UNIVERSITI PUTRA MALAYSIA

EFFECT OF COPPER ON THE NON-SPECIFIC IMMUNE RESPONSE OF *PUNTIUS GONIONOTUS* CHALLENGED WITH *AEROMONAS HYDROPHILA*

P.A.H.L. JAYAWARDENA

FPSS 1996 3

EFFECT OF COPPER ON THE NON-SPECIFIC IMMUNE RESPONSE OF *PUNTIUS GONIONOTUS* CHALLENGED WITH *AEROMONAS HYDROPHILA*

P.A.H.L. JAYAWARDENA

MASTER OF SCIENCE
UNIVERSITI PERTANIAN MALAYSIA
1996

EFFECT OF COPPER ON THE NON-SPECIFIC IMMUNE RESPONSE OF *PUNTIUS GONIONOTUS* CHALLENGED WITH *AEROMONAS HYDROPHILA*

BY

P.A.H.L. JAYAWARDENA

Thesis Submitted in Partial Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Fisheries & Marine Science, Universiti Pertanian Malaysia.

December 1996

To the scientists who would enrich production for human life
whilst ensuring protection of human life.

ACKNOWLEDGEMENTS

I am truly indebted to my chairman Prof. Dr. Mohd. Shariff Mohd. Din for his guidance, patience and encouragement throughout the period of my stay at UPM.

I am grateful to the members of the supervisory committee, Prof. Dr. Law Ah Theem and Associate Professor Dr. Fatimah Md. Yusoff for their continuous guidance and fruitful suggestions during the study.

It is a pleasure to thank Dr. Rohana Subasinghe for his fruitful suggestions and guidance .

My special thanks to En. Rosdi Abd. Ghani for his technical assistance. I must also thank my course-mates, specially Mr. Joselito R. Somga for his kind help and good work relations.

My sincere gratitude to International Development Research Centre (IDRC), Canada for extending financial assistance for the programme.

Last, but not lease, my special gratitude to my beloved wife Gayani for her constant source of encouragement, assistance and inspiration.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
APHA	American Public Health Association
DO	Dissolved Oxygen
EDTA	Ethylenediamine tetraacetic acid
EIFAC	European Inland Fisheries Advisory Commission
EPA	Environmental Protection Agency
FAO	Food and Agricultural Organization
h	Hours
IPNV	Infectious Pancreatic Necrosis Virus
MATC	Maximum Allowable Toxicant Concentration
PBS	Phosphate Buffer Saline
S.D.	Standard Deviation
TSA	Trypticase Soy Agar
V/V	Volume by volume
Wt.	Weight

Abstract of the thesis submitted to the Senate of Universiti Pertanian Malaysia in partial fulfilment of the requirement for the degree of Master of Science

EFFECT OF COPPER ON THE NON-SPECIFIC IMMUNE RESPONSE OF *PUNTIUS GONIONOTUS* CHALLENGED WITH *AEROMONAS HYDROPHILA*.

by

P.A.H.L. Jayawardena
December 1996

Chairman: Prof. Mohamed. Shariff Mohamed. Din, Ph.D.

Faculty: Fisheries & Marine Science

This study is focused on toxicity of copper and its effects on the immune response of *P. gonionotus*. The median lethal concentration of copper (LC_{50}) on *P. gonionotus* (56.9±4.5 g) at 12, 24, 48, 72 and 120 hours was estimated as 2.17, 0.91, 0.57, 0.53, 0.42 mg/L respectively.

Fish were intraperitonealy injected with three different doses (4.5×10^5, 4.5×10^7 and 4.5×10^9 CFU/mL) of formalin killed *A. hydrophila* (0.1 mL) and the immune responses were monitored using haematological and serological assays over a period of 30 days post-injection. The assays used were total WBC counts, lysozyme, NBT, total protein, total immunoglobulin and haematocrit levels. The immune responses with the highest magnitude were observed among fish injected with 4.5×10^9 and the lowest among 4.5×10^5 CFU/mL. The responses were observed to be both time and dose dependent.

To find the effect of copper on the immune response, fish were exposed to different sublethal concentrations of copper, determined at 10%, 20%, and 30% of 96 hour LC_{50} for 71 days. After 56 days of initial exposure, fish were challenged with 0.1 mL of 4.5 x 10^5 CFU/mL formalin killed *A. hydrophila*. The immune responses were monitored using the same immunological assays mentioned above withdrawing non-lethal blood samples at 14, 28, 56, 61, 66 and 71 days of exposure to copper. Though a depletion of protein and immunoglobulin titres in plasma were observed at the initial exposure to copper, it did not influence the increased titres demonstrated against the bacterial challenge in any of the treatments. In contrast, the WBC counts increased during the initial exposure to copper. WBC counts, lysozyme and NBT assays showed suppressed immune responses after challenging with bacteria compared to the control group. But the responses observed at the lowest concentration (0.05 mg/L) of exposure to copper, were similar to that of the control in most of the cases. This suggests the absence of immunosuppressive effects at 0.05 mg/L of copper.

Abstrack thesis dikemukakan Senat Universiti Pertanian Malaysia,
sebagai memenuhi syarat untuk mendapat Ijazah Master Sains.

KESAN KUPRUM KE ATAS TINDAKABALAS IMUNO *PUNTIUS GONIONOTUS* DICABAR DENGAN *AEROMONAS HYDROPHILA*

oleh

P.A.H.L.Jayawardena

Disember, 1996

Pengerusi : Prof. Mohamed Shariff Mohamed Din, Ph.D

Fakulti : Perikanan dan Sains Samudera

Kajian ini berfokus ke atas ketoksikan kuprum dan kesannya terhadap tindakbalas imuno dalam *Puntius gonionotus*. Kepekatan lethal median kuprum (LC_{50}) ke atas *P. Gonionotus* (56±4.5g) untuk jangkamasa pendedaham 12, 24, 48, 72, and 120 jam adalah 2.173, 0.910, 0.527, 0.420 mg/L masing-masing.

Ikan disuntik secara intraperitoneal dengan tiga dos *Aeromonas hydrophila* (yang dibunuh dengan formalin) yang berbeza ($4.5x10^5$, $4.5x10^7$ and $4.5x10^9$ CFU/mL). Tindakbalas imuno dikesan dengan menggunakan ujian haematologikal dan serologikal melebihi 30 hari selepas suntikan. Ujian-ujian yang digunakan adalah pengiraan jumlah WBC, lisizim, NBT, jumlah protein, jumlah imunoglobulin dan paras hematokrit. Tindakbalas imuno yang tertinggi didapati dalam ikan yang disuntik dengan ($4.5x10^9$ CFU/mL) dan yang paling rendah dalam ikan yang disuntik dengan $4.5x10^5$ CFU/mL). Tindakbalas adalah bergantung kepada kedua-dua masa dan dos.

Untuk menentukan kesan kuprum ke atas tindakbalas imuno, ikan didedahkan kepada tiga kepekatan sublethal kuprum yang berbeza iaitu 10%, 20%, dan 30% daripada nilai LC_{50} 96 jam selama 71 hari. Selapas 56 hari, ikan dicabar dengan 0.1 mL 4.5 x 10^5 CFU/ml *A. hypdrophila* yang telah dibunuh dengan formalin. Tindakbalas imuno dikesan dengan menggunakan ujian imunologikal dengan mengambil darah secara tidak lethal pada 14, 28, 56, 61, 66, dan 71 hari selepas pendedaham dengan kuprum. Pengurangan protein dan titer imunoglobulin dalam plasma telah diperhatikan di peringkat permulaan pendedaham terhadap kuprum. Walaubagaimanapun, pendedahan permulaan ini tidak mempengaruhi peningkatan titer apabila dicabar dengan bakteria dalam semua rawatan.

Sebaliknya, bilangan WBC meningkat dengan pendedahan permulaan terhadap kuprum. Bilangan WBC, ilsozim dan NBT menunjukkan tindakbalas imunotindasan selepas dicabar dengan bakteria berbanding dengan kawalan. Tindakbalas pada kepekatan yang paling rendah (0.05 mg/L) kuprum adalah sama dengan kawalan dalam kebanyakan kes. Ini menunjukkan ketiadaan kesan imunotindasan pada 0.05 mg/L kuprum.

CHAPTER I

GENERAL INTRODUCTION

Copper is a heavy metal commonly present in aquatic ecosystems, with a capacity to act as an environmental stressor, which sometimes contribute to the outbreak of infectious diseases in fish (Snieszko, 1974). Furthermore, it is capable of exhibiting toxic effects on certain flora and fauna (Leland and Kunabara, 1984).

During the nineteenth century, the presence of copper in the environment as a hazard for life and vegetation seems to have passed unnoticed probably due to the trivial contribution to it made by the human industry. The global figures show a marked increase in copper level since the beginning of the twentieth century. This is probably due to a variety of industrial efforts undertaken by man, thus increasing the global levels of aquatic copper content (Mance, 1987).

Copper is found in natural waters as a trace metal, usually below 5mg/L, but can also be present at much higher concentrations as a result of mining activities and other industrial processes which could be to the detrimental to fish. Moreover, indiscriminate discharge of copper associated waste products into the natural environment, use of pesticides, fungicides, algaecides, molluscicides, weathering of

soil and rocks due to volcanic eruptions are some of the sources of copper contamination in natural ecosystems (EIFAC/FAO, 1976).

As a result of intensive agricultural production and industrial development during the last few decades, increased concentrations of copper in rivers and lakes have adversely affected fishes. Some of these effects are recognizable by interference with their normal bio-chemical and physiological mechanisms (Schreck and Lorz, 1978; Waiwood and Beamish, 1978; Anderson and Spear, 1980).

Furthermore, heavy metals, including copper, may produce general stress in fishes such as increasing catecholamine, blood sugar and cortisol concentrations in blood plasma (Nakono and Tomlinson, 1967; Schreck and Lorz, 1978). Moreover, environmental pollution also produces alterations in cardio-respiratory responses and several blood parameters of fish (Hughes, 1981).

Copper exhibits a few oxidation states. But in natural aquatic systems with aerobic conditions, the only stable oxidation state is the cupric form. It forms a wide variety of complexes with inorganic and organic substances and gets absorbed to particulate matter in water. The proportion of the presence of its free ion stage depends on factors like pH, alkalinity, and presence of organic matter. Physical and chemical qualities generally tend to fluctuate in less alkaline and soft water and enhances the toxic effects of metals when compared to hard water (Alabaster and Lloyd, 1981).

Disease is an outcome of a complex interaction between host, pathogen and environmental factors. Low dissolved oxygen content, extremes of pH, temperature

fluctuations, and pollutants are examples of the environment factors. These factors could act directly on the physiology of fish or could cause stress (Ferguson, 1991).

Sublethal levels of copper has been identified as a factor causing stress associated immunosuppression in fish (Ellis, 1981), resulting in increased susceptibility of fish to infectious diseases (Mazeaud et al., 1977).

Immunity is an important physiological phenomenon of animals which provides protection against infections through preservation of internal homeostasis. It can be either non-specific natural immunity which is an innate mechanism rendering the host resistance to, or an acquired specific process which is induced in response to a foreign agent. Non-specific defense against pathogens includes the skin, mucus and various substances in serum (Ingram, 1980).

Although many aspects of immune mechanisms in fish are similar to those of mammals, significant differences also exist. Fish possess all the characteristics of adaptive immunity, both humoral and cell mediated (Ellis, 1982).

The research executed early this century on fish revealed the fact that fish are capable of producing antibodies as a response to antigenic stimuli (Corbell, 1975). Studies regarding the direct influence of metals and other pollutants in aquatic ecosystems on immune responses of tropical fish is limited (O'Neil, 1981b).

In addition, it is evident from the literature that stress and resistance to infectious diseases are two closely related factors involving the immune system. This is one of the vital areas of front-line research (Ellis, 1981).

Aeromonas hydrophila is an ubiquitous and heterogeneous organism which produces disease under stress conditions or in concert with infection by other

pathogens (Roselynn and Stevenson, 1988). It is considered as one of the constituents of the natural microflora of aquatic animals and plants and has been experimentally recovered from various types of aquatic vertebrates and invertebrates (Trust and Sparrow, 1974).

Aeromonas hydrophila is a Gram-negative, motile, short rod shaped bacterium with a single polar flagellum, classified taxonomically under the family Vibrionaceae. It also exhibits positive results for the biochemical tests such as oxidase, catalase and glucose fermentation (Kabata, 1985; Austin and Austin, 1987). Among *A. hydrophila*, both virulent and avirulent forms have been identified and different strains with various virulence properties have been described (De Figueiredo and Plumb, 1977; Janda et al., 1985). Systemic fish diseases such as motile aeromonad septicaemia as well as localized secondary infections at sites of injury or parasitic attachments could also be caused by this organism (Roselynn and Stevenson, 1988).

As discussed earlier, the global copper concentration in water is gradually increasing due to modern industrial efforts. Exposure of fish to copper at sublethal levels has been recognized as a stressor causing immuno-suppression in fish. Further, the fish immune system has a capacity to be used as a tool to assess the toxicity of different heavy metals (Weeks et al., 1990). Most of the research associated with the effect of copper on fish immune system has been undertaken in temperate regions associated with the temperate fish species. Research data on the capacity of fish immune system in assessing the toxicity of heavy metals in tropical areas is scarce.

In order to understand the effect of sublethal levels of copper, comparable to what is present in the natural aquatic ecosystems, on the immune response of *Puntius gonionotus*, the following objectives were considered in the present study :

1. Determine the median lethal concentration (LC_{50}) of copper on *P. gonionotus* at 96 hours.
2. Evaluate the hematology and serology of *P. gonionotus* challenged with formalin killed *Aeromonus hydrophila.*
3. Evaluate the effect of copper on the immune response of *P. gonionotus.*