies on the development of lymphoid organs and immune responses in the Teleost, *Tilapia mossambica* (Peters).
Thesis, Madurai University, pp 106.

Sailendri, K., and Muthukkaruppan, VR. (1975a),
Morphology of Lymphoid Organs in a Cichlid Teleost, *Tilapia mossambica* (Peters),
Journal of Morphology, 147, pp 109 - 1223

Sailendri, K., and Muthukkaruppan, VR. (1975b),
The immune response of the teleost *Tilapia mossambica* to soluble and cellular antigens.
Journal of Experimental Zool., Vol. 191, pp 371 - 382

Saito Saburo (1950)
The embryological Study of Fishes
I-General observation on the Early Development of the Dog - Salmon, Oncorhynchus Keta Walbaum.
J. of Faculty Agriculture Hokkaido University, Vol. 48, pp 267 - 297.

Sakai , D.K. (1981),
Heat inactivation of Complements and Immune hemolysis Reactions in Rainbow trout, *Masu salmon*, *Coho salmon*, Gold fish and *Tilapia*. Bull. of Japanese Society of Scientific Fisheries., Vol. 47(5), pp 565 - 571.

Salkind, J. (1915),
Contributions histologiques a la biologie comparee du thymus B-Poissons, Archives De Zoologie Experimentale et Generale, Vol. 55, pp 278 - 297.

Sanchez, G.A., Martak, G. and De Ioannes, A.E. (1980)
IgM-Like Natural Hemagglutinin from Ratfish serum. Isolation and Physico-Chemical characterization (*Callorhynchus callorhynchus*) Dev. & Comp. Imm., Vol. 4, pp 667 - 678.

Schiffman, R.H., and Fromm, P.O. (1959),
Measurement of some of the physiological parameters in rainbow trout (*Salmo gairdneri*). Can. J. Zool., 37, pp 25 - 32.

Schneider Barbel (1983),
Ontogeny of Fish lymphoid Organs
Dev & Comp. Imm., Vol. 7, pp 739 - 740.

Scolley, R., Bartlett, P., and Shortman, K. (1984),
T-cell development in the Adult Murine Thymus : Changes in the Expression of the Surface Antigen. Ly_2, L_3 T_4 and B_2 A_2 during development from Early Precursor cells to Emigrants.
Immunological Reviews No. 82.

Secombes, C.J. (1981),
Comparative studies on the structure and function of Teleost Lymphoid organs., Ph.D. Thesis, University of Hull.

Secombes C.J. and Manning M.J. (1980),
Comparative studies on the immune system of fishes and Amphibians. antigen localization in the carp (*Cyprinus carpio L.*). Journal of Fish Diseases, Vol. 3, pp 399 - 412.

Secombes, C.J. and Manning M.J. (1982a),
Histological changes in lymphoid organs of carp following injection of soluble or particulate antigens.
Dev. & Comp. Immunology, Supplement 2, pp 53 - 58.

Secombes, C.J., Manning, M.J. (1982b),
The effect of Primary and Secondary immune response on the lymphoid tissues of the carp *Cyprinus carpio*.
J. Experimental Zool., 220 (3), pp 277 - 288.

Secombes, C.J. & Resink, J.W. (1984),
The immune response of carp, *Cyprinus carpio* L. following injection of antigen - antibody complexes.
J. of Fish Biol. Vol. 24, pp 193 - 200.

Secombes, C.J., Manning, M.J., and Ellis, A.E., (1981),
Antigen - Trapping in the carp *Cyprinus carpio*.
Aspects of Dev. & Comp. Immunology by Solomon J.B.
Pergamon Press, Oxford, New York, pp 465.

Secombes, C.J., Manning, M.J. and Ellis, A.E. (1982),
Localization of immune complexes and heat - aggregated immuno-globulin in the carp *Cyprinus carpio L.*
Immunology, Vol. 47.

Secombes, C.J., van Gisningen, J.J.M., and Egberts, E. (1983a),
Separation of lymphocyte subpopulation in carp *Cyprinus carpio* L. by Monoclonal antibodies. Immunohistochemical studies.
Immunology, Vol. 48, pp 165 - 175.

Secombes, C.J., van Groningen, J.J.M., van Muiswinkel, W.B. and Egberts, E. (1983b),
Ontogeny of the immune system in carp *Cyprinus carpio* L.
The appearance of antigenic determinants on lymphoid cells detected by mouse anti-carp thymocyte monoclonal antibodies.
Dev. Comp. Immunol., Vol. 7, pp 455 - 464.

Sekretaryuk, K.V. (1983),
Electron Microscopy of contacting structures in the parasite host system when phyllometra inhabit carp skin.
Sov. Agric. Sci. (I), pp 44 - 46.

Semba, R., (1979),
Contributions to semi-thin sectioning on a conventional Rotary Microtome.
Stain Technology, Vol. 54, No.5.

Sergio, B., Constantini,M., Kunkl, A., & Manca, F. (1984),
A simple procedure for freezing and storing lymphocyte panels in trays.
Journal of Immunological Methods, 69, pp 17 - 21.

Serero, M. & Avtalian, R.R. (1978),
Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates.
III Establishment of immunological suppression in fish.
Dev. Comp. Immunol., Vol. 2, pp 87 - 94.

Shaw, E.S. (1951),
Oral incubation in *Tilapia macrocephala*
Bull Am. Mus. Nat's History, 103, pp 381 - 415.

Shellam, G.R., & Nossal, G.J.V. (1968),
Mechanism of Introduction of Immunological Tolerance,
IV. The effects of Ultra-Low Doses of Filagellin
Immunology, Vol. 14, pp 273.

Sheridan, P.H., Youngs, L.J., Krieger. N.R. and Selzer, M.E. (1984),
Glycerine uptake by lamprey Petromyzon marinus spinal neurons demonstrated by light microscopic autoradiography.
Journal of Comp. Neurol., 223(2), pp 252 - 258.

Shohat, M., Janossy G., & Dourmashkin, R.R. (1973),
Development of rough endoplasmic reticulum in mouse splenic lymphocytes stimulated by mitogens.
European Journal of Immunology, Vol. 3, pp 680 - 687.

Smith, A.C. (1977),
Reactions of Fish Red Blood Cells with Mucus and Sera from other fish(es).
Calif. Fish & Game, Vol. 63(1) pp 52 - 57.

Smith, P.D., Brown-Nesje, R. (1982),
Cell mediated immunity in the salmon : lymphocyte and macrophage stimulation and the production of lympho Kine like factors by stimulated lymphocytes.
Dev. Comp. Immunol. Suppl., 233 - 238.

Smith , R.T., Miescher , P.A., & Good, R.A. (1966),
Phylogeny of Immunity,
Gainesville University of Florida.

Smith, A.M., Potter, M. & Merchant, E.B. (1967),
Antibody - forming cells in the pronephros of the .....
Journal of Imm., Vol. 99, pp 876 - 882.

Snieszko, S.F. (1970),
Immunisation of Fishes : A Review.
Journal of Wild Life Diseases, Vol. 6, January.

Solomon, J.B. (1981),
Aspects of Dev. & Comp. Immunology,
Aberdeen University - Perganon Press, Oxford, New York, 1st edition.

Solomon, J.B. & Horton, J.D. (1977),
Developmental Immunobiology, Elsevier/North-Holland.

Stave, J.W., Roberson, B.S., & Hetrick F.M. (1983),
Chemi luminescence of Phagocytic cells isolated from the pronephros of striped bass.
Dev. & Comp. Imm., Vol. 7, pp 269 - 276.

Stavitsky, A.B. (1954),
Micromethods for the study of proteins and antibodies.
1. Procedure and general applications of hemagglutination and hemagglutination - Inhibition reactions with Tannic acid and Protein treated Red Blood cells. Journal of Immunology, Vol. (72), pp 360 - 375.

Stolen, J.S., & Makela, O. (1975),
Carrier preimmunization in the anti-hapten response of a marine fish.
Nature, Lond., pp 254, 118.

Stryer, L.(1981),
Biochemistry, 2nd edition.
W.H. Freeman & Company, San Francisco.

Sypek, J.P. & Burreson E.M. (1983),
Influence of Temperature on the Immune response of Juvenile Summer Flounder, *Paralighthys dentatus,* and its role in the Elimination of trypano plasma *Bullocki* Infections.
Dev. & Comp. Imm., Vol. 7, pp 277 - 286.

Tacha, D.E. & Richards, T.C. (1981)
Comparison of Glycol Methacrylate - and paraffin embedded tissues for identification of tritium - labelled cells after emulsion.
Radioautography Journal of Histotechnology, Vol.4, No.2 June .

Tamura Eimitsu, Honma Yoshiharu & Kitamura Yutaka (1981),
Seasonal changes in the thymus of the *viviparous surfperch,* Ditrema temmincki, with special reference to its Maturity and Gestation.
Japanese Journal of Ichthyology, Vol.28, No.3

Tatner, M.F. (nee´ Grace) (1983),
Growth of the lymphoid organs in Rainbow Trout, *Salmo gairdneri* from one to 15 months of age.
Journal of Zool. Lond. 199, pp 503 - 520.

Tatner, M.F. (nee´ Grace) (1985),
The migration of labelled thymocytes to the peripheral lymphoid organs in the Rainbow trout, *Salmo gairdneri Richardson.*
Dev. & Comp. Immunology, Vol. 9, pp 85 - 91.

Tatner, M.F. & Horne, M.J. (1983),
Susceptibility and immunity to *Vibrio anguillarum* in post hatching rainbow trout fry, *Salmo gairdneri* , Richardson 1836.
Dev. Comp. Immunol., Vol. 7, pp 465 - 472

Tatner, M.F. & Manning, M.J. (1983),
The ontogeny of cellular immunity in the rainbow trout, *Salmo gairdneri* in relation to the stage of development of the lymphoid organs.
Richardson., Dev. & Comp. Immunol.,Vol. 7, pp 69 - 76.

Tatner, M.F. & Horne M.T. (1984),
The effects of early exposure to *Vibrio-anguillarum* vaccine on the immune response of the fry of the rainbow trout. *Salmo gairdneri*, Richardson., Aquaculture, Vol. 41, pp 193 - 202.

Till, J.E., & Blaylock, B.G. (1976),
The chemical and radiological toxicity of Plutonium to developing Embryos of fish. Radiation Research, Vol 67(3), pp 626 - 627.

Tochinai, S. and Katagiri, C. (1975),
Complete abrogation of immune response to skin allografts and rabbit erythrocytes in the early thymectomised *Xenopus*.
Development, Growth and Differentiation, Vol. 17, pp 383.

Tokumaru, M., Ferri, A.G. (1970),
Histochemistry of the pigments found in the.....
Revista Brasileivade Biologia, Vol 30(2), pp 163 - 171.

Tokumaru, M. & Godinho, H.(1968),
Morphologic studies of Pigments found in liver.
Revistra da Faculdade de medicina veterinaria, Universi dade de Saa paulo. Vol 7(4), pp 833 - 840.

Toro, I., & Olah, I. (1966),
Electron Microscopic study of Guinea-Pig thymus
Acta morphologica Academical Scientiarum.
Hungaricae Tomus 14 (3 - 4) pp 275 - 290.

Triplett E.L., & Barrymore, S. (1960),
Tissue specificity in Embryonic & Adult Cymatogaster aggregata studied by scale transplantation.
Biol. Bull. 118, pp 463.

Trump, G.N. & Hildermann, W.H. (1970),
Antibody responses of gold fish to Bovine serum albumin.
Immunology, Vol. 19, pp 621 - 627.

R J

Turner, R[illegible]., & Manning, M.J. (1973),
Response of the Toad, *Xenopus laevis,* circulating antigens.
I. Cellular changes in the spleen.
Journal of Experimental Zool.,183, pp 21 - 34.

Turner, R.J. & Manning, M.J.(1974)
Thymic dependence of amphibian antibody responses.
European Journal of Immunology, Vol.4, No.5pp 346 - 349.

Valembois, P., Roch, Ph. & Du Pasquier, L. (1981),
Evidence of a MLR-like Reaction in an invertebrate, the earthworm *Eisenia Fetia.*
Aspects of Developmental and Comparative Immunology
by J.B. Solomon. Pergamon Press, New York.

Van Furth R. and Thedal, L. (1981),
In vitro determination of phagocytosis and intracellular killing by polymopho-nuclear and mononuclear phagocytes.
Handbook of Experimental Immunology I by Weir, 3rd edition.

Van Loon J.J.A., Secombes, C.J., Egberts E., & Van Muiswinkel, W. (1982),
Ontogeny of the immune system in fish.
Role of the thymus.
Advances in Exp. Med. & Biol., Vol. 149. Plenum Press, New York.

Van Loon J.J.A., Van Oosterom R., & Van Muiswinkel, W.B. (1980)
Development of the Immune System in Carp *(Cyprinus Carpio)*
Aspects of Dev. & Comp. Immunology, Vol. 1
by Solomon, J.B., pp 469 - 470
Perganon Press, Oxford.

Van Muiswinkel, W.B., & Cooper, E.L. (1982),
"Immunology and Immunization of fish".
Develop Comp. Immunol. Suppl.2, Pergamon Press, Oxford.

Van Wijhe, J.W. (1926),
Thymus, spiracular sense organ and fenestra vestibuli (ovalis) in a 63 mm, long embryo of *Heptanchus cinereus.*
Ver Hande Lince N-Akad, Wet, Amsterdam. Vol. 26.

Volpe, E.P., and Tompkins, R. (1982),
Intraembryonic origin of lymphoid stem cells in amphibians.
In Development Immunology: Clinical Problems and Aging.
(Eds. Cooper, E.L. & Brazier, M.A.B), Academic Press,
New York. pp 139 - 149.

Volpe, E.P., Tompkins R., & Reinschmidt, D.C. (1980),
Evolutionary modifications of Nephrogenic mesoderm to establish the embryonic centers of hemopoiesis.
Aspects of Developmental and Comparative Immunology edited by J.B. Solomon, Pergamon Press, Oxford, New York, Toronto.

Von Hagen, F.(1936),
Die Wichtigsten Endokrinendes Flussaals. Thyreoidea, Thymus und Hypophyse in Lebenszyklus des Flussaals (Anguilla vulgaris) nebst einiger Untersuchungen uber das chromophile und chromophobe Kolloid der thyreoidea. Zool. Jahrb, 61, pp 467 - 538.

Ward, P.D., Tatner, M.F., and Horne, M.T. (1985),
Factors influencing the efficacy of vaccines against vibrosis caused by *Vibrio anguillarum.*
In "Fish Immunology" Edt. by Manning, M.J. & Tatner, M.F.
Academic Press, London, New York.

Wardle C.S. (1971),
New observations on the lymph system of the plaice *Pleuronectes platessa* and other teleosts.
Journal of marine biol. Ass. U.K., pp 977 - 990.

Walters, M.N.I. & Papadimitriou, J.M. (1978),
Phagocytosis : A critical review in toxicology [CRC] ,
Vol. 5, page 377.

Warr, G.W. & Marchalonis, J.J. (1977),
Lymphocyte surface Immunoglobulin of the Goldfish differs from its serum counterpart.
Dev. & Comp. Imm., Vol. 1, pp 15 - 22.

Warr, G.W., De luca, D., Decker, J.M., Marchalonis, J.J. and Ruben, L.N.(1977),
Lymphoid heterogeneity in Teleost fish : Studies on the genus Carassius.
Dev. & Comp. Immunobiology, Eds. by J.B. Solomon., J.D. Horton.
Elsevier/North Holland Biomedical Press, pp 241 - 248.

Warr G.W., Simon R.C. (1983),
The mitogen response potential of lymphocytes from the rainbow trout *Salmo-gairdneri* re-examined.
Dev. & Comp. Immunology, Vol. 7(2), pp 379 - 384.

Wedemeyer, G.A., & McLeay D.J. (1981),
Methods for determining the tolerance of fishes to Environmental Stressors, page 247.
"Stress & Fish" by Pickering, A.D., Academic Press London, New York, Toronto, Sydney, San Francisco.

Weir, D.M. (1978),
Handbook of experimental Immunology 3rd edition. Blackwell Scientific ublications, Oxford.
Assays for immunoglobulin - secreting cells by Dresser.

Weir, D.M. (1983),
Immunology,
Outline for student of Medicine and Biology,
5th edition. Edinburgh & London. Churchill, Livingstone.

Weisbart, M. (1981),
In vitro incubation of pronephric tissue from the pacific hagfish. Eptatresus - Stouti.
American Zool. 20(4), pp 764.

Weisbart, M., Youson J.H., & Weibe J.P. (1978),
Biochemical, Histochemical & Ultrastructural Analyses of presumed steroid - producing tissues in the sexually mature sea lamprey, Petromyzon marinus L.
General & Comp. Endocrinology, Vol. 34, pp 26 - 37.

Weissman, L., Irving, M.D. (1967),
Thymus cell migration,
Journal of Experimental Medicine, Vol. 126, pp 291 - 304.

Wekerle, H. & Ketelsen, U.P. (1980),
Thymic nurse cells in bearing epithelium involved in T-lymphocyte differentiation,
Nature 283, pp 402 - 404.

Welsch, U., & Storch, V. (1982),
Light & Electron Microscopical observations on the caecilian spleen.
A contribution to the evolution of lymphatic organs.
Dev. & Comp. Immunology, Vol. 6, pp 293 - 302.

White, R.G. (1963),
Functional Recognition of Immunologically Competent cells by means of the Fluorescent Antibody Technique.
The immunologically competent cell by Wolstenholme, G.E.W. & Knight, J. (Eds), Boston : Little, Brown & Co. pp 6 - 16.

White, A ., Fletcher, T.C., Pepys, M.B., and Baldo, B.A. (1981),
The effect of inflammatory agents on C-reactive protein and serum amyloid P-component levels in plaice (*Pleuronectes platessa* L.)
Serum. Comp. Biochem. Physiol. Vol. 69C, pp 325 - 335.

Whitear, M. (1970),
The skin surface of body fishes,
Journal Zool. London (160), pp 437 - 454.

William, C.L., & Leslie, G.A. (1982),
Phylogeny of Immunoglobulin Structure and Function xv.
Idiotypic Analysis of Shark Antibodies.
Dev. & Comp. Immunol. Vol. 6, pp 463 - 472.

Wilson, J.G.M. (1976),
Immunological aspects of fungal disease in fish.
In "Recent Advances in Aquatic Mycology". Edt. by Gareth, Jones, E.B., Elek, Science, London, pp 573.

Winkelhake, J.L. & Chang, R.J. (1982),
Acute Phase (C-Reactive) Protein - like Macromolecules from Rainbow Trout (*Salmo gairdneri*).
Dev. & Comp. Imm., Vol. 6., pp 481 - 489.

Wishkovsky, A., & Avtalian, R.R. (1982),
Induction of helper and suppressor functions in carp (*Cyprinus carpio*) and their possible implication in seasonal disease in fish.
Dev. Comp. Immunol. Suppl. 2, pp 83 - 91.

Wislocki, G.B. (1980),
The action of vital dyes in teleosts,
Anatomical Record 3, pp 399 - 412.

Wojdani, A., Katz, E., Shahrabani., R. & Avtalion, R.R. (1979),
Influence of environmental temperature, on the fate of soluble and bacterial antigens in carp, Fla, USA, Dec. 27 - 30,
AM. Zool. (19), (3), pp 932.

Wrathmell, A.B., and Parish N.M. (1980),
Cell surface receptors in the Immune response in fish.
Phylogeny of Immunological Memory (ed.) by M.J. Manning,
Elsevier/North Holland, Biomedical Press, pp 143 - 152.

Wright, R.K,. and Cooper, E.L. (1982),
Origins of lymphocytes, cellular immunity and the major histocompatibility system. In "Developmental Immunology : Clinical Problems and Aging". Edt. by Cooper, E.L. and Brazier, M.A.B., Academic Press, New York, pp 13 - 26.

Yazulla, S. (1981),
Gamma Amino Butyric, Acid Ergic,
Synapses in the Goldfish Carassius auratus Retina an
autoradiographic study of Tritium labelled Musimol and Tritium Labelled
and amino Butyric acid.
Journal of Comp. Neurol. Zool. (1), pp 83 - 94.

Yocum, D., Cuchens, M.A. and Clem, L.W. (1975),
The hapten-carier effect in teleost fish.
J. Immunol. 114, pp 925 - 927.

Yoffey, J.M. and Manch M.D. (1929),
A contribution to the study of the Comparative histology and
physiology of the spleen with reference chiefly to its cellular constituents.
I - in fishes.
Journal of Anatomy, Vol. 63, pp 314 - 344.

Young, J.Z. (1981),
The life of Vertebrates,
3rd edition, Clarendon Press, Oxford.

Yunis, E.J., Hang, R.G., and Good, R.A. (1967),
Post thymectomy Wasting Associated with autoimmune phenomena
Journal of Exp. Medicine, Vol.125, pp 947 - 965.

Zapata, A. (1979),
Ultrastructural Study of the Teleost Fish kidney,
Dev. and Comp. Imm., Vol. 3, pp 55 - 65.

Zapata, A. (1980),
Ultrastructural of Elasmobranch lymphoid tissue 1.
Thymus and Spleen.
Dev. and Comp. Imm., Vol.4, pp 459 - 472.

Zapata, A. (1981),
Lymphoid Organs of Teleost Fish
I- Ultrastructure of the Thymus of *Rutilus rutilus*
Dev. & Comp. Immunology, Vol. 5, pp 427 - 436.

Zapata, A. (1981b),
Lymphoid Organs of Teleost Fish,
II - Ultrastructure of Renal Lymphoid tissues in *Rutilus rutilus* and
Gobio gobio,
Dev. & Comp. Immunology, Vol. 5, pp 685 - 690.

Zapata, A. (1982),
Lymphoid organs of Teleost Fish,
III. Splenic Lymphoid Tissue of *Rutilus rutilus* and *Gobio gobio*
Dev. & Comp. Immunology, Vol. 6, pp 87 - 94.