TOLERANCE OF EARLY LIFE STAGES OF TILAPIA (CICHLIDAE: TILAPIINI) TO METAL STRESS

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BY

P.P.G.S.N.SIRIWARDENA (BSc. special, MSc.)

INSTITUTE OF AQUACULTURE
UNIVERSITY OF STIRLING
STIRLING
SCOTLAND

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DECLARATION

This thesis embodies the results of scientific experimental investigations carried out by P.P.G.S.N. Siriwardena at the Institute of Aquaculture, University of Stirling, during the period October 1990 to November 1993. The thesis has been composed independently by P.P.G.S.N. Siriwardena and no part of this work has been submitted for any other degree.

Candidate:

P.P.G.S.N. Siriwardena

In memory of

my father:
who took such pride in my undertaking this study
but sadly knew not of its results.

and

son Parami:
who never saw me

Dedicated

to

my mother, wife Ranganie and son Lahiru Sachithra

ABSTRACT

Ecophysiological indices that characterise animals fitness directly or indirectly measure the components of protein turnover and its associated metabolic costs. Therefore, more likely protein turnover and associated metabolic costs may play a major role in underlying stress tolerance mechanisms. In this thesis a flow-through system was designed and developed to overcome some of the existing basic design flaws in such systems and used to determine responses of different species of tilapiine fishes (Cichlidae: Tilapiini) under lethal and non-lethal stress using cadmium and copper.

A significant variation in tolerance capability between mouth brooding and substrate spawning tilapia yolk sac-fry to lethal cadmium and copper stress was observed. There was concordance between the relative tolerance capabilities of these two groups to the two metals suggesting a general response. Similarly, tolerance capabilities were in concordance with early life-history growth traits and associated metabolic costs measured under non-stressed (control) conditions suggesting individuals with higher growth rates and low maintenance metabolic costs are better capable of tolerating metal stress than the individuals with low growth rates and higher maintenance metabolic costs. Lower cadmium body burden levels were observed in the sac-fry of the more tolerant substrate-spawner *T. zillii* than those of in the more sensitive mouth-brooder *O. niloticus*. Variations in growth performances between mouthbrooders and substrate spawners were attributed to the difference in their developmental rates. Therefore, genetically based phenotypic variations for early life history traits translate into variations in stress tolerance.

Similarly *O. niloticus* yolk sac-fry originating from small eggs were more tolerant to cadmium stress and had lower body burdens than larger conspecifics originating from large eggs. The early life history growth traits and associated metabolic costs measured under non-stress conditions were in concordance with the tolerance capabilities of the two size groups supporting the correlation between higher tolerance and low maintenance metabolic cost. The size of the yolk sac-fry was influenced by maternal age and size, and hence, by egg size. Therefore, translation of pre-determined phenotypic variations for early life history traits into variations in tolerance capabilities to metal lethal stress was supported.

Starvation-induced reductions in metabolic rate of tilapia sac-fry carried a fitness advantage by reducing cadmium uptake under lethal stress. Therefore, post adapted physiological acclimation to one type of stress may carry a fitness advantage over metal stress. In all cases tolerance capability to metal stress was correlated to the metabolic status of yolk sac-fry.

Using the most sensitive mouth brooder *O. niloticus* and most tolerant substrate spawner *T. zillii*, which demonstrated the largest difference in their lethal tolerance to cadmium and copper, the effects of non-lethal cadmium stress were investigated. Significant differences in stress tolerance between the two species was observed. The effects of cadmium on growth and associated metabolic costs were similar for both species suggesting a general response under non-lethal cadmium stress. There was evidence that both species showed an increase in protein turnover, and hence, an increase in maintenance metabolic costs. It was found that cadmium did not affect the energy supply, but reduced protein growth which appears to be due to investment of

more supplied energy on increased protein turnover, and hence, increased maintenance metabolic cost than deposition as growth energy. Therefore, the predicted fitness advantage for lethal cadmium stress was observed for non-lethal cadmium stress.

In conclusion, in tilapia yolk sac-fry there was a general response to lethal as well as non-lethal stress. The tolerance capability have been brought about either by genetically pre-determined or physiologically post-adapted variations in early life history traits of tilapia yolk sac-fry. The observed concordance between the range over which differences in responses occur in terms of more sensitive non-lethal stress indices and lethal tolerance capability of *O. niloticus* and *T. zillii* yolk sac-fry suggests there may be a possible link between responses to lethal and non-lethal cadmium stress.

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DEFINITION OF TERMINOLOGY USED IN THIS THESIS

Embryo:	The developing fish prior to hatching.
Yolk sac-fry:	The developing fish between hatch and completion of yolk absorption and initiation of feeding.
Fry:	The developing fish after initiation of exogenous feeding.
Starved fry:	The developing fish after initiation of exogenous feeding stage to which no external source of food was made available.
Body:	The somatic tissues of the developing yolk-sac fry including the yolk-sac but excluding the yolk.
Gill filament:	Refers to primary lamellae.
Lamellae:	Refers to secondary lamellae.
Stress:	Refers to the stimulus.
Stress response:	Refers to the reaction of the biological system to the stress.
Lethal response:	Refers to the response determined by immobility.
Non-lethal response:	Refers to responses determined by other than immobility.
Effective concentration:	Refers to the concentration at which the measured parameter impairs significantly from the control value.

TAXONOMIC CLASSIFICATION

Throughout this thesis the tilapia names are used according to the taxonomic classification of Trewavas (1983) and not according to the author of the cited publication. *Tilapia nilotica* (Linaeus), *Tilapia mossambica* (Peters) and *Tilapia aurea* (Steindacner) are referred to as *Oreochromis niloticus* (Lineaus), *Oreochromis mossambicus* (Peters) and *Oreochromis aureus* (Steindachner) respectively. Rainbow trout (*Salmo gairdneri*, Richardson) is referred to as *Oncorhynchus mykiss* (Walbaum) according to the recent classification.

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CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

There are over 50,000 known chemicals produced in quantities greater than one ton per annum, and since up to 1000 new chemicals are launched on the market annually (Bourdeou, 1984), the control and evaluation of these substances has become one of the major environmental issues of present times. Over the past two decades increasing emphasis has been placed on the toxic evaluation of the impact of industrial and municipal effluents on the aquatic environment and the organisms which dwell within it, since the aquatic environment increasingly serves as a receiving sink for various chemicals. For a comprehensive evaluation of the environmental hazards associated with a specific chemical, the determination of its toxic potential along with fate of the substance in the environment, is needed. Toxic effects of a substance on organisms is largely determined by the extent to which the organisms could tolerate the stress caused by the chemical. This concern prompted the development of a new scientific discipline, ecotoxicology, which can be broadly defined as the science which is concerned with the effects of potentially toxic chemicals on the functioning of populations, communities and ecosystems, through effects on individual organisms.

1.1 Environmental stress, response and stress tolerance

1.1.1 Concept of biological stress

The concept of biological stress has been defined at different times by various authors. These definitions of stress, however, share the common premise of a 'stimulus' acting upon a biological system and the subsequent reaction or the 'response' of the

biological system to the stimulus (Pickering, 1981). When defining the term stress, some workers (Meir, 1972; Leffler, 1978; Ulanowicz, 1978) used the 'stimulus' as stress while others (Selye, 1956; Esch and Hazen, 1978; Lugo, 1978) used the 'response' as stress and the stimulus as stressor. For the purpose of this thesis, unless otherwise specified the term 'stress' will be used to describe a stimulus, the reaction of the biological system being defined as the stress 'response'.

The approaches of defining stress are based on the changes in performance capacity of an organism (or Darwinian fitness, see below) and the energy demand caused by a stress. Brett (1958) defined stress as any environmental factor which extends the normal adaptive response of an animal, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced. This definition is useful when referring to individuals but it is not so readily applicable to populations in which, under certain circumstances, a decrease in the probability of survival of one individual may directly increase the probability of survival of other members of the population by means of reduced, intraspecific competition (Pickering, 1981). Sibly and Calow (1989) defined stress in general as an environmental condition that, when first applied, impairs Darwinian fitness, which is applicable to higher levels of organization. Impairment of Darwinian fitness can involve reductions in survivorship, fecundity or an increase in the time interval between life-cycle events. Grime (1979) used the term "stress" only to describe factors that inhibit production and the term "disturbance" to describe factors that impair survivorship to which Sibly and Calow (1989) referred as growth stress and mortality stress respectively. Bradshaw and Hardwick (1989) considered that stress is anything which reduces growth or

performance, thus a reduction in fitness. Koehn and Bayne (1989) consider the effects of stress as the physiological energetics of the organism, i e, the physiological traits such as feeding, absorption and the metabolic costs of maintenance, growth and reproduction, that together reduces the performance capacity of the individual due to the energy costs associated with trying to maintain homeostasis.

In terms of energy demand resulting from stress, Ivanovici and Webe (1981) defined stress more specifically as "a significant reduction of adenylate energy charge (AEC) which is induced by an environmental perturbation". Adenylate energy charge is an index calculated from measured amounts of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) where by (Atkinson, 1977; Hochachka and Somero, 1984)

$$AEC = (ATP + 1/2\ ADP)/(ATP + ADP + AMP).$$

However, such biochemical measurements need to be interpreted with caution as they may not translate into effects at individual and population levels because of homeostatic compensation (Calow, 1989). Irrespective of the definition and source, stress is now generally accepted as an interference with the normal functioning of a biological system which reduces its structural and functional integrity, and results in an increase in energy demand, and, reduces fitness (Hoesch and Rosenberg, 1981).

1.1.2 Response to toxic stress: Advantages of using physiological and biochemical responses in toxic stress.

In his classic work on the morphological and physiological responses of animals to

stress, the Austrian physician Hans Selye (1950; 1956; 1959) suggested that in mammalian systems there is a recognisable group of physiological responses which he termed the General Adaptation Syndrome (GAS) to environmental influences. This group of physiological responses operates in three successive stages: alarm reaction, resistance and exhaustion, which influence the health of the individual concerned (Selye, 1973). Selye's concept of stress differs from others in that stress (response) is considered to be an essential component of normal metabolism (Pickering, 1981), and there is no reference to a non-stressed state which makes quantitative and qualitative predictions difficult (Barber, 1990).

Organisms function at many levels in an hierarchy of organized states from the cell to the community and the search for measures of response to stress has proceeded at all levels (Bayne, 1985). Neural and neuro-endocrine responses which occur at the cytological level and their resulting physiological consequences are known as primary and secondary responses, respectively (Mazeaud, Mazeaud and Donaldson, 1977). Tertiary responses includes changes in behaviour, decreased growth rate and increased susceptibility to disease and are resultants of biochemical and physiological changes in the organism (Wedemeyer and McLeay, 1981). Therefore, integration of biochemical and physiological responses will be a useful tool to quantify an organism's performance under conditions of environmental stress. These responses meet the majority of criteria of responses to be considered as useful measures of biological effect in toxicological or environmental monitoring programmes (Widdows, 1985)

-they are sensitive to environmental stress and have a large scope for response

throughout the range from optimal to lethal conditions.

-they reflect a quantitative or otherwise predictable relationship with the stress or pollutant.

-they have a relatively short response time, in the order of hours to weeks, so that stress impact may be detected in its incipient stages.

- they represent non-specific (general) responses to the sum of environmental stimuli, thus providing measurements of the overall impact of environmental change and complementing the more contaminant-specific responses at the cellular level.

-they are measurable with precision and with a high "signal to noise" ratio so that the effect of stress may be detected above the "noise" of natural variability.

- they are ecologically significant and can be shown, or convincingly argued to be related to an adverse or damaging effect on growth, reproduction or the survival of the individual and the population.

1.1.3 Proposed stress tolerance mechanisms

The ecophysiological indices used to characterise the fitness of an animal under given environmental conditions directly or indirectly measure components of protein turnover and its associated metabolic costs. Many of these indices, such as oxygen:nitrogen (O:N) ratio (Widdows, Phelps and Galloway, 1981; Correa, 1987), net growth efficiency (Widdows *et al.*, 1981), scope for growth (Widdows, Bakke, Bayne, Donkin, Livingstone, Lowe, Moore, Evans and Moore, 1982; Stickle, Rice and Moles, 1984; Moore, Livingstone and Widdows, 1987) and adenylate energy charge

(Ivanovici and Wiebe, 1981; Giesy, Duke, Bingham and Dickson, 1983) have been used as stress indices. Recently, the interpretation of protein turnover as a possible general mechanism for stress tolerance has been offered by several authors for both plants and animals (Cooke, Oliver and Davies, 1979; Cooke and Davies, 1980; Hawkins, Wilson and Bayne, 1987; Koehn and Bayne, 1989).

1.1.3.1 Proposed mechanisms of stress tolerance based on protein turnover

There is a continuous need to repair and replace the damaged molecules and tissues (protein turnover) throughout the life time of an organism and this is known as protein turnover (Sibly and Calow, 1989). This equilibrium state which is referred to as the dynamic steady state of the body (Schoenheimer, 1946) requires energy for its maintenance at a certain level, known as maintenance metabolic cost. Proposed response mechanisms based on protein turnover and associated metabolic costs are given below.

i) High inherent metabolic cost.

A priori, high rates of protein turnover, though incurring high metabolic costs, have been proposed to be an advantage under conditions of stress (Koehn and Bayne, 1989). This was proposed on the basis, that inherent high protein turnover is sufficient to repair the protein damage caused by toxicants, and bearing the energy cost involved in repair. Therefore, the cost involved in protein turnover under stress should not vary significantly from that under non-stressed conditions, as the cost of tolerance is carried all the time.

ii) Reduction in metabolic cost

One way that an organism may increase its tolerance to a range of environmental stresses is to reduce its metabolic energy requirements via a reduction in metabolic rate during exposure (Hoffmann and Parsons, 1989). Such a mechanism is used by animals that hibernate or diapause to avoid periods of environmental stress (Hochachka and Somero, 1984). Supporting this hypothesis, stress tolerance is known to be reduced under stress conditions that increase metabolic rate (Hoffmann and Parsons, 1989). For example, when the metabolic rate was increased at higher temperatures, adult *Drosopila melanogaster* showed a reduction in tolerance to various stress factors, including anoxia, starvation, high concentrations of ethanol, and desiccation (Matheson and Parsons, 1973): the strategy adopted here is to reduce overall activity to save energy. Protein turnover is a primary factor affecting maintenance metabolic energy demand and therefore considered to be an important component of maintenance energy requirements. Therefore, organisms under such types of stress should reduce the protein turnover to reduce its metabolic energy requirement. The prediction of reduction in metabolic rate to tolerate stress may not apply to all cases of stress tolerance, particularly those associated with chemical stress (Hoffmann and Parsons, 1989). Tolerance for chemical stress could be associated with increased metabolic rate because of an increase rate of detoxification. This leads to the third proposed mechanism.

iii) Increase in metabolic rate

Changes in environmental conditions may affect the dynamic steady state of the body by increasing the rate of protein damage (Garlick, Millward, James and Waterlow,

1975; Hawkins *et al.*, 1987; Houlihan, Hall, Gray and Noble, 1988). This will lead to an increase in the rate of repair process of the damaged proteins to regain a new equilibrium at higher rates of both processes: thus the organism can tolerate the damage caused by the environmental change. This process is expensive in terms of energy, as it leads to a higher maintenance cost, using resources in maintenance which might otherwise have been invested in production (growth and/or reproduction) (Sibley and Calow, 1989). These increased maintenance costs have been documented in several organisms subjected to environmental stress such as fast flowing water (Fox and Simmonds, 1933), osmotic stress (Stearns, 1980) and chemical pollution (Widdows *et al.*, 1981).

It can be seen from the previous section that there is no single generalized mechanism to stress tolerance. It is also known that very distinctive responses can evolve to resist stress, each related to the nature of the particular stress: eg. physiological acclimation, behaviourial changes within the life span of an animal to resist the stress or leading to natural selection for an increased resistance, resulting in genetic adaptation to the stress, have been documented as other possible resistance mechanisms (Klerks, and Levinton, 1989). These genetic adaptations may include special detoxification mechanisms and structural alterations of the site of action to reduce the body burden

1.2 Advantages of using fish early life stages in ecotoxicity studies over complete life cycle ecotoxicity studies

Olsen and Foster's (1956) summary on sodium dichromate toxicity to the eggs, fry and early juvenile stages of salmonids is one of the earliest reports of non-lethal exposure to consecutive fish life stages. Following this report studies on complete life-cycle toxicity tests (embryo to embryo) were conducted with the fathead minnow (*Pimephales promilas*) by Mount and Stephen (1967; 1969) and Mount (1968), and the effects of the toxicant on survival, growth and reproduction were measured quantitatively. Such quantitative data were used to determine the maximum acceptable toxicant concentration (MATC), which is defined as the geometric mean between the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) (Mount and Stephen, 1967). Such studies were also conducted for many other fish (Akiyama,1970; Eaton,1970; McKim and Benoit, 1971, 1974; Smith, 1973; Schimmel and Hansen, 1974; Holcombe, Benoit, Leonard and McKim, 1976; Spehar, 1976; Hansen and Parrish, 1977; Middaugh and Dean, 1977). The fish complete life-cycle toxicity test is considered by many aquatic toxicologists to be the ultimate test in establishing long term "safe" environmental concentrations of toxic chemicals for both vertebrate and invertebrate aquatic populations (McKim, 1985).

A disadvantage in complete life-cycle toxicity studies is that, for many fish species, a minimum of six to twelve months of concentrated effort is required. Some of the complete life-cycle non-lethal toxicity tests can last for up to two years (Benoit,Puglisi and Olsen, 1982). The increasing rate of production of new products in recent years

has created the urgency for more rapid, less costly and reliable toxicity tests than complete life-cycle tests for determining safe environmental concentrations of toxic chemicals.

Hynes (1960) and Tarzwell (1967) emphasized the necessity of conducting toxicity tests with the most susceptible life stages of dominant species. During early life stages many critical development events take place in a very short period of time including dramatic biological, metabolic and morphological changes from embryo to yolk sac-fry (Christensen, 1975) and these stages are particularly sensitive to low level environmental disturbances (Vailati, Calamari and Marchetti, 1975; Macek, Lindberg, Sauter, Buxton and Costa, 1976; von Westernhagen, 1988) than later developmental stages (von Westernhagen, 1988; Rosenthal and Alderdice, 1976; McKim, 1985). Short-term exposures of embryos and newly hatched fry provided an adequate estimate of safe concentrations over the complete life cycle of those fish (McKim, Arthur and Thorslund, 1975; Vailati *et al.*, 1975). The studies used shorter early life stage toxicity tests (Pickering and Gast, 1972; Eaton, 1974; McKim *et al.* 1975; McKim, 1977; Mckim, Eaton and Holcombe, 1978; Eaton, McKim and Holcombe, 1978) and strongly supported the usefulness of the early life stage toxicity test as a quick reliable method for predicting long term non-lethal effects (Benoit *et al.*, 1982). McKim (1977) analysed data from 56 life cycle toxicity tests completed during the 1960's and early 1970's with 34 organic and inorganic chemicals and four species of fish. He concluded that the embryo-larval and early juvenile life stages were the most or among the most sensitive in their responses to chemical insult. Macek and Sleight (1977) concluded after a review of much of the available data that critical life stages

(embryos and developing fry) exposed to toxicants provides estimates of chronically safe concentrations remarkably similar to those empirically derived from complete life-cycle chronic toxicity studies. Woltering (1984) reviewed 173 toxicity studies. The proportion of these studies which included early life stages as well as adult stages, reproduction and adult growth as most sensitive end point however, was only 30%.

Apart from the reduction in cost and time, toxicity tests with critical early life stages can provide information on potential long term effects in situations where lethal toxicity (LC50) is not observed (Macek and Sleight, 1977).

1.3 Test organism selection: Tilapia as a possible test organism in early life stage toxicity testing in tropics

A number of fresh water and salt water species of fish have been used in early life stage toxicity tests (see Appendix I). Tilapias (Cichlidae: Tilapiini) have not received much attention as a test species in toxicity testing compared with most other fresh water fish largely because they have been traditionally regarded as a "hardy" fish. The sensitivity of tilapias to toxic compounds is poorly studied. A few metal toxicity studies using juvenile or adult tilapias were reported (Table 1.1). Except the studies that have been done by Pandya and Rao (1986), De Silva and Ranasinghe (1989) (with pesticides) and Fu and Lock (1990) (with metals) in acute toxicity tests no toxicity studies have been reported on the early life stages such as eggs, sac-fry and feeding fry of tilapia. Thus the most sensitive early life stages have rarely been subjected to testing. Despite their reputation as "hardy" fish, tilapias fulfil most of the

Table 1.1 Summary of representative metal toxicity studies using tilapia species corresponding to different sensitive end points

Toxicant	Species	Size (g)/ stage	Concentration μgl^{-1}	End point	Reference
Aluminium	*Oreochromis aureus*	5.2	330	96h-LC50	Phillips & Saleh, 1988
cadmium	*Oreochromis mossambicus*	10.00	5000	behaviour	Rani and Ramamurthi, 1987
cadmium	*O. mossambicus*	Eggs, sac-fry	10	biochemical	Fu & Lock, 1990
cadmium	*O. mossambicus*	16-25	10	metabolism	Fu *et al*., 1990
cadmium	*O. mossambicus*	19-28	10	biochemical	Fu *et al*.,1989
Cadmium	*O. mossambicus*	8.5	224,008	96h-LC50	Kaviraj & Konar, 1982
Cadmium	*O. mossambicus*	10.0	50,000	48h-LC50	Rani & Ramamurthi, 1987
Chromium	*O. mossambicus*	8.5	218,000	96h-LC50	Kaviraj and Konar, 1982
Mercury	*O. mossambicus*	8.5	400	96h-LC50	Kaviraj & Konar, 1982
Zinc	*O. mossambicus*	adult	6000	48h-LC50	Mukaopadhyay, 1983

criteria used in selection of a test organism for toxicity studies.

Tilapia are widely available, occur in a wide range of aquatic habitats, including pools, lagoons, river margins and flood plains, reservoirs and lakes. Due to the keen interest in tilapia as an aquaculture species, it has been widely introduced into tropical areas other than its native Africa and can now be found in over one hundred and five countries (Balarin and Hatton, 1979; Philippart and Ruwet, 1982). Thus, tilapias could be considered as a representative fish in the tropics.

Adequate background information does exist on the biology and ecology of tilapias to establish laboratory rearing. The fry of tilapia unlike marine fry, also are amenable to hatchery rearing (Rana, 1986b; 1988). The relative ease of culture of tilapia and its rapid growth rate under tropical conditions (Balarin and Hatton, 1979; Balarin and Haller, 1982) show that tilapia are well adapted to laboratory rearing. Rana (1986a) reported high hatchability rates and subsequent fry survival rates for *Oreochromis* species (around 85%) in artificial incubation containers. The abnormalities in yolk sac-fry during intensive breeding, which are observed in other species (Doroshev, 1970; Piron, 1978), were below 2% in hatchery reared tilapia species (Rana, 1986b). The ability to spawn readily throughout the year and produce high quality fry in large numbers under laboratory conditions will supply sufficient numbers of eggs and fry for toxicity testing. The early embryology of tilapia is known. The incubation and complete yolk absorption periods are as short as five to twelve days post hatch (Rana, 1986b; 1988). Therefore, the short early life stages, which ensure a reduction in cost and time involving in toxicity tests, make tilapia useful fish for laboratory early-life stage toxicity tests.

1.4 Ecotoxicological importance of cadmium

Cadmium was selected in this thesis as a study compound due to its ecotoxicological importance. Cadmium is a biologically non-essential, non-degradable cumulative pollutant, interfering with the metabolism of some of the essential metals in animals and human beings (Allen, Grimshaw, Parkinson and Quarmby, 1973). The total world production and the total anthropogenic emissions of cadmium have increased greatly between 1920 and 1980 (Nriagu, 1979; Korte, 1982). Apart from geological sources additional cadmium enters the aquatic environment as a result of many industrial activities such as zinc refining and mining, cadmium plating, alloy, battery, aluminium solder, smoke bombs, arms and ammunitions, paint and pigment manufacturing, and from pesticides and fertilizer sources (von Westernhagen, Rosenthal and Sperling, 1974). It may contaminate the drinking water by its leakage from galvanized copper or plastic pipes (Saxena and Parashari, 1983).

Among heavy metals cadmium as a pollutant represents a great hazard to human health and has been implicated in the "itai-itai" disease in Japan (Kobayashi, 1971). Cadmium is normally present in natural water, both in soluble fractions and bound to particles. Under circumneutral pHs, particulate cadmium has no known toxic effect on fish but with a decrease in pH it may become unbound and bioavailable, unleashing a series of non-lethal and lethal effects (Haux, 1985). As shown in Table 1.2 levels of dissolved cadmium in natural fresh water are usually low, but can be considerably higher in areas affected by human activities and polluted water (see Table 1.3). The cadmium concentrations in many waters in Atlantic Canada that

Table 1.2 Cadmium concentrations in natural fresh waters

Natural fresh waters	Country	Cadmium concentration (ug/l)			Refernce
		Min.	Max.	Mean	
New South Wale fresh waters	Australia	0.02	0.12	-	Doolan and and Smythe, 1973
Upper neckar river		-	-	0.10	Lodemann and Bukenberger, 1973
Lake Washington	USA	-	-	0.02	Schell and Nevissi, 1977
St. Lowrance river	Canada	-	-	0.27	Bewers and yeats, 1977
Lake Suwa	Japan	-	-	0.02	Sugawara, 1978
Lake Constance and Upper Rhine	Germany	0.05	0.24	-	Nriagu, 1980
Po near Persepino		-	-	0.02	Nriagu, 1980
Sweden fresh waters	Sweeden	-	-	1.00	Haux, 1985
Conway river	Wales, UK	0.00	1.20	0.5	Elderfield *et al.*, 1971

Table 1.3 Cadmium concentrations in polluted fresh water.

Natural fresh waters	Country	Maximum concentration ($\mu g l^{-1}$)	Reference
Siberian fresh waters	USSR	3.0	Ududov and Parilov, 1961
Cuyahoga river	USA	120.0	Kopp and Kroner, 1968
Conway river	UK	95.0	Thornton *et al.*, 1975
Carron river	UK	20.0	Thornton *et al.*, 1975
River Rheidol	UK	4.7	Abdulla and Royle, 1972; 1974
River Tean	UK	36.0	Cooper and Solbe, 1978
River Ystwyth	UK	3.8	Abdulla and Royle, 1972; 1974
Jintsu river	Japan	9.0	Goto, 1973
Lower Rhine	Germany	16.4	Kempf, 1973
Guantin reservoir	China	0.7	Forstner and Wittman, 1981

provide important habitat for the Atlantic salmon are in the range of 1 to 1.4 $\mu g l^{-1}$ (Mercer-Clarke and Lord, 1979) which is well in excess the safe limits of 0.3 $\mu g l^{-1}$ (Anon, 1978) to 0.4 $\mu g l^{-1}$ (Anon, 1974) recommended for salmonids in soft waters.

The toxicity of cadmium to teleosts has several interesting aspects. One of these is delayed mortality often observed in lethal toxicity tests (Alabaster and Lloyd, 1980; Haux, 1985). Accumulation factors, ie., ratio of cadmium in the tissues to level of cadmium in the water, of more than 10000 have been reported (Pascoe and Mattey, 1977). On the other hand cadmium seems to be extremely slowly eliminated from tissues that contain the major body burden of cadmium (Benoit, Leonard, Christensen and Fiandt, 1976; Pentreath, 1977; Calamari, Gaggino and Pachetti, 1982). Furthermore non-lethal effects initially only recorded in high dose groups may subsequently also develop in low dose groups (Bengtsson, Carlin, Larsson and Svanberg, 1975; Larsson, 1975). This suggests that cadmium may lack a distinct threshold where no effect is observed, which indicates that it is hard to provide a safe level of cadmium in water (Haux, 1985). Hence, a comprehensive approach to evaluate the effects of cadmium on a wide range of species is needed in order to delineate its toxicity in the environment.

1.5 The effects of cadmium toxicity on early life stages of fish.

The major concern of research into heavy metal in general, and cadmium in particular, in the past has been primarily with direct toxicity, with much of the work oriented towards ascertaining the lethal levels. Less attention had been given to aspects of

indirect toxicity such as long-term non-lethal effects. Very little work has focused on elucidating the underlying resistance mechanisms of fish to cadmium insult.

The term non-lethal is not used in this thesis as a synonym for "sub-lethal". The term "sub- lethal" is not easily defined. Rosenthal and Alderdice (1976) defined "sub-lethal" as those responses to environmental changes that may be induced in one stage of development but be expressed at a later stage of development in terms of reduced survival potential. Sub-lethal effects might not necessarily be persistent. Particularly during the very early cleavage stages of embryonic development, irregularities caused by different stresses being adjusted in the course of development (von Westernhagen, 1988). Therefore, to avoid confusion, the term "non-lethal" was used in this thesis to include stresses that may or may not persist until later development stages. The term "lethal" refers to the lethal response determined by the non-recovered immobility upon gentle prodding.

1.5.1 Lethal toxicity of cadmium on early life stages of fish.

There are a number of extensive reviews on the toxicity of cadmium to fresh water fish (Alabaster and Lloyd, 1980; Nriagu, 1980; 1981; Mance, 1987; Sorensen, 1991). In aquatic environments, the presence of relatively low and high levels of cadmium is reported to be lethal to early life stages of teleost fish (Table. 1.4). This is exemplified by the 96-hr LC50 value for rainbow trout, which was estimated to be as low as 1 ug/l (Ball, 1967).

The exact mechanisms causing death due to cadmium poisoning in fish has not been

Table 1.4 Summary of representative lethal toxicity (LC50) of cadmium for early life stages of different fresh water fish species or fresh water early life stages.

Common name	ELS	Water quality			Exposure period (h.)	Concentration (μgl^{-1})	Reference
		T°C	pH	Hardness (mg/L)			
rainbow trout	embryo-sac-fry	10.0	7.6	50.0	96	9200.0	Van Leeuwen, *et al.*, 1985
rainbow trout	fry	10.0	7.6	50.0	96	30.0	Van Leeuwen, *et al.*, 1985
Atlantic salmon	embryo	5-10	7.3	28.0	-	800.0	Rombough & Garside, 1982
Atlantic salmon	fry	5-10	7.3	28.0	24	2700.0	Rombough & Garside, 1982
rainbow trout	fry	15.5	7.2	20.0	48	91.0	Calamari *et al.*, 1980
rainbow trout	fry	15.5	7.2	80.0	48	358.0	Calamari *et al.*, 1980
rainbow trout	fry	15.5	7.2	320.0	48	3698.0	Calamari *et al.*, 1980
brook trout	fry	12.0	7.7	44.0	96	2.4	Carrol *et al.*, 1979
brook trout	fry	12.0	7.7	340.0	96	26.0	Carrol *et al.*, 1979
medakafish	fry	24.0	-	100.0	96	350.0	Canton & Slooff, 1982
medakafish	fry	24.0	-	200.0	96	130.0	Canton & Slooff, 1982
common carp	fry	-	-	55.0	96	240.0	Rehwoldt *et al.*, 1972
fathead monnow	embryo-sac-fry	20.0	7.7	101.6	192	125.0	Birge *et al.*, 1985
fathead minnow	embryo-sac-fry	28.0	7.7	101.6	192	87.0	Birge *et al.*, 1985
zeebrafish	sac-fry	25.0	7.0	40.0	48	330.0	Dave, 1985
zeebrafish	sac-fry	25.0	5.0	40.0	48	1100.0	Dave, 1985

resolved (Sorensen, 1991). Several possible causes for death due to cadmium stress have been suggested. Among those, the most commonly suggested are physiological disturbances to respiration which result in hypoxia, ionoregulatory disturbances resulting in body ion depletion and necrotic damages. no attempts have been made to identify the underlying mechanisms behind species-specific differences in cadmium toxicity in fishes.

1.5.2 Non-lethal effects of cadmium on early life stages of fish.

The tolerance capabilities of early life stages to cadmium stress may vary with the developmental stage and species. Early-life stages that were considered in this review are the developmental stages from fertilization to fry stage of fish. Gamete production and fertilization processes were considered as events of reproduction.

1.5.2.1. Tolerance of fish embryos and yolk sac-fry to non-lethal cadmium stress

Early embryonic development of Baltic herring was affected by low concentrations (5.0 $\mu g l^{-1}$) of cadmium (Ojaveer, Annist, Jankowoski, Palm and Raid, 1980). The most sensitive embryonic developmental stage of Japanese medaka (*Oryzias latipes*) to cadmium toxicity was found to be early cleavage stage up to thirty two cell stage (Michibata, Najima and Kojima, 1987). In contrast, Van Leeuwen, Griffioen, Vergouw and Maas-Diepeveen (1985) reported that the sensitivity of rainbow trout embryos to cadmium increased during embryonic development. Cadmium in high concentrations may have a indirect detrimental effect on embryogenesis by altering the properties of egg membrane and jelly coat and impeding oxygen exchange (Alderdice, Rao and Rosenthal, 1979; Von Westernhagen, Dethlefsen and Rosenthal, 1975). Chorion

strength of herring embryo during late development is reduced by cadmium (Rosenthal and Sperling, 1974; von Westernhagen, *et al.*, 1974; Alderdice, Rosenthal and Velson, 1979a,b). This weak chorion may be the reason for increased sensitivity to cadmium stress as reported by Van Leeuwen *et al.*, (1985). Up to 98% of the cadmium can be associated with the chorion, rather than with the developing embryo (Beattie and Pascoe, 1978, Michibata, 1981, Peterson, Metcalfe and Ray, 1983). Generally greater resistance of eggs (embryos) than sac-fry to metal poisoning has been attributed to a measure of protection afforded by the egg capsule rather than to some change in the intrinsic sensitivities of the various stages (Rosenthal and Sperling, 1974; von Westernhagen and Dethlefsen, 1975; Beattie and Pascoe, 1978; Michibata, 1981). These observations are difficult to reconcile with the chorionic function described by Rombough and Garside (1982), who reported that dechorionated Atlantic salmon embryos were more resistant to cadmium and took up less of the metal than did embryos with intact chorions (Weis and Weis, 1991). Therefore, the underlying mechanism for the resistance of embryos to cadmium remains uncertain.

Due to its resistance, the embryo stage has been omitted in many early-life stage toxicity studies (Crossland, 1985; USEPA, 1989). Metal toxicity to early life-stages of fish expresses itself most sensitively in sac-fry than embryo, with significant inhibition of growth in Atlantic salmon upon exposure to cadmium levels as low as 0.47 $\mu g l^{-1}$, while a significant reduction in viable hatch is noticed at high cadmium concentration (300-800 μg^{-1}) (Rombough and Garside, 1982). Low concentrations of methylmercuric, cadmium and lead ions appear to cause little "biochemical stress" on brook trout embryos, but cause definite changes in sac-fry (von Westernhagen, 1988).

The response variation in tolerance to cadmium among early-life stages attributable to phenotypic variations such as, size and age has been overlooked. Natural fish communities are composed of a mixture of species and phenotypes. Therefore, an integrated approach to determine interspecific and phenotypic sensitivity would be useful to predict impacts on biodiversity.

1.5.2.2 Effects of non-lethal cadmium stress on morphology of embryos and yolk sac-fry

The most conspicuous non-lethal effects observed due to cadmium on embryos and sac-fry are abnormal development of the spinal column, head and eye and irregular proliferations from the main body over the yolk surface (von Westernhagen, 1988). Deformations of the eye are common in sac-fry hatched from eggs that were exposed to cadmium. Eye diameter of the newly hatched sac-fry incubated in cadmium contaminated water tends to decrease with increase in cadmium concentrations from 50.0 to 500.0 $\mu g l^{-1}$ (Ojaveer *et al*., 1980) or > 1000.0 $\mu g l^{-1}$ (von Westernhagen, *et al*., 1974) irrespective of the salinity level. Subtle deviations from the normal are displayed by herring sac-fry from embryos exposed to cadmium (Rosenthal and Sperling, 1974, von Westernhagen *et al*., 1974).

Cadmium causes vertebral damage in developing fish embryos at concentrations between 8.0 $\mu g l^{-1}$ (Eaton, 1974) and 300 μg^{-1} (Rombough and Garside, 1982) in fresh or brackish water (Voyer, Wentworth, Berry and Hennekey, 1977) but higher concentrations of between 1000.0 and 2000.0 $\mu g l^{-1}$ in sea water (von Westernhagen *et al*., 1974, 1975). Cadmium concentrations up to 1000 $\mu g l^{-1}$ with varying salinities

(10 to 30 ppt) and temperatures (5°C to 10°C), had a deleterious impact on the embryonic development of winter flounder that manifested itself at the time of hatching (Voyer *et al.*, 1977). Eaton (1974) reported that bluegill embryos when incubated in cadmium contaminated water (780 $\mu g l^{-1}$) developed severe physical abnormalities including pericardial and abdominal edema, lordosis dorsally of more than 90° from normal, delayed yolk sorption, shorten and deformed caudal fin, peduncle and microcephalia. Cardiac malformations were also reported in rainbow trout embryos subjected to 10-100 $\mu g l^{-1}$ cadmium treatment (Woodworth and Pascoe, 1982).

Since cadmium interferes with calcium metabolism it may also impair the calcification process directly (von Westernhagen, 1988). Abnormalities were also seen in perichord and chorda leading to a bent body in Baltic herring sac-fry hatched from eggs incubated in cadmium contaminant water (Djaveer *et al.*, 1980). These gross deformities due to cadmium lead to abnormal behaviour during embryonic and sac-fry stages and may affect their survival (von Westernhagen, 1988).

1.5.2.3 Effects of non-lethal cadmium stress on physiology of embryo and yolk sac-fry

In addition to morphological effects, activity, hatching success and growth of embryos and yolk sac-fry have received considerable attention as sensitive end points in early-life stage toxicity tests.

Activity

Many workers have used the change in the rate of heart beat of embryos as an end point in non-lethal toxicity tests to determine the effects of cadmium on embryonic activity (von Westernhagen *et al.*, 1975; Dial, 1978). Cadmium concentrations around 1000

$\mu g l^{-1}$ in the incubation medium of garpike eggs caused a reduction in heart beat rate of the developing embryos (von Westernhagen *et al.*, 1975). The depressed heart beat was more pronounced with advancing development and at low salinity levels. Cadmium influences the normal "wriggling" movement of late embryos of herring (von Westernhagen *et al.*, 1974; von Westernhagen, Dethlefsen and Rosenthal, 1979) and pectoral fin movement, responsible for circulating the perivitelline fluid, of late embryos of garpike (von Westernhagen *et al.*, 1975). Implication of these effects on embryo movements would be altered hatching success. The detrimental effect of cadmium on embryonic activity was most apparent in diluted sea water or in fresh water.

Sac-fry hatched from eggs incubated in high concentrations of cadmium remain still as seen in bluegill and cod (Eaton, 1974; Swedmark and Granmo, 1981) or show reduced swimming activity as seen in winter flounder up to 100 $\mu g l^{-1}$ (Voyer, Cardin, Heltsche and Hoffman, 1982). These responses suggest a potential long term effect on sac-fry yolk utilization, growth and susceptibility to predation (Rosenthal and Alderdice, 1976; von Westernhagen, 1988). Therefore, the implication of lowered larval activity would be reduced fitness.

Hatching success

Cadmium can cause both shortening and lengthening of the incubation time depending the concentration in the medium. Rosenthal and Sperling (1974), von Westernhagen *et al.*, (1974), Mounib, Rosenthal and Ersan, (1976) and Woodworth and Pascoe (1982) reported that cadmium shortened the incubation period of herring eggs and caused premature hatch, while Servizi and Martens (1978) reported a prolonged incubation period for salmonids. Premature hatch is often associated with a reduction in chorion strength. Rosenthal and Sperling (1974) attributed the decreased hatching time to reduced embryonic activity preventing the normal distribution of hatching enzyme, resulting in it concentrating in one region, thus facilitating the rupture.

Effects of toxic metals on hatchability and viable hatch are dependent on the stage of development, the species and type of toxicant. The viable hatch is regarded as a more sensitive indicator of toxic effects than hatchability because only the viable and normal sac-fry are eventually recruited to the adult population (Rosenthal and Sperling, 1974; Mazmanidi and Bazhashvili, 1975; Voyer *et al.*, 1977; Ojaveer *et al.*, 1980; von Westernhagen, 1988). Cadmium exerts detrimental effects on hatchability and viable hatch at lower concentrations in fresh water or diluted salt water than in salt water (Table. 1.5). A negative relationship was observed between cadmium toxicity and salinity level on the effects of viable hatch of salt water species. The enhanced toxicity at lower salinity may be due to greater uptake of water, and, therefore, of the metal (Weis and Weis, 1991). It is also known that at the low salinities and fresh water, less cadmium ions may go into complex ion formation and more will be available as free ions. Unlike salinity, temperature has contrasting effects

Table 1.5 Effect of cadmium toxicity and salinity on viable hatch of different fish species.

Species	Early life stage	Water quality				Reference
		T°C	pH	Salinity (ppt)	concentration (μgl^{-1})	
Clupea harengus	embryo-sac-fry	10	7.5-8.0	5; 16	500; 5000	Von Westernhagen *et al.*, 1974
Clupea harengus	embryo-sac-fry	10	6.8-7.3	20	5000-10,000	Rosenthal & Sperling, 1974
Salmo salar	embryo-sac-fry	5	6.5	0	270	Rombough and Garside, 1982
Pseudopleuronectes americanus	embryo-sac-fry	5	-	10; 30	1750; 2110	Voyer *et al.*, 1977
Clupea harengus pallasi	embryo-sac-fry	5	6.5	20	10,000	Mounib *et al.*, 1976
Oncorhyncus mykiss	embryo-sac-fry	10	7.5-8.0	0	124	Woodworth & Pascoe, 1982
Pseudopleuronectes americanus	embryo-sac-fry	10	-	10; 30	320; 1100	Voyer *et al.*, 1977

Table 1.6 Effects of temperature on cadmium toxicity of early life stages of different fish species

Species	Early life stage	Water quality			End point	Concentration (μgl^{-1})	Reference
		T°C	pH	Salinity (ppt)			
Salmo salar	embryo-sac-fry	5	6.5-7.3	0	viable hatch	270.0	Rombough & Garside, 1982
Salmo salar	embryo-sac-fry	10	6.5-7.3	0	viable hatch	800.0	Rombough & Garside, 1982
Pimephales promelas	embryo-sac-fry	20	7.70	0	LC50	125.0	Birge *et al.*, 1985
Pimephales promelas	embryo-sac-fry	28	7.70	0	LC50	87.0	Birge *et al.*, 1985
Pseudopleuronectes americanus	embryo-sac-fry	5	-	10	viable hatch	1750.0	Voyer *et al.*, 1977
Pseudopleuronectes americanus	embryo-sac-fry	10	-	10	viable hatch	320.0	Voyer *et al.*, 1977
Pseudopleuronectes americanus	embryo-sac-fry	5	-	30	viable hatch	2110.0	Voyer *et al.*, 1977
Pseudopleuronectes americanus	embryo-sac-fry	10	-	30	viable hatch	1110.0	Voyer *et al.*, 1977

(Table. 1.6) on cadmium toxicity. Rombough and Garside (1982) reported a greater embryonic toxicity of Atlantic salmon at low than high temperature, while Birge *et al*., (1985) reported opposite toxicity of cadmium to embryos of fathead minnow with increasing temperature. The former attributed the higher toxicity of cadmium at low temperature to a prolongation of sensitive stage by the reduced developmental rate at the lower temperature whereas the latter suggest an increased metabolic rate at higher temperature.

Growth

Growth of the newly-hatched fry from the eggs that were subjected to cadmium stress, has been used as a sensitive non-lethal end point in early-life stage toxicity tests (Eaton, 1974; Rosenthal and Sperling, 1974; Ojaveer *et al*., 1980). Reduced growth in terms of length was reported to be more sensitive in fresh water than in salt water (Table 1.7). Reduced length of newly hatched larvae is frequently correlated with larger yolk sac sizes (von Westernhagen, 1988). A large or deformed yolk sac is taken as an indication for metabolic or osmotic disturbances that may be caused by mitochondrial malfunction, induced by heavy metals or petroleum hydrocarbons (von Westernhagen, 1988)

Increased yolk sac volume has been reported for herring embryos incubated in high cadmium concentrations (von Westernhagen, *et al*., 1974) and with decreasing salinity levels (Rosenthal and Mann, 1973). Since metabolism and mobilization of yolk proteins involve formation of calcium complexes with those proteins (Black, Pritchard and Fraser, 1971), their utilization may be less efficient in yolk sac-fry exposed to

Table 1.7 Effect of cadmium toxicity on growth of yolk sac-fry hatched from cadmium treated eggs of fresh and salt water fish species.

	Water quality				
Species	**T°C**	**pH**	**Salinity (ppt)**	**Concentration (μl^{-1})**	**Reference**
Salmo salar	8-10	6.8-7.3	0	5	Rombough & Garside, 1982
Clupea harengus	8-10	6.8-7.3	20	1000	Rosenthal and Sperling, 1974
Oncorhyncus mykiss	10	7.5-8.0	0	124	Woodworth & Pascoe, 1982
Clupea harengus	10	7.5-8.0	5	>100	Von Westernhagen *et al.*, 1974;

cadmium (Westernhagen *et al.*, 1974; Rombough and Garside, 1982; Peterson *et al.*, 1983). It has been reported that the impairment of yolk utilization occurs in Atlantic salmon yolk sac-fry when reared through yolk absorption in low (0.47 ugl^{-1}) cadmium concentrations (Rombough and Garside, 1982). In addition to the said growth-related physiological effects, osmotic disturbances in the perivitelline fluid have occurred when fish embryos were exposed to cadmium (Rosenthal and Sperling, 1974; Alderdice *et al.*, 1979b).

Growth is the resultant surplus energy in the balanced energy equation given below and despite the awareness that it could represent an important index of environmental stress, no attempts have been made to investigate the effects of cadmium toxicity on growth in terms of energy.

$$\underset{\text{(production energy)}}{Pr + Pg} = \underset{\text{(supplied energy)}}{C} - \underset{\text{(expended energy)}}{(TM + U + F)} \quad \ldots\ldots 1.1$$

where, Pr = reproductive production

Pg = somatic production

C = food energy,

TM = total metabolic energy

U = energy loss in excretory matter

F = energy loss in faecal matter.

$$AE = C - (F+U) \quad \ldots\ldots 1.2$$

where, AE = assimilated energy.

Component Pr is not involved in early life stages such as yolk sac-fry. Therefore,

$$Pg = AE - TM \quad \ldots\ldots 1.3$$

Most studies employing growth as an index of cadmium toxic effect used the direct measurement of growth in terms of length or weight rather than determining it together with the effects on different elements of the balanced energy equation. Determination of the available energy for growth based on the physiological and biochemical analysis of components in the balanced energy equation proves particularly useful in assessing the biological impact and tolerance mechanisms to stress at the individual, population and ecosystem level and permits the application of modelling (Kooyman and Metz, 1984). Moreover, linking stress tolerance capability to available energy for growth (see section 1.1.3) may reveal whether there is a general tolerance response to metals.

1.5.2.4 Effects of cadmium stress on biochemical activity of embryos and yolk sac-fry.

Cadmium can cause a significant decrease, increase or inhibition in enzyme activity in fish embryos and yolk sac-fry (Christensen, 1975). The activity of four important carbon dioxide- fixing enzymes, namely, propionyl coenzyme A (Co A) carboxylase, nicotinamide adenine dinucleotide (NAD) enzyme, NADP malic enzyme and

phosphoenolpyruvate (PEP) carboxykinase, decreased in Pacific herring eggs when exposed to 10,000 $\mu g l^{-1}$ cadmium (Mounib *et al.*, 1976). It is also known to inhibit the proteolytic functions of enzymes (Hagenmaier, 1974) in developing fish embryos. In view of the important role played by the carbon dioxide fixing enzymes in biosynthetic processes, the effect of cadmium in depressing enzyme activity during the developmental stages may result in lethargic embryos and small and inactive yolk sac-fry (von Westernhagen, 1988). Cadmium also significantly affected the activity of enzymes such as glutamic-oxaloacetic transaminase (GOT), alkaline phosphate (ALP), acetylcholine esterase (ACH) and adenosine triphosphate (ATP), causing either a significant decrease in activity late embryos or increase in yolk sac-fry of brook trout (Christensen, 1975). Cadmium not only affects the activity of enzymes but also affects the substrates of enzymatic reactions (Scoppa, 1975; Zaba and Harris, 1978). The reduced growth which is often observed in yolk sac-fry at hatching may be due to decreased enzyme activity, which has been noted in cadmium exposed embryos (Weis and Weis, 1991).

Cadmium may also influence hormonal activity in embryos and yolk sac-fry. Fu and Lock (1990) demonstrated an increase in fractional volume of prolactin cells in *Oreochromis mossambicus* embryos, indicating a higher synthesizing capacity. They suggested that the higher levels of synthesised prolactin have a decisive role in newly hatched fry to counteract adverse effects of cadmium.

A few recent investigations suggested that even without obvious external damage, pollutants such as metals may adversely affect cellular or sub-cellular and tissue

organization by disrupting intracellular structure in organs, causing damage to mitochondria, ribosomes and endoplasmic reticulum (von Westernhagen, 1988). This in turn may affect biochemical and related physiological reactions and may give rise to delayed responses ultimately affecting survival. Terms such as cellular or sub-cellular ultrastructure and biochemical functions have been used only superficially to describe the possible causes to the observed responses instead of establishing clear cause-effect relationships.

From the above review it was revealed that interspecific comparisons in sensitivity are difficult due to large variation in experimental conditions and exposure durations (see Tables 1.4 and 1.8). Most early-life stage toxicity studies with different fish species have focused on easily visible responses such as gross morphological abnormalities and growth (see Table 1.9 and Appendix II) in terms of differences in length or weight increments to cadmium stress, ignoring the question why are there differences in sensitivities? The extrapolation of these responses at the individual level to effects at the population level has not been attempted. Yet unless stress can be shown as having effects at the population level it is unlikely to be ecologically significant (McIntyre and Pearce, 1981). These morphological and growth responses may be considered as ultimate responses to stress. Moreover, a possible relationship between lethal and non-lethal metal stress responses in early life stages has been overlooked. Investigations into a possible relationship between lethal and non-lethal stress responses may yield a possibility of uncovering a general response or a mechanism of metal tolerance. The range of species on which this kind of knowledge is available is extremely narrow.

1.6 Aims of the study

Despite the awareness of increasing metal pollution in the tropics (Berg, Kribus and Kautsky, in press.), published data on the toxicity of cadmium to fish early-life stages largely relates to temperate species (Alabaster and Lloyd 1980). Consequently, we are no further towards either providing a suitable system for assessing toxicity levels of cadmium in tropical aquatic environments, or selecting the most sensitive stage and species to adopt as a representative of such environments, or understanding the tolerance mechanisms to cadmium stress. To address these short comings the aims of this thesis were to -

1) design a flow-through system suitable for early life stage toxicity testing using tilapias,

2) investigate the interspecific and intraspecific phenotypic variations of tilapias in tolerance to lethal and non-lethal cadmium stress,

3) investigate the correlation between the early life history growth traits and associated physiological and biochemical parameters and stress tolerance capability of tilapia to cadmium,

4). investigate the correlation between the cadmium body burden of tilapias and the tolerance capability, and,

5). investigate whether there is a relationship between the tolerance capabilities of tilapias to lethal and non-lethal cadmium stress.

Table 1.8 Summary of representative non-lethal concentration of cadmium for early life stages of different fresh water fish species or fresh water early life stages.

		Water quality					
Comman name	**Early life stage**	**T°C**	**pH**	**Hardness (mg/L)**	**concentration (μgl^{-1})**	**End point**	**Reference**
White sucker	embryo-Juvenile	10	7.6	45	12.0	growth	Eaton *et al.*, 1978
Northern pike	embryo-juvenile	10	7.6	45	12.9	growth	Eaton *et al.*, 1978
smallmouth bass	embryo-juvenile	10	7.6	45	12.7	growth	Eaton *et al.*, 1978
coho salmon	embryo-juvenile	10	7.6	45	12.5	growth	Eaton *et al.*, 1978
lake trout	embryo-juvenile	10	7.6	45	12.3	growth	Eaton *et al.*, 1978
brown trout	embryo-juvenile	10	7.6	45	11.7	growth	Eaton *et al.*, 1978
brown trout	embryo-juvenile	10	7.6	45	3.7	growth	Eaton *et al.*, 1978
coho salmon	sac-fry	10	7.6	45	3.4	growth	Eaton *et al.*, 1978
Atlantic salmon	sac-fry[a]	8-10	7.3	28	8.2	metabolism	Rombough & Garside, 1984
Atlantic salmon	sac-fry[a]	8-9	7.3	28	270.0	growth	Rombough & Garside, 1982
Atlantic salmon	sac-fry	8-12	6.8	13?	2.0	growth	Rombough & Garside, 1982
Atlantic salmon	embryo	5	6.5	19?	270.0	hatchability	Peterson *et al.*, 1983
Atlantic salmon	fry	8-12	6.8	13?	2.0	growth	Peterson *et al.*, 1983
rainbow trout	embryo-sac-fry	11.5	7.9	89	124.0	incubation time	Woodworth & Pascoe, 1982
flagfish	embryo-sac-fry	25	7.8	44	31.0	growth	Spehar, 1976
fathead minnow	embryo	16-27	7.6	204	57.0	hatchability	Pickering & Gast, 1972
brook trout	embryo-sac-fry[a]	-	-	37	6.0	growth, survival	Sauter *et al.*, 1976
brook trout	embryo-sac-fry[a]	-	-	188	7.0	growth, survival	Sauter *et al.*, 1976
channel catfish	embryo-sac-fry[a]	-	-	185	17.0	growth	Sauter *et al.*, 1976
garpike	embryo	15	-	-	2000.0	hatchability	Von Westernhagen *et al.*, 1975

a- Hatched from cadmium treated embryos

Table 1.9 The occurrence of different sensitive end points used in fish early life stage toxicity studies corresponding to non-lethal cadmium stress (n = 57)

Sensitive end point	Occurrence (%)
Hatching success	29.80
Growth	28.08
Morphology	17.54
Metabolism	8.77
Embryonic activity	7.03
Biochemical	5.26
Behaviour	3.52

CHAPTER 2

GENERAL MATERIALS AND METHODS

Techniques common to all sections of the present study are described below. Materials and methods specific to individual experiments are outlined in the relevant chapters.

2.1 Procurement of eggs and fry

2.1.1 Brood stock.

Six tilapia species, *Oreochromis niloticus* (Linnaeus), *Oreochromis mossambicus* (Peters), *Oreochromis aureus* (Steindachner), *Sarotherodon galilaeus* (Linnaeus), *Tilapia zillii* (Gervais) and *Tilapia rendalli* (Boulenger) used during the course of this investigation were procured from the tropical hatchery at the Institute of Aquaculture, University of Stirling. The brood fish were maintained in a recirculatory system in the tropical aquarium facility of the Institute of Aquaculture. The recirculatory system comprised of 12 glass aquaria (250 l), which individually received water at a rate of 2 l $min.^{-1}$ through delivery pipes from the header tank. Water quality was maintained by an extensive system of biofiltration. The outflows from individual tanks were channelled to sump tanks through a series of settling tanks, interdigitated with bio-filter tanks which contained bio-filter rings. A submersible pump was used to pump the water from the sump tank to the header tank from which water was fed into the fish holding tanks by gravity. The efficiency of the bio-filters was regularly monitored by measuring NH_4-N and NO_3-N in the tank water. The system was cleaned and fresh water added at regular intervals. Water temperature was regulated (25.5 and 27°C) using thermostatically- controlled heaters in the header tank. A 12:12 h light and dark regime was maintained using electronically preset lights. Individual fish holding tanks

were aerated using diffusers and additional aeration was provided in the header and sump tanks to cope with the oxygen demand generated by the bacterial activity in the bio-filters. The oxygen level in the tanks was always maintained above 6 mg/l. The fish were fed daily with trout pellet (Omega, no.3; protein content-47%, Ewos Bakers, Bathgate, Scotland).

2.1.2 Supply of eggs and fry

Eggs and yolk sac-fry for the experiments were obtained from genetically pure species (McAndrew and Majumdar, 1983). Artificially obtained eggs/yolk sac-fry stocks were used in all experiments. For the artificially obtained yolk sac-fry stocks, ovulated eggs were manually stripped from females into clean dry petri dishes and fertilized with milt pooled from three conspecific males. Five minutes were allowed for fertilization. The eggs were then rinsed in a net container with clean warm (28°C) water. Prior to incubation 50 randomly sampled eggs from each clutch were weighed on a balance (Mettler AE100). The egg clutches of the mouth-brooding *Oreochromis* and *Sarotherodon* species were then transferred to round bottom jar incubators described by Rana (1986a), until used in the experiment. The eggs from each clutch of the substrate-spawning *Tilapia* species were placed in petri dishes before transferring to the chambers of the incubation system.

2.2 Dilution water.

In order to carry out metal exposures under consistent conditions, an artificial exposure medium was used for all trials as recommended by ASTM (1980). Soft

synthetic dilution water at temperature 27+ 1°C was prepared according to the ASTM (1980) chemical recipe (see Appendix III) using nano pure water (conductivity < 0.05 $\mu s\ cm^{-1}$). Nanopure water was obtained from a filtration system (Barnstead D 4752). Dilution water was prepared and continuously aerated for at least 24 h before use. The water hardness, temperature, pH and dissolved oxygen were monitored by standard methods (APHA, 1989).

2.3 Toxicant solution.

Cadmium chloride (Anhydrous $CdCl_2$ Analar grade, Sigma Chemicals Ltd.) was used to prepare the metal stock solution. The stock solution (one l) of required concentration was made up in nanopure water and stored in stoppered and labelled amber colour glass bottles (2.5 l volume). The required concentration of toxicant solution was prepared by mixing the appropriate amounts of $CdCl_2$ stock solution and pre-aerated dilution water. Toxicant solutions were not aerated during any experiment.

2.4 Preparation of the system for experiments.

New chambers were cleaned to remove any chemical or dirt residues remaining from manufacture or accumulated during construction and storage. Hypochlorite at 200 mg/l (5 ml of household bleach in 1 litre of water) was used followed by rinsing with water to disinfect and remove any organic matter in the system. 5% nitric acid was used to remove mineral deposits and metal residues that may have an adverse effect on the test organism. Finally the system was washed with dilution water. Then the flow-

through system was run for 12 h, continuously before eye-fry were introduced into the exposure chambers. Physical and chemical water parameters were monitored during this time to ensure that the system was operating efficiently. Acceptable limits of stability were usually achieved within 24 h and maintained with in limits thereafter.

2.5 Monitoring metal concentration.

The nominal concentrations of the metals used in the present study were maintained by regulating the concentration of the metal in the stock solution and flow rate per minute of the toxic solution into an individual exposure chamber. The actual concentration of the metal in the exposure chamber was monitored regularly with each experiment. Samples of water taken at regular intervals from individual exposure chambers during each experiment were acidified (1% HNO_3) and analyzed using an atomic absorbtion spectrophotometer (Perkin-Elmer 2280) (Golterman, Clymo and Ohnstad, 1978). Then actual concentration of the metal in the water was calculated from a standard curve. The actual concentrations of the respective metals in the exposure chambers did not differ significantly ($df = 79$, $t = 0.722$, $P > 0.05$) from the nominal concentrations (Figure 2.1 & 2.2). The actual concentrations of the metal in the exposure chambers were always within 10% of the nominal concentrations. The lowest detection levels of the metals, cadmium and copper, used in the present study were 0.5 $\mu g l^{-1}$ (W. Struthers, Institute of Aquaculture, University of Stirling, personal communication).

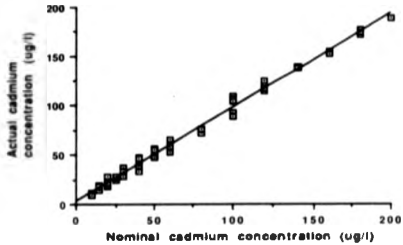


Fig. 2.1 Relationship between nominal and actual cadmium concentration at t=0 h. Actual Cd=2.863+0.955 X nominal Cd (n=81).

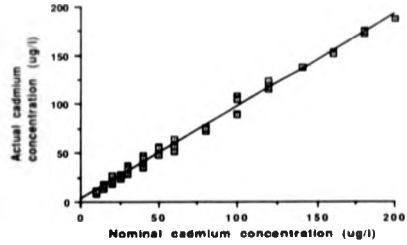


Fig. 2.2 Relationship between nominal and actual cadmium concentration at t=96 h Actual Cd = 2.518+0.951 X nominal Cd (n=81)

2.6 Monitoring important water quality parameters.

The temperature, oxygen, pH, hardness and ammonia were monitored according to APHA (1989). These parameters were analyzed during evaluation of the flow-through system (see Chapter 3), primarily to ensure the system did not allow the build-up of the harmful levels of ammonia or deplete oxygen.

2.7 Flow rate of the system.

The flow of the water through the test chambers minimised the accumulation of metabolic products, prevented build-up of organic matter which could be a nutrient source for bacteria and maintained acceptable dissolved oxygen content of solution in the exposure chamber. The water flow through the exposure chambers was always maintained to achieve 90% replacement time within each exposure chamber every 4 h. This was above the recommended minimum replacement time in a flow-through toxicity system (Sprague, 1976).

2.8 Acclimatization of experimental fish.

Sac fry were removed from the round bottom jars (see section 2.1.2) and transferred to the exposure chambers of the flow-through system and held for a minimum of 24 h before the commencement of any experiment. This procedure of acclimatization remained the same throughout the study.

CHAPTER 3

DESIGNING A FLOW-THROUGH TOXICITY TESTING SYSTEM FOR EARLY LIFE STAGES OF TILAPIA

3.1 Introduction

There is a considerable discrepancy between results obtained between responses to toxic stress when tested under static and flowing test conditions (Mance, 1987). The advantages of a flow-through system over a static system for toxicity testing have been described by several authors (Buikema, Niederlenher and Cains, 1982; Pascoe and Edwards, 1989). The use of flow-through systems in aquatic toxicity testing is superior to static systems, not only because they prevent the build up of test animal waste products, but also they allow a constant exposure level to be maintained over the test period. This latter advantage is particularly important when dealing with so-called 'difficult substances' (i.e. poorly water soluble, volatile or biodegradable compounds).

3.1.1 Physical and chemical considerations

The movement of the water itself through the test chamber in a flow-through toxicity testing system, influences the results of a test. Fluctuation in the quantity of toxicant introduced into the test chamber may affect the validity of test results more significantly than fluctuations in other physical and chemical test conditions. Hence, the toxicant delivery system in a flow-through toxicity testing system plays a critical role in the validity of the test results. Many systems consist of two devices to deliver the dilution water and toxicant stock solution. A number of devices designed to deliver dilution water have been described previously, and early devices include those operated by hydrostatic force (Abram, 1960; Grenier, 1960; Mount and Warner, 1965;

Stark, 1967) and electromechanical devices (Herbert and Merkens, 1952; Merkens, 1957, Alabaster and Abram, 1965). Benoit, Mattson and Olson (1982) developed a space saving gravity operated system which can also be installed in a compact vented enclosure to permit safe testing of hazardous volatile chemicals.

Warner (1964), was the first to introduce a truly continuous flow serial diluter. The serial diluter and the proportional diluter described by Mount and Warner (1965) and Mount and Brungs (1967) respectively, were the first types of intermittent flow-through systems successfully used (Garton, 1980; Benoit *et al.*, 1982). Serial diluters continuously dilute a toxicant from a stock solution by successive addition of water to provide series of concentrations of a toxicant, while proportional diluters consist of dosing apparatus which can maintain a series of constant concentrations of toxicant in flowing water. A majority of designs that have been used for toxicant delivery systems are offshoots of the serial diluter and the continuous flow diluter (McKim, 1985). Interest in developing the early life stage fish toxicity test concept introduce by McKim (1977) led to design of the compact continuous-flow mini-diluter exposure systems (Benoit *et al.*, 1982).

Unfortunately, the systems which are presently in use have four basic design problems. Firstly they are complex, often requiring excessive amounts of space, large water volumes (and hence potentially large amounts of waste water for disposal) (Reish and Oshida, 1986). In many cases, flow-through systems incorporate many movable components, some of which are expensive or fragile and may not be readily or commercially available. These delicate assemblies will require frequent cleaning,

adjustment and repairs to maintain reliability (Chandler, Sanders and Walsh, 1974). System complexity can thus lead to increased variability in toxicity data and reproducibility (*sensu* Calow, 1992). Hence, when designing a flow-through toxicity testing system, special consideration should be given to readily available components, small space, low flow rate and simplicity.

Second, in multichannel systems, particularly those using proportional diluters of split-channel designs, channels within the system are not truly independent of one another, since they derive from a common reservoir. Thus, if a channel needs to be closed, or otherwise taken off-line for maintenance, this cannot be done without affecting the function of the remaining channels. In addition, channels within the system are non-independent, producing pseudoreplicated data which are unsuitable for inferential statistical analysis (Hurlbert, 1984).

Third, many toxicity testing systems use separate reservoirs to deliver toxic stock solution and dilution water. Since the homogenous mixing of toxicant and dilution water in exposure chamber would be difficult to achieve, most toxicity testing systems use a chamber between the toxicant delivery system and the exposure chamber, to promote mixing of toxicant and dilution water for each concentration (Benoit and Puglisi, 1973). These additional units not only increase the complexity of the system but also the surface area in the system for toxicant adsorption. This is one of the potential problems that was overlooked in toxicity tests which has the tendency to reduce the toxicity of the substance under testing by altering the concentration. Delivering toxicant stock solution has been done by using either a Mariotte bottle or

a dosing pump. Even though the Mariotte bottle has been widely used as a toxic stock solution delivery device, it is difficult to fill, may require insulation where ambient temperature vary considerably (Chandler *et al.*, 1974), limits the amount of stock solution and would be a disadvantage in long term experiments (Garton, 1980). Dosing pump systems for the delivery of toxic stock solution (Graton, 1980) can be simple, compact, easy to set up, and portable. High accuracy could be obtained if high quality pumps are used and such pumps will last a long time with proper maintenance (USEPA, 1982).

Finally, most toxicity testing systems are inflexible, offering the user a relatively poor choice of test parameters for measurement. In toxicity testing, particularly where this may involve work with so-called 'difficult substances' (see above), flexibility of operation is necessary in order to customise test designs appropriately (within acceptable regulatory limits, e.g. OECD, 1993).

3.1.2 Biological considerations

The usefulness of an ecotoxicity test in evaluating the hazard potential of a test toxicant is of questionable value unless the survival rates in the controls are high. For this reason a minimum survival of 80% in controls has been set for fish early life stage toxicity tests(USEPA, 1982; OECD, 1993). The test system should be designed to minimise stress to the organism due to crowding. Hence, the biological loading should be limited to assure that a) the concentration of dissolved oxygen and test substance do not decrease below acceptable levels, b) and that waste metabolites (unionised ammonia, nitrite and nitrate) accumulation does not exceed acceptable

levels.

In working with laboratory studies on the toxicity of metals to early life stages of mouth brooding tilapia, the churning movement provided in the mothers buccal cavity for eggs and early yolk sac-fry should be provided in the exposure chambers to obtain natural survival rates. To stimulate the churning movement of the naturally reared eggs and early yolk sac-fry, various investigators have used conical upwelling containers (Mires, 1973; Rothbard and Hulata, 1980; Rana, 1986a,b) and shaking tables (Shaw and Aronson, 1954; Rothbard and Pruginin, 1975; Snow, Berrios-Hernendez and Ye, 1983). Losses in these systems have been reported as high as 40%. The flow-through type round bottom jar incubation system described by Rana (1986a,b) used 500 l of water day^{-1} 1000 yolk sac-fry^{-1}. However, the yolk sac-fry in exposure chambers of the present system were not subjected to churning movement as such movements for yolk-sac fry are not essential (eg. Rana, 1986a).

The techniques mentioned above used for mass rearing of eggs and sac-fry included air-water interphase to agitate them. Flow-through systems using these techniques will require large volume of water per day and facilitate loss of toxicant into air, especially those volatile in nature. Hence, a system is needed to test toxic effects of chemicals on the early life stages of mouth brooding fish such as *Oreochromis* and *Sarotherodon* species, with closed exposure chambers throught to facilitate the measurements of physiological responses which require minimum water

3.1.3 Aim of the design of system.

The aim was to design a flow-through system which minimizes variability in the testing procedure and enhances reproducibility by overcoming the four basic design flaws outlined above with special reference to yolk sac-fry of tilapia to be used in toxicity studies. To meet this challenge using cadmium as the study compound the following were ascertained to select optimum conditions and requirements of the system for tilapia yolk sac-fry and then evaluate the efficiency of the design:-

1) the quantity of the toxicant introduced by the toxicant delivery system from one addition of toxicant to the next,

2) the adsorption of cadmium by the materials used,

3) the loading density and survival rates of tilapia yolk sac-fry under control exposure, and,

4) the important chemical parameters such as oxygen and ammonia levels.

3.2 Materials and methods

3.2.1 Design of the flow-through system

The flow-through system designed and developed to test the effects of cadmium on yolk sac-fry of tilapia is represented in schematic plan view in Figure. 3.1.

Toxicant reservoirs

Fifteen 2.5 l amber coloured glass bottles were used to deliver four different concentrations of cadmium solutions and ASTM dilution water (see chapter 2) as the control treatment. The reservoirs were filled with freshly prepared toxic solutions of different concentrations and dilution water in a randomised design at the commencement of each experiment and thereafter replaced with freshly prepared toxic solutions twice daily.

Toxicant delivery system

Toxicant solutions were delivered to individual exposure chambers using a 16-channel variable speed Watson-Marlow Peristaltic pump (model 202 U/AA). Siliconized tubing (manifold pump tubing of 1.52 mm internal diameter, Watson-Marlow, UK) connected to individual channel frames (cassettes) was used to deliver the toxicant. These short tubings were placed in such a fashion in the individual channel frames, that, during the rotation of the 8 rollers of the pump, the tubing would be pressed in between the roller and the channel frame at regular intervals. The speed of the pump and the internal diameter of the tubing determined the flow rate. One free end of the tubing was connected to an individual reservoir containing toxic solution or ASTM soft

dilution water (see Chapter 2) and the other end to an individual exposure chamber using translucent silicone tubing (1.52 mm internal diameter). These connections were secured with small plastic straight tubing connections. The outlet from the pump carrying the toxicant solution was connected to the inlet tubing of an individual exposure chamber.

Exposure chamber unit

The yolk sac-fry exposure chambers were 260 ml Nunclon polystyrene plastic stoppered tissue culture flasks (Nunc, Inter Med, Denmark). To ensure the exposure chamber was completely filled with toxicant solution at all the times clumps were connected to the outlet tubing from the individual exposure chambers to regulate the outflow in a such a way to equal to the inflow. Each chamber was placed in a plastic spill container (12.0 cm x 7.0 cm x 4.0 cm).

Waste collector

The discharged toxic solution from both the outlets and spill containers drained into a 75 l plastic container for disposal

3.2.2 Efficiency of the system.

The source, procedure for maintenance and pre acclimation of *O. niloticus* yolk sac-fry, unless otherwise mentioned, were similar to those described in chapter 2. To evaluate the efficiency of the system a series of trials were conducted to establish the consistency of the chemical composition of dilution water, flow rates of the dosing pump, the oxygen content of the reservoirs and exposure chambers, ammonia content

of the exposure chambers, amount of cadmium adsorbed and optimum loading density of the system were investigated.

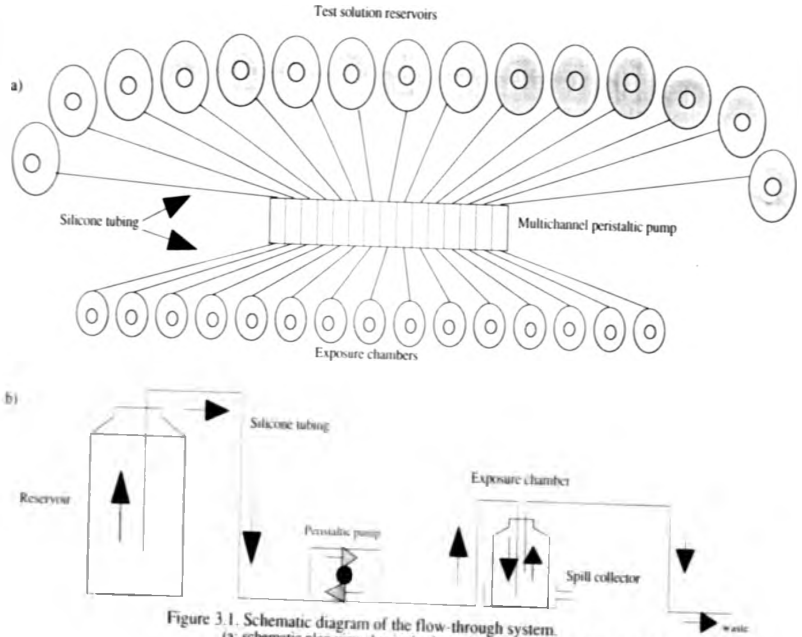


Figure 3.1. Schematic diagram of the flow-through system.
(a: schematic plan view; b: single channel of the system in elevation view)
Not to scale.

3.2.2.1 Experimental protocol.

Experiment 1: Evaluation of flow rates of the multichannels of toxicant solution dosing pump

Dilution water was filled into each of the 15 reservoirs, and collected from each inlet to exposure chambers into pre weighed 5 ml sample tubes for a duration of 3 minutes. Final weights of each tube were determined. Difference between the final and initial weights of the tubes was used to determine the flow rate of each channel. This procedure was repeated 5 times and the mean was taken as the flow rate.

Experiment 2: Evaluation of oxygen concentrations of toxicant solution reservoirs.

Fifteen reservoirs were filled with pre-aerated dilution water and their oxygen concentration was determined in each reservoir with a Strathkelvin dissolved oxygen meter (model 781). Thereafter, oxygen concentrations in each reservoir were recorded for 24 h at 4 hour intervals. The temperature of the dilution water was recorded to allow estimation of the oxygen saturation level.

Experiment 3. Determination of the chemical composition of the dilution water.

Samples were collected from outlets of exposure chambers and stored at 4°C in acid washed polythene bottles, in a matrix of 1% (V/V) nitric acid for determination of total Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}. Total levels of each were determined using atomic absorption spectroscopy as described in Golterman *et al.*, (1978).

Experiment 4: Cadmium adsorbed by the testing system.

Prior to using any toxic substance the system was operated without yolk sac-fry with 5 cadmium concentrations (0.5, 1.0, 10.0, 100.0 and 500.0 μg/l), each in triplicate, for 24 h. Triplicated samples from each replicate at 0 , 6, 12 and 24 h were acidified and analysed with an atomic absorbtion spectrophotometer. The actual cadmium concentration in the water was calculated from a standard curve. The actual concentrations at 6, 12, and 24 h were plotted against the initial actual concentrations. Regression analysis was performed to see the significant differences in the actual cadmium concentrations between initial and the final at 6, 12 and 24 h.

Experiment 5: Evaluation of the survival rates and loading density of yolk-sac fry of *O. niloticus* in the exposure chambers.

This experiment was designed to test the following aspects.

a) Whether acceptable survival rates could be achieved in the exposure chambers by rearing one day old yolk sac-fry for 15 days in the dilution water.

b) The loading density of yolk sac-fry to ensure that, 1) the concentration of dissolved oxygen does not decrease below acceptable levels, 2) the waste metabolite accumulation does not reach above unacceptable levels and 3) that yolk sac-fry are not stressed due to crowding.

To establish the optimum loading density for sac-fry in dilution water five loadings (70.0, 104.0, 140.0, 174.0 and 210.0 mgl^{-1} wet weight) of one-day old *O. niloticus* yolk-sac fry in triplicate were used. Mortality in each exposure chamber was recorded daily up to 15 days. Water samples were collected from each exposure chamber on

3, 6, 9, 12 and 15 day of the rearing period and analysed for ammonia (NH_3) concentration on a Technicon Sampler IV autoanalyser and oxygen concentrations were measured with a Strathkelvin oxygen meter (model 781) .

3.2.3 Statistical analyses

One way analysis of variance (ANOVA) and Tukey HSD multiple range tests, were used to compare the arcsine transformed values of % survival, NH_3 concentrations and arcsine transformed values of % saturation oxygen obtained at each loading density (Zar, 1984).

3.3 Results

Mean flow rates from the 15 channels are given in Table 3.1. The maximum coefficient of variation within a channel for five measurements was 0.8 %. The maximum % variation between flow rates of 15 channels was 6.0%. The mean pump flow rate was 1.12 ml min^{-1} (SE±0.0075). Total amount of dilution water required for 15 channels was 24.2 l day^{-1}.

The daily maximum and minimum saturation level of oxygen in each reservoir is shown in Figure 3.2. The maximum variation of oxygen saturation day^{-1} between reservoirs was 1.6%. The saturation level of oxygen day^{-1} in any reservoir did not fall below 95.0%. The results of the chemical composition of dilution water is presented in Table 3.2.

The relationship between initial and final actual concentrations of cadmium in exposure chambers is shown in Figure 3.3. The R^2 value (= 1.0) indicated the system material did not cause any variation between initial and final actual concentrations.

The temporal mean survival (%) for *O. niloticus* yolk-sac fry reared at various loading densities is shown in Figure 3.4. Mean survival (%) of fry reared at 70 mg (20 fry), 104 mg (30 fry), 140 mg (40 fry), 174 mg (50 fry) and 210 mg (60 fry) $l^{-1}day^{-1}$ did not fall below 88 %. There were no significant difference ($P>0.05$) between survivals among the three lowest loadings (70, 104 and 140 mgl^{-1}) while the survival of fry in the two highest loadings differ significantly ($F=16.67$, 4,10 d.f., $P<0.05$) from the

Table 3.1 Mean flow rates of each channel of the flow-through system

Channel number	Mean flow rate (ml min^{-1})	Coefficient of variation (%)	n
1	1.14	0.123	5
2	1.14	0.176	5
3	1.13	0.100	5
4	1.17	0.100	5
5	1.10	0.080	5
6	1.12	0.080	5
7	1.11	0.081	5
8	1.10	0.082	5
9	1.10	0.454	5
10	1.06	0.800	5
11	1.11	0.081	5
12	1.11	0.670	5
13	1.12	0.080	5
14	1.15	0.078	5
15	1.12	0.063	5

Table 3.2 Ionic composition and total hardness of dilution water used in the system.

Parameter	Mean (mgl^{-1})	Range (mgl^{-1})	CV (%)	n
Na^{+}	2.42	2.80-2.20	7.12	30
K^{+}	0.97	1.05-0.93	3.10	30
Ca^{++}	8.29	8.92-7.38	5.02	30
Mg^{++}	5.45	6.72-5.12	7.24	30
Total hardness as $CaCo_3$	43.30	48.4-40.40	3.84	30

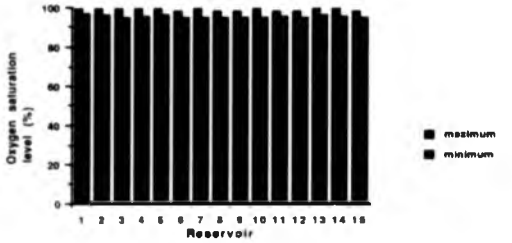


Fig. 3.2 Maximum and minimum oxygen saturation level recorded in each reservoir over 24 h.

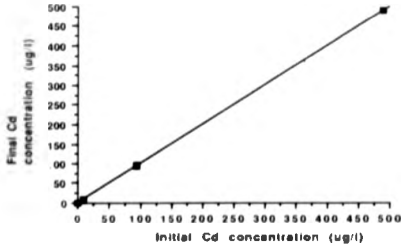


Fig.3.3 Relationship between initial and final Cd concentrations in the system without fry. Final Cd=-2.033e-4+0.999 X Initial Cd R=1.0 (n=45)

three lower loadings, but not from one another ($P>0.05$).

The temporal variation of total NH_t-N at different loading densities is shown in Fig. 3.5. The total NH_t-N showed a positive relationship with loading density. A triphasic behaviour of total NH_t-N concentration was observed at all loading densities. The first phase consisted of an increase for the first 6 days followed by the second phase of a decrease towards the end of yolk absorption stage. Third phase was the slight increase of total NH_t-N towards the 15th day during starvation. The minimum NH_t concentration (76.93 μgl^{-1}, SD±4.84) was observed after one day of rearing at the lowest loading density, while the highest (219.88 μgl^{-1}, SD±3.46) was observed on 6 day of rearing at the highest loading density. The ammonia concentrations at 5 loading densities were significantly different ($F=408.43$; 4,10 d.f.; $P<0.05$) from one another except at 140 and 174 mgl^{-1} loadings.

The temporal changes in saturation level of oxygen in exposure chambers at 5 loading densities is shown in Fig. 3.6. At all stocking densities the saturation level of oxygen decreased towards 9 day of yolk absorption and then increased towards the completion of yolk absorption and during subsequent starvation. However, the oxygen saturation did not fall below 60% during the rearing period at any loading density except for 210 mgl^{-1} day^{-1}. The oxygen saturation levels at 5 different loadings were significantly different ($F=105.11$; 4,10 d.f., $P<0.05$) from one another, except for the lowest two loadings (0.07 and 0.104 $gl.^{-1}$), over the period of the experiment.

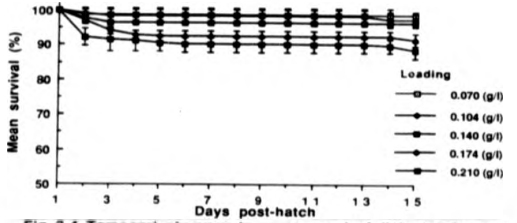


Fig 3.4 Temporal changes in mean survival (%) at 5 loading densities (means given with SE)

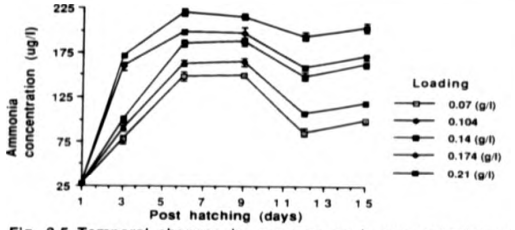


Fig. 3.5 Temporal changes in mean ammonia concentration at 5 loading densities (means given with SD)

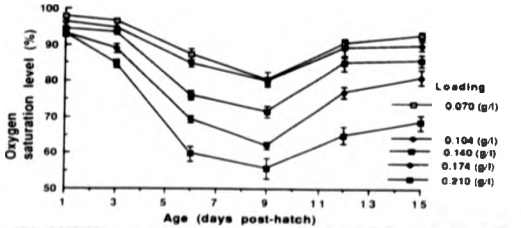


Fig. 3.6 Temporal changes in oxygen saturation at 5 loading densities (means given with SD)

3.4 Discussion

Many toxicity testing system requirements were established upon the available information supporting specific methods or conditions. Requirements of a system for toxicity testing should always take into consideration the optimum requirements for the test species. Therefore, several aspects of the present testing system were studied and discussed in this section to evaluate its suitability for early life stage toxicity testing with tilapia yolk-sac fry and cadmium.

3.5.1 Physical and chemical aspects of the system

The present system is simple in design, based on inexpensive off-the shelf components, which are potentially disposable if required. One of the important features of the system is that the test solution reservoirs, delivery system and exposure units including outlets are truly independent of one another. These independent channels can be taken off-line without affecting the rest of the system, and also avoid pseudoreplication by allowing true randomisation of experimental units.

In these studies the mean flow rate and the amount of dilution water required were 1.12 ml min^{-1} and 24.2 l day^{-1} respectively. These flow rates and daily water requirement were lower than those used by other workers in early life stage toxicity tests (see Table 3.3). As there is no general agreement on the flow rates used in flow-through systems for toxicity testing, flow rate should be carefully selected to cater to the needs of the species and life stage under test. This will avoid arbitrary selection of high flow rates leading to high water requirements. A rate of two to three l g^{-1} and

Table 3.3 Summary of flow rates used in early life stage toxicity tests using flow-through systems

ELS	Species	Flow rate (ml min.$^{-1}$)	Reference
Alevins;fry	*Salmo salar*	716-692	Peterson *et al.*, 1983
Embryo	*Salvelinus fontinalis*	222.20**	Hunn *et al.*, 1987
Embryo; larvae	*Pimephales promelas*	75.00	McCormick & Jensen, 1989
Embryo;fry	*Menidia peninsulae*	66.66**	Goodman *et al.*, 1983
Embryo;fry	*Menidia beryllina*	66.66*	Goodman *et al.*, 1985a
Embryo;fry	*Leuresthes tenuis*	66.66**	Goodman *et al.*, 1985b
Embryo; larvae	8 fresh water spp.[a]	42.18*	McKim *et al.*, 1978
Embryo; larvae	7 fresh water spp.[b]	37.50*	Eaton *et al.*, 1978
Larvae	4 fresh water spp.[c]	34.72*	McKim *et al.*, 1975
Eggs; larvae	*Menidia menidia*	15.00	Voyer *et al.*, 1979
Eggs; larvae	*Pimephales promelas*	15.00	Benoit *et al.*, 1982
Embryo;fry	*Cyprinodon variegatus*	7.00	Schimmel *et al.*, 1974
Yolk sac-fry	*O. niloticus*	1.12	present system

a *Oncorhyncus mykiss, Catostomus commersoni, Coregonus artedi, Salvelinus fontinalis, S. namaycush, Salmo trutta, Esox lucius, Micropterus dolomieui*

b *Salvelinus fontinalis, S. namaycush, Salmo trutta, Esox lucius, Catostomus commersoni, Micropterus dolomieui, Oncorhyncus kisutch*

c *Pimephales promelas, Esox lucius, Micropterus dolomieu, Catostomus* sp.

* Calculated from the turn-over rate and the capacity of water in an exposure chambers

** Calculated from the duration of a cycle and amount water delivered into an exposure chamber

one l $0.1g^{-1}$ (Alabaster and Abram, 1965; USEPA, 1982) of testing organism were recommended.

Sprague (1969) recommended a flow rate which provides 90% replacement time of 8-12 h. The turnover rate of 6.5 day^{-1} of the system is well within Sprague's recommendation.

One factor often overlooked in evaluating the efficiency of a toxicity testing system is adsorption of the toxicant to the walls of the exposure chambers. This could contribute substantially to inaccuracy in test results. However, the materials used in the present system did not produce significant variability in observed actual concentrations of cadmium after 24 h. of exposure to system material.

The present system gives maximum flexibility in terms of mixing protocols and chamber design, allowing customising to the particular chemical/species requirements of the user. It can also be used for on-line monitoring of early life history and metabolic parameters such as survival, growth, yolk utilization, respiration and excretion. Thus it offers a good choice of test parameters for measurement. One of the problems of most of the flow-through systems used to study the effects of toxicants on the energy budgets of early life stages of fish species is undetectable excretory ammonia concentrations due to high flow rates. In the current design the flow rates used enabled the accurate monitoring of NH_4 excreted by the sac-fry. The rate of ammonia production was so small in Mussisi's (1984) experiments with developing young stages of *Oreochromis mossambicus* that the flow-through respirometer had to

be stopped for several hours to allow the ammonia to build up to levels that could be reliably measured. Although this is a common practice, it entails the following two hazards (Brafield, 1985). The ammonia may build up to levels that harm the fish and oxygen deficiency may occur if the water is not aerated, yet if it is aerated some ammonia may escape (Weiler, 1979). However, aerating a toxic medium is not advisable as it may result loss of toxicant through evaporation.

The temporal fluctuations in ammonia concentrations in exposure chambers in the present study could be related to the pattern of yolk nutrient utilization. Generally, carbohydrate, lipid and protein are consumed prior to hatching, while protein and lipid catabolism predominate after hatching (Heming and Buddington, 1988). It is evident that after hatching a larger proportion of yolk protein is utilised for energy production and thus, yolk protein quantity is decreased as growth proceeds and the metabolic rate increases (Heming and Buddington, 1988). This trend has been reported for other fish species (Kamler, 1976; Zeitoun, Ullney, Bergen, and Magee, 1977; Buckley, 1981; Dabrowski and Luczynski, 1984). The higher utilization rate of yolk protein during first 6 days compared with that of 6 to 9 days after hatching may have resulted in an increase of ammonia production followed by a decrease. Subsequent increase of ammonia production could be attributed to the resorption of body protein during starvation.

Dissolved oxygen is an important parameter in the toxic medium since exposure of fish to a toxicant may increase the rate at which they consume oxygen or microorganisms if present in the test chamber may also create a demand for the

oxygen. A minimum level of 60% saturation of dissolved oxygen has been recommended for test solution water used in early life stage toxicity studies (USEPA, 1982; OECD, 1984; 1993). Minimum dissolved oxygen requirements for warm water species are generally not as critical as those for cold water species (Doudoroff and Shumway, 1970). The system used in the present study allows loadings of 174 mg $l^{-1}day^{-1}$ in keeping the recommended level of oxygen saturation for early life stage toxicity tests.

Cost of all the components of the test system are inexpensive except for the relatively high cost multichannel pump (87.50% of the total capital cost (see Table 3.4). However, the depreciated cost of the pump could be considered as low. The precision and high reproducibility provided by the multichannel pump justifies its initial cost.

3.5.2 Biological aspects of the system.

Survival of 80% (USEPA, 1982) or 90% (Buikema *et al*., 1982; OECD, 1984, Mance, 1987) for controls has been considered as acceptable for lethal toxicity tests. Notwithstanding this, there are long term experiments reported with considerably lower control survivals. How these particular survival levels were chosen is not clear. The acceptable control survival level should be species dependent and not be less than those typically determined historically. The survival rates observed in the present study could be compared with those observed by others for the yolk absorption period of *O. niloticus*. Survival percentages between 80 and 90, have been observed during hatchery rearing for fry production (Rana, 1986a; 1986b; Rana and Macintosh, 1987). The optimal survival of the fry to the completion of yolk-sac stage has been observed

Table 3.4 Cost estimate of the present flow-through system

A. Capital cost

Item	Cost (£ Sterling)	% of total cost
Peristaltic pump	1600.00	87.50
Manifold pump tubing	69.00	3.77
Waste drainage system	68.00	3.74
Reservoirs	45.00	2.46
Exposure chambers	20.40	1.15
Tubing connectors	15.00	0.82
Translucent precision tubing	10.20	0.56
Total cost	**1827.60**	**100.00**

B. Cost after depreciation

Item	Depreciated cost (£ Sterling)
Peristaltic pump	200.00
Manifold pump tubing	69.00
Waste drainage system	8.55
Reservoirs	5.62
Exposure chambers	20.40
Tubing connectors	1.87
Translucent precision tubing	1.27
Total cost after depreciation	**306.71**

at loading densities between 70 to 174 mg l^{-1} day^{-1} (20-50 yolk-sac fry per chamber) in keeping with acceptable dissolved oxygen and unionised ammonia levels. Despite therecorded low saturation level of oxygen at the highest loading, acceptable survivals indicated that crowding might not caused stress. The highest concentration of NH_3-N, the main waste metabolite, recorded was 219.88 $\mu g l^{-1}$ at the maximum loading density tested. This level is well below the acceptable highest level, when compared with those of other authors. Total ammonia and nitrite concentrations of 6200 $\mu g l^{-1}$ and 7200 $\mu g l^{-1}$, respectively, for *O. niloticus* (Rana, 1988) and *O. mossambicus* (Subasinghe, 1986) were considered as sublethal concentrations as they showed histopathological changes in the gills and kidney.

As percentage survival, oxygen saturation levels and ammonia concentration are kept well within the acceptable levels, loading densities up to 174 mg l^{-1} day^{-1} could be recommended for tilapia yolk sac-fry in the present test system. The test system satisfied important requirements set by other agencies for toxicity testing. It also provides the flexibility to use species other than tilapia as test species and other toxicants other than metals. Therefore, the present system could be used as a reproducible system to evaluate lethal and non-lethal responses of early life stages of fish to toxicants stress.

CHAPTER 4

MECHANISMS OF INTERSPECIFIC VARIATIONS IN TOLERANCE TO CADMIUM STRESS IN TILAPIA YOLK SAC-FRY

4.1 Introduction.

There are two possible reasons why an organism exhibits resistance to a toxicant. This could be first, due to pre-adaptive resistance: pre-adaptive resistance is the potential ability of an organism to perform its normal biological functions under a wide range of environmental fluctuations such as temperature, oxygen and performing other activities necessary for the organism to survive and ultimately reproduce. This may be the resistance Schreck (1981) referred to as potential performance capacity of an organism. This type of genetically-based resistance evolves by means of natural selection. Second, post-adaptive resistance: post-adaptive resistance could also be genetically based but may need to be induced by the environment. Even though post-adaptive resistance is a somewhat reduced version of pre-adaptive resistance Shreck (1981), it could carry fitness advantages through physiological acclimation; eg., resistance acquired through physiological acclimation during exposure to sublethal concentrations of a toxicant at some prior period of their life. For many studies that report on resistance of organisms to heavy metals, it is often not clear whether this involves an adaptation (pre-adaptive) or physiological acclimation (post-adaptive) (Klerks and Weis, 1987). The distinction between phenotypic and genetic basis of tolerance is somewhat arbitrary, and difficult to distinguish as the capacity for a physiologically plastic response must ultimately have an underlying genetic basis (Mulvey and Diamond, 1991)

Species specific differences in mode of life may translate into differences in fitness

and this may explain differences between sensitivities to stress. Differences in mode of life could fall into either pre-adaptive or post-adaptive resistance. Higher whole body cadmium levels were observed in bottom dwelling (benthic) fish species than in free swimming fish species (Ney and Van Hassel, 1983). The association of fish with sediments seems to play a critical role in their sensitivity to cadmium. The position of an organism is of particular relevance to tidal water. Sub-tidal and intertidal animals often have different levels of contaminants (eg. De Wolf, 1975).

Differences in developmental rate and ontogeny may be important in reducing abiotic stress, although they may have evolved for other reasons (eg. to avoid predation). This type of fitness advantage is more likely to be pre-adaptive. However, the difficulty in distinguishing between pre-adaptive and post-adaptive tolerance may be due to a common ultimate mechanism underlying them. Developmental rate influences the growth pattern of an organism. There is evidence to suggest that growth rates of organisms are not always at their potential maximum levels as costs of growing fast in the juvenile phase can increase mortality (Williams, 1966; Lack, 1968; Case, 1978). It is hypothesised that there are mortality costs in growing faster, since growing faster might involve taking more risks to obtain more food, and, faster growth may imply that less energy is being invested in maintenance and repair (Sibly and Calow, 1989). There is little information on the relationship between mortality and growth rate. A positive correlation was shown between growth and mortality rates in echinoderms (Ebert, 1985) and a similar relationship has been demonstrated for fish (Beverton and Holt, 1959). It is supported that mortality rate is positively correlated with growth rate (Sibly and Calow, 1989). Therefore, animals should "hurry through" the vulnerable

early life-stages (Williams, 1966, Lack, 1968). Similarly in early life stages, growth rates should increase with vulnerability, so that species with high off-spring mortality should have higher growth rates than those with a lower mortality level (Sibly and Calow, 1989). On the other hand maximising growth rates would minimise developmental time and thus the exposure of early developmental stages to abiotic stress factors. Therefore, under exposure to chemical stress faster developing species may have a fitness advantage over slower developing species by minimising the exposure period of sensitive or vulnerable early life stages. This hypothesis is explored in the present study by examining interspecific variation in tilapia early life stage response to cadmium exposure.

Tilapias which belong to the tribe Tilapiini are a group of fish which exhibit considerable interspecific variation in their early ontogeny (Noakes, 1991). The most recent classification of this group recognises three genera, *Tilapia*, *Sarotherodon* and *Oreochromis*, which differ in their reproductive behaviour. It is suggested that the more primitive substrate spawners belonging to the genus *Tilapia* have given rise to two distinct groups of mouth brooders, the genera *Oreochromis* and *Sarotherodon*. Substrate spawners maintain their brood on or close to the substrate, from the time of spawning until the young become independent, while mouth brooders carry their off-spring in their buccal cavity until first feeding, a possible mechanism to reduce predation mortality on eggs and sac-fry (biotic stress) (Noakes and Balon, 1982, Ranu, 1990). As a consequence of these differences in their reproductive behaviour, substrate spawning and mouth brooding species differ markedly in the development of their early life history stages. Fry in the former group having a distinct larval stage (indirect

development) (Youson, 1988; Flegler-Balon, 1989), whereas those of the latter undergo direct development without a larval stage (Noakes, 1991). In addition, mouth brooders produce much larger, yolkier eggs than substrate spawners, and their offspring have a more protracted early developmental period than those of the latter. To test the hypothesis that offspring from tilapia species with faster developmental rate are more resistant to metal stress than those with slower developmental rate, yolk sac-fry of six species of Tilapiini belonging to three genera, *Tilapia, Sarotherodon* and *Oreochromis* were exposed to heavy metal stress using cadmium as a reference metal.

To establish the variation in stress tolerance among substrate-spawning and mouth brooding tilapias, lethal toxicity tests were performed to provide a rapid screening for interspecific sensitivity and to provide the information for a detailed non-lethal toxic assessment. It is often assumed that differences in non-lethal toxicity will translate into larger differences in lethal toxicity (Kenga, 1982). Hence, lethal tests can be used initially to establish whether species have different tolerances, and if so, to rank species in order of sensitivity to stress. Also life- history traits could be used to predict how animals are likely to respond to different levels of stresses as they are expected to be correlated with the availability of metabolic energy (Hoffmann and Parsons, 1989). As described earlier (Chapter 1) the availability of metabolic energy provides a general measure of the environmental stress that can be tolerated by organisms. Therefore, life-history growth traits were measured in tilapia yolk sac-fry under non-stressed (control) conditions in the present study to predict the possible underlying mechanism to withstand metal stress.

4.2 Aims of the study.

The aims were to establish:

1) whether the difference in developmental rates of early life stages of different species of tilapia yolk sac-fry offer any advantage in resisting lethal cadmium stress,

2) whether the early life-history traits and in particular growth performance, of tilapia yolk sac-fry correlates with the tolerance capability to lethal cadmium stress, and,

3) to select the most and least tolerant tilapia species tested for further study to determine the underlying mechanisms of cadmium stress tolerance.

4.2 Materials and Methods.

4.2.1 Lethal toxicity tests.

All lethal toxicity tests were performed according to established OECD (1992) guidelines. However, to minimise the differences in tolerance due to genetic variability within any one species the same tagged brood fish were used to provide yolk sac-fry in repeated experiments.

Initial range-finding experiments were carried out for cadmium and copper with 5 test concentrations, 0, 1, 10, 100, 1000 $\mu g l^{-1}$. If any of the species tested did not fall into this range, or if control mortality was greater than 20%, the test was repeated , using a different toxicant range if necessary. The test end point was non-recovery immobilization based on response to gentle prodding with a pipette, hence in the strict sense, an LC50 was calculated, as this was considered as indicative of a lethal response.

4.2.2 Preparation of cadmium and copper test concentrations.

Each test concentration was prepared by serially diluting a stock solution as described below.

A 10 mg l^{-1} stock solution of cadmium (Cd^{+2}) was prepared by dissolving 16.32 mg anhydrous cadmium chloride ($CdCl_2$) (Analar grade) in 1 litre of ASTM dilution water. A 20 mg l^{-1} stock solution of copper (Cu^{+2}) was prepared by dissolving 53.70 mg dihydrated cupric chloride ($CuCl_2$) (Analar grade) in 1 litre ASTM dilution water. Four test concentrations were then prepared by serial dilutions of the stock solutions.

Fresh stock solutions were prepared on the day of use. Whenever possible each test concentration was analyzed on the day for the actual concentration. If this was not possible a 100 ml sample from each concentration was acidified with 1% Aristar nitric acid and stored in cleaned plastic bottles kept in a refrigerator (4° C) for analysis at a later date.

4.2.3 Experimental protocol.

Experiment 1. Assessment of interspecific variation in sensitivity to lethal cadmium stress.

The 6 species belonging to the tribe Tilapiini used in the experiment were *O niloticus*, *O mossambicus*, *O aureus*, *S galilaeus*, *Tilapia zillii* and *T rendalli*. Five-day old yolk-sac fry of each *Oreochromis* and *Sarotherodon* species and 1-day old *Tilapia* species were obtained from the incubation system in the tropical aquarium facility. Fry of each species used in the experiment were originated from a single clutch. Four hundred and fifty fry of each species were divided among fifteen 260 ml exposure chambers filled with ASTM dilution water (see chapter 2). After 24 hours of acclimation, when yolk sac-fry of *Oreochromis*, *Sarotherodon* and *Tilapia* were 6- and 2- day old respectively the yolk-sac-fry of mouth brooders and substrate spawners were of equivalent developmental stages (See discussion). After acclimation fry in exposure chambers were randomly allocated, using random number tables, to one of 5 triplicated treatment levels (including control). Each exposure chamber was checked for immobilization and the number of fry responding was recorded at 24 hour intervals for a 96 h period.

Experiment 2. Assessment of interspecific variation in sensitivity to lethal Cu stress.

Previous work (Baird, Barber, Bradley, Soares and Calow, 1991) indicated that in *Daphnia* genotypes, variability in resistance to cadmium was much greater (greater than three orders of magnitude concentration) than resistance to copper and zinc (less than one order of magnitude concentration). It was speculated that response to cadmium, a non-essential toxic metal, was more variable since this trait was not under 'continuous selection' from an evolutionary standpoint (unlike copper and zinc). For this reason copper, an essential metal, was also used to screen for acute sensitivity, in order to compare and contrast interspecific responses.

The previous experiment was repeated with same age of fry obtained from egg clutches of same brooders used in the previous experiment to evaluate the consistency of the response.

Experiment 3. Assessment of early life-history growth traits under non stressed (control) conditions.

This experiment was carried out to determine whether there is concordance between early life history growth traits and lethal heavy metal stress tolerance of six tilapia species.

At hatching, fry from an individual egg clutch were stocked in 15 exposure chambers at a density of 40 yolk sac-fry. To eliminate the influence of reduced stocking density three exposure chambers were randomly selected and all fry sampled at 3, 6, 9, 12 and

15 days post-hatch. The fry were killed in benzocaine (1 : 10,000 solution in water) and rinsed in nanopure water to minimise shrinkage. The bodies of half of the each sample were dissected from their yolk-sacs (if present) under a dissecting microscope (Olympus) and the bodies and fry (bodies + yolk, if present) were weighed after removing excess surface moisture with absorbent paper. The samples were oven dried overnight at 60° C. The dried samples were then cooled in a desiccator to room temperature and reweighed to an accuracy of 0.0001g on a top pan balance (Mettler AE 100). Similarly, the initial body weight and whole fry weight was performed on the triplicated samples of fry at hatch obtained from the same egg clutch.

4.2.4 Statistical analyses.

A proportional mortality response based on measured actual rather than nominal concentrations was calculated using a standard probit procedure (Finney, 1971) to estimate the 50% lethal concentration (LC50). Goodness-of-fit for each data set to the probit model was assessed by comparison with critical Chi-square-value (P= 0.05). One way ANOVA, and Tukey HSD multiple range tests (Zar, 1984), were used to compare the values of specific growth rates and yolk utilization efficiencies obtained for the species. Correlation coefficients were used to compare the stress tolerance and early life history growth traits. Where appropriate, data were first normalised using an appropriate transformation (arcsine transformation).

4.3 Results

4.3.1 Interspecific variation in sensitivity to lethal metal stress

There was significant interspecies variations in lethal tolerance to both cadmium and copper. These differences were consistent over time. In all cases, concentration response data fitted the probit model adequately (Chi-square $P > 0.05$ in all cases). The predicted LC50 value, together with its calculated 95% confidence limits, for each species is displayed in Figures 4.1 and 4.2 as a normal probability density function (midpoint = LC50; kurtosis = intensity of the response). Two things are apparent from the density functions given in Figures 4.1 and 4.2. First, there was significant ($P < 0.05$) variation in the response to the metals among the species tested, of almost an order of magnitude (20.64 to 143.47 $\mu g l^{-1}$ and 70.55 to 439.30 $\mu g l^{-1}$ for cadmium and copper respectively). The yolk sac-fry of mouth brooding species, as predicted, were consistently more sensitive than those of the substrate spawners (Table 4.1). Second, the response of individuals within each of the species tested was remarkably consistent, giving extremely steep responses (hence the leptokurtotic, or 'spiked' appearance of the density functions). The correlation coefficient showed that there was a clear concordance ($r = 0.999$; $P = 0.00$) in the rank order of species response for the two toxicants.

4.3.2 Early life-history growth traits under non-stressed (control) conditions.

The time taken to attain maximum weight, maximum length and to complete utilization of yolk in mouth brooding species was about twice that of substrate spawning species and the mean growth characteristics of yolk sac-fry of the 6 species

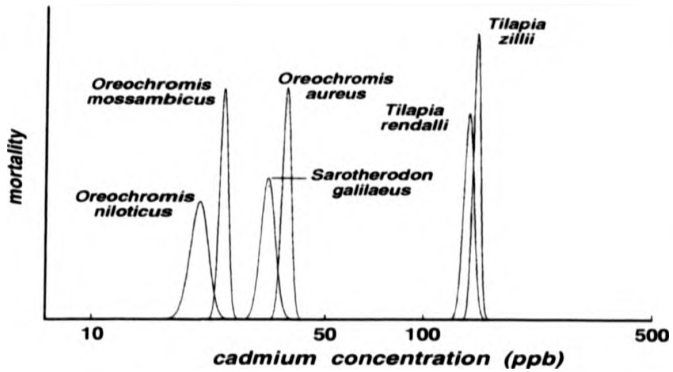


Fig 4.1 Interspecific variation in lethal responses of 6-day old tilapia yolk-sac fry to cadmium stress. Responses are expressed as probability density functions of the 96h LC_{50} (mean and 95% confidence limits)

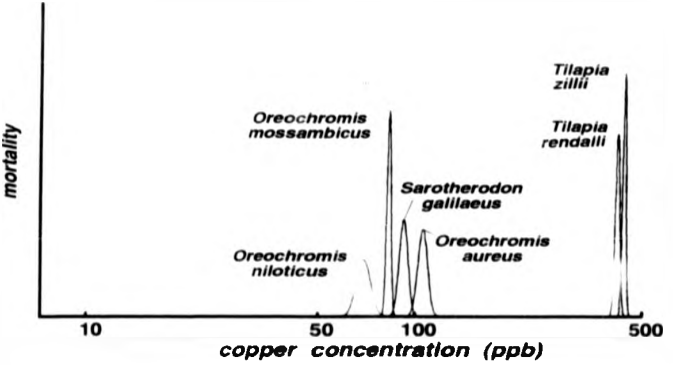


Fig 4.2 Interspecific variation in lethal responses of 6-day old tilapia yolk-sac fry to copper stress. Responses are expressed as probability density functions of the 96h LC_{50} (mean and 95% confidence limits)

Table 4.1 The rank order of lethal toxicity of cadmium and copper to the six species of tilapia (95% confidence intervals: upper and lower limits are given in parenthesis)

Species	96-hr. LC50	
	cadmium (μgl^{-1})	copper (μgl^{-1})
O. niloticus	20.64 (22.06,19.31)	70.55 (74.66,66.57)
O. mossambicus	26.95 (29.67,24.49)	84.29 (87.96,80.77)
S. galilaeus	35.09 (37.04,33.24)	93.57 (97.53,89.76)
O. aureus	37.02 (38.92,35.21)	106.74 (111.76,101.95)
T. rendalli	136.28 (141.79,130.99)	419.34 (426.93,411.83)
T.zillii	148.47 (148.52,138.58)	439.30 (446.61,432.11)

Table 4.2 Growth characteristics of fry of the six tilapia species tested developing solely on their yolk reserves

Growth traits	Species					
	O. niloticus	*O. mossambicus*	*O. aureus*	*S. galilaeus*	*T. zillii*	*T. rendalli*
Dry body weight of fry (mg):						
at the beginning of exposure	1.45 ±0.025	1.04 ±0.012	1.37 ±+0.015	1.14 ±0.046	0.20 ±0.007	0.21 ±0.005
at the beginning of exposure (% of maximum)	80.50	77.68	76.53	74.27	58.97	58.00
Yolk reserves:						
End of yolk sac stage (days)	12	12	15	15	6	6
Age at maximal body weight (days)	9	9	9	9	5	5

are given in Table 4.2. Temporal changes in mean body and yolk dry weights of fry of the 6 species are shown in Figure 4.3 and 4.3.1.

The specific growth rate and yolk utilization efficiency for the periods hatching to maximum body weight attainment of the tested 6 tilapia species are shown in Figures 4.4. and 4.5. The interspecific variation in mean specific growth rate for the period from hatching to maximum body weight attainment period ($df = 5,12$; $F = 999.99$; $P < 0.05$) and yolk utilization efficiency for the period from hatching to maximum body weight attainment ($df = 5,12$; $F = 21.196$; $P < 0.05$) were significantly different.

In general significantly ($P < 0.05$) higher specific growth rate and yolk utilization efficiency values were obtained for yolk sac-fry from substrate spawners than those of mouth brooders. The specific growth rate and yolk utilization efficiency values between the substrate spawners, *T. rendalli* and *T. zillii*, were not significantly different ($P > 0.05$). Among the mouth brooders the lowest values for both specific growth rate and yolk utilization efficiency were observed for *O. niloticus*.

The rank order for specific growth rate and yolk utilization efficiency values for all 6 species are given in Tables 4.3 and 4.4. Even though the mean values for specific growth rate of *O. niloticus* yolk sac-fry from hatching to maximum body weight were significantly ($P < 0.05$) lowest, the other mouth brooding tilapia yolk sac-fry showed insignificant variation ($P > 0.05$) from one another.

There was a significant correlation between cadmium LC50 values and specific

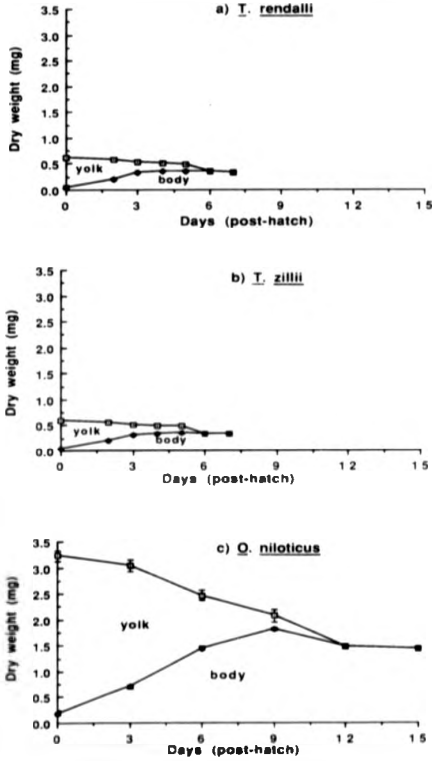
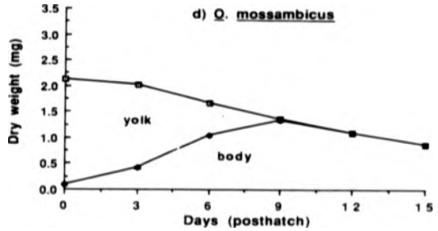
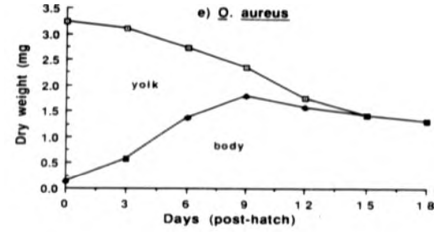


Fig. 4.3 Temporal changes in body and yolk mean dry wt. of tilapia yolk sac-fry (means given with SD)





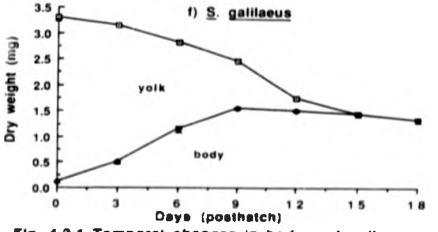


Fig. 4.3.1 Temporal changes in body and yolk mean dry wt. of tilapia yolk sac-fry (means given with SD)

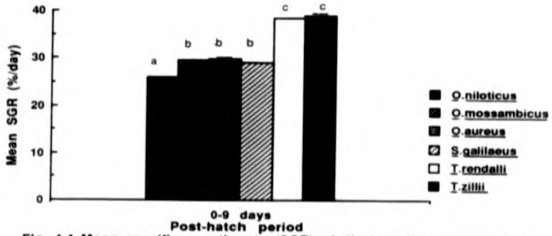


Fig. 4.4 Mean specific growth rate (SGR) of tilapia yolk sac-fry from hatching to maximum body wt. (means given with SD and with different superscripts are significantly different. P<0.05)

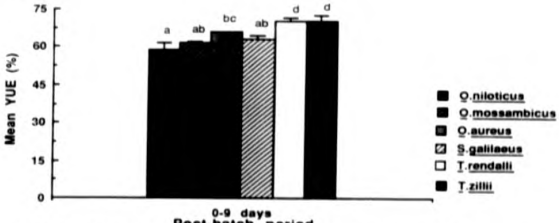


Fig 4.5 Mean yolk utilization efficiency of tilapia yolk sac-fry from hatching to maximum body wt. (means given with SD and with different superscripts are significantly different. P<0.05)

Table 4.3 The rank order of specific growth rates (% day $^{-1}$) of the six species of tilapia (means given with SD)

Hatching to maximum weight	species
25.86 ± 0.15	*O. niloticus*
29.37 ± 0.10	*S. galilaeus*
29.47 ± 0.15	*O. mossambicus*
29.82 ± 0.16	*O aureus*
38.45 ± 0.15	*T. rendalli*
39.02 ± 0.45	*T. zillii*

Table 4.4 The rank order of yolk utilization efficiency (%) of the six species of tilapia (means given with SD)

Hatching to maximum weight	Species
58.73 ± 2.88	*O. niloticus*
61.65 ± 0.40	*O. mossambicus*
63.16 ± 2.60	*S. galilaeus*
65.72 ± 0.21	*O. aureus*
70.05 ± 2.38	*T. zillii*
70.20 ± 1.50	*T. rendalli*

growth rate (df= 4, r= 0.981, P< 0.001) and between cadmium LC50 values and yolk utilization efficiency (df= 4, r= 0.990, P< 0.001) of tilapia yolk sac-fry (Figures 4.6 and 4.7). Similarly a significant correlation between copper LC 50 values and specific growth rate (df= 4, r= 0.977, P< 0.001) and between copper LC50 values and yolk utilization efficiency (df= 4, r= 0.986, P< 0.001) was observed (Figures 4.8 and 4.9)

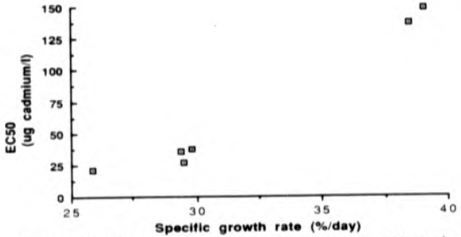


Fig. 4.6 Relationship between specific growth rate and cadmium EC50 of tilapia yolk sac-fry (r=0.981)

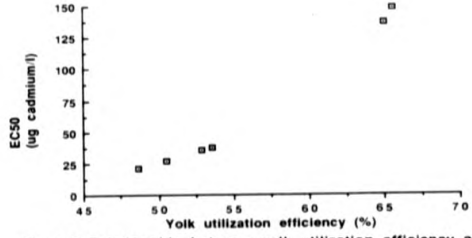


Fig. 4.7 Relationship between yolk utilization efficiency and cadmium EC50 of tilapia yolk sac-fry (r=0.990)

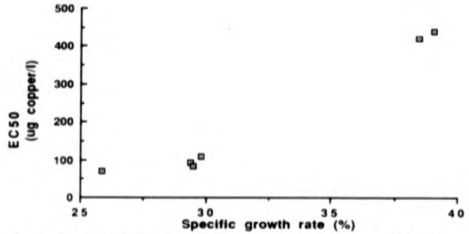


Fig. 4.8 Relationship between specific growth rate and copper EC50 of tilapia yolk sac-fry (r=0.977)

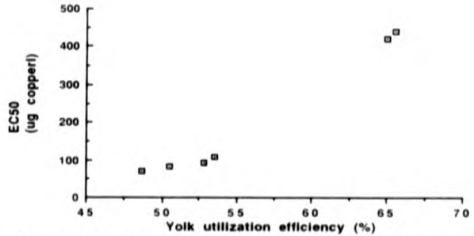


Fig. 4.9 Relationship between yolk utilization efficiency and copper EC50 of tilapia yolk sac-fry (r=0.986)

4.4 Discussion

The present study investigated the tolerance of tilapia species belonging to two distinct groups, substrate spawners and mouth brooders, to metals. The tilapia species in these groups markedly differed in developmental rates of their early life stages. Thus, here, "equivalent" developmental stages of the two groups rather than age were used to compare the tolerance to metal stress. Six-day old yolk sac-fry of mouth brooders were considered to be the "equivalent" developmental stage to two-day old yolk sac-fry of substrate spawner. These observations were based on the ontogenical studies of Ranu (1986b), Gulman and Avtalion (1989) and Mutsekwa (1989).

The results obtained here indicate that interspecific differences in sensitivity are significant for both cadmium and copper. The 96h LC50 for cadmium ranged between 20.64 $\mu g\ l^{-1}$ and 148.47 and for copper 70.55 and 439.30 $\mu g l^{-1}$. No published values comparable to the test conditions of the present study were found in the literature for lethal toxicity of cadmium and copper to early life stages of tilapia and therefore any comparison with previous studies would be dubious. The only similar study found in literature was the effects of cadmium and 3,4 dichloroanilline (DCA) on 9 clones of *Daphnia magna* (Baird *et al.*, 1991). The remarkably consistent individual responses to both cadmium and copper lethal stress observed in the present study, were perhaps not surprising, given that all the individuals tested within each species were obtained from the same parent. A similar response patterns was also reported for *Daphnia* clones (Baird *et al.*, 1991), raised in a common environment. These studies, however, served to underline the fact that the observed interspecific differences reflected

genetically based,

and life history dependent pre-adaptation to pollutant stress.

Despite their reputation as "hardy" fish, mouth-brooding tilapia sac-fry proved very sensitive to cadmium and copper exposure, with LC50 values lower than those recorded for equivalent stages of salmonids (Calamari, Marchetti and Vailati, 1980) and comparable to those obtained for *Daphnia magna* (Baird *et al.*, 1991). This high degree of sensitivity suggests that tilapia yolk sac-fry may be an ideal candidate species for ecotoxicity testing in tropical countries.

The variability in response and the rank order of tolerance of tilapia species were the same for cadmium and copper. Contrary to this observation lack of concordance in acute tolerance of *D. magna* within metals have been recorded. The intraspecific variation of *Daphnia* in response to metals was generally within an order of magnitude, with the notable exception of cadmium which varied over three order of magnitude (Baird *et al.*, 1991). Baird *et al.*, (1991) attempted to attribute this exceptional response to cadmium to its non-essential metal category when compared to the other essential metals tested. In contrast, in the present study, the inter-specific variation of tilapia response to cadmium and copper was around an order of magnitude. *Daphnia* species are among the oldest and possibly the most often used test organism in ecotoxicology because of low genetic variability maintained by asexual reproduction. There are, however, genetic differences between clones of *D. magna* that are used for ecotoxicological testing (Soares, 1989; Baird *et al.*, 1991). If the differences in lethal tolerance to cadmium of over three orders of magnitude in

Daphnia can result from intraspecific variability in genetically-based pre-adapted tolerance alone, the expectation here was to observe a similar large variation in response to cadmium when shifted to larger levels of generic variation, as levels of genetic variation will be determined by opposing selection pressure (Hoffman and Parsons, 1989). Therefore, the observed variation in interspecific tolerance to cadmium lethal stress in the present study suggests the underlying mechanism may not be a compound-specific genetically-based pre-adaptation.

What inferences could be made from the observed generality in toxic responses?. The generality in the response and rank order of tolerance to cadmium and copper between the six species tested suggests that either the mode of action of the two toxicants or the mechanism of detoxification could be similar. The observed differences in tolerance between mouth brooders and substrate spawners may reflect a difference in general stress response between the two groups, which may be related to differences in modes of life between mouth brooders and substrate spawners.

Proposed models of stress tolerance (see Chapter 1) suggest that the existence of genetically based differences in growth may account for genetically based differences in stress tolerance. The efficiency with which yolk is transformed to body tissue would give an indication how effectively resources are allocated for growth and other metabolic activities. High growth rate and yolk utilization efficiency means less resources being used for maintenance than for growth. Thus, higher growth rates may indicate reduced energy investment in maintenance compared with growth, hence a trade-off could be taking place between growth and maintenance to meet the higher

maintenance cost under stress conditions. This is in agreement with the findings of the present study that faster growing yolk sac-fry of substrate spawning *Tilapia* species (having significantly higher specific growth rate and yolk utilization efficiency than mouth brooding yolk sac-fry) were more tolerant than slower growing mouth brooding *Oreochromis* and *Sarotherodon* species. The rank order of tolerance among species tested within mouth brooders showed the similar rank order of growth performance (measured as specific growth rate from hatching to maximum weight attainment).

To conclude, there were significant differences in lethal tolerance between the tilapia species tested for both cadmium and copper. Moreover, a concordance in response to the two metals was observed, suggesting that the observed differences in tolerance may relate to differences in modes of life between mouthbrooding and substrate spawning species. A reduction in the exposure period of sensitive developmental stages of early life stages could cause a reduction in uptake, and hence increase tolerance. An increased body burden of cadmium in the relatively slow developing mouth brooder sac-fry compared with the faster developing substrate spawner sac-fry could be expected as the sensitive early developmental stages of the former will be exposed for longer periods than the latter. Clearly, in order to test this hypothesis further, it will be necessary to demonstrate differences in the metal uptake and its partitioned body burden between the two groups of fishes. Under non-stressed conditions, the substrate spawning and mouth brooding yolk sac-fry showed significant ($P<0.05$) differences in their patterns of growth and these differences were found to correlate with the tolerance capabilities of the two groups. The correlation between tolerance capability and growth performance suggested the tolerance

capability may relate to the energy investment in maintenance relative to growth. As growth performance is influenced by the size and nutritional status of an animal (Jobling, 1993), a variation in the energy investment on maintenance relative to growth may be expected between different sizes and nutritional status of intraspecific tilapia yolk sac-fry. Thus, a variation in tolerance response to metal stress may be expected between these intraspecific tilapia groups.

CHAPTER 5

INFLUENCE OF TILAPIA YOLK SAC-FRY AGE AND SIZE ON THE TOLERANCE TO CADMIUM STRESS

5.1 Introduction.

The previous chapter demonstrated significant differences between tilapia species. A key feature of these differences between the three genera, however, is the obvious size difference in eggs produced between substrate spawners and mouth brooders. This chapter explores any general advantage that egg size, and therefore yolk sac-fry size, and age may offer to lethal cadmium tolerance.

Variation in physical characteristics such as egg size and weight can be higher (Bagenal, 1971) than the biochemical composition of major constituents in eggs between females of the same species originating from populations of mixed ages or from different geographical locations (Rana, 1986b). Intraspecific variation in egg size and weight and hence, quality of emergent sac-fry, could be attributed to either the genetic variation (Kirpichnikov, 1981; Rana, 1986b) or to the age and size structure of the breeding population (Blaxter and Hempel, 1963; Gall, 1974; Hulata, Mouv and Wohlforth, 1974). In general egg weight, and hence emergent yolk sac-fry are dependant on maternal fish age and size for many fish species (Kamler, 1976; Kamler, Zuromska and Nissinen, 1982; Docker, Medland and Beamish, 1986) including tilapias (e.g. Rana, 1985,1990).

Physiological processes are controlled by age and adjusted to body size (Kamler, 1992). Thus, intraspecific differences in egg size, and hence size and age of yolk sac-fry have the potential to influence their tolerance to metal stress. Natural populations are composed of a wide range of maternal ages and sizes of a single species

producing offspring with a wide range of ages and sizes. Variations in age and size response to metal toxicity are often eliminated by use of a narrow range of size and/or age classes to enhance the precision of the toxicity study (Newman and Heagler, 1991). Hence, to describe, understand and predict laboratory-based toxic effects of metals to field populations, an understanding of ecotoxicological allometry and age is needed. Physiological differences between intraspecific sac-fry of different ages and sizes can be inferred to reflect their different ability to resist metal exposure. Therefore, comparative studies using sac-fry of different ages and sizes may yield information about intraspecific variation in metal tolerance.

5.1.1 Intraspecies age-specific tolerance of sac-fry

Changes in physiological activities with age of a particular early-life stage can be identified by the differences in oxygen consumption. The oxygen consumption rate of fish embryos increases slowly during the cell cleavage stage and then accelerates several fold to hatching (Davenport and Lonning, 1980). A distinct increase in the relative respiratory rate after hatching has been reported for endogenously feeding sac-fry of many fish species (Davenport and Lonning, 1980; Ozernyuk and Lelyanova, 1987). The peak relative rate of oxygen consumption usually coincides with the onset of exogenous feeding fry (Kamler, 1992) and then declines with the depletion of yolk reserves in unfed fry. As the oxygen consumption rate declines with depletion of yolk the metabolic rate of yolk sac-fry may also to decrease. Many species of fish fry are capable of surviving long periods of food deprivation by reducing metabolic rates (Jobling, 1993). Thus, unfed sac-fry with limited or deprived yolk reserves may be expected to experience reduced metabolic levels and decreased oxygen consumption

rates.

The reduction in oxygen consumption which accompanies depleted yolk reserves and starvation may partly be due to a reduction in activity and partly due to a reduction in protein synthesis activity, since protein synthesis is the major contributor to maintenance energy metabolism (Houlihan, Mathes, Foster, 1993).

Based upon the results of growth studies with brown trout, Brown (1946) as early as mid 1940's suggested that the metabolism of the fish declined as the food supply was reduced. Brown (1946) hypothesised that the nutritional status of the fish was an important determinant of the level of energy expenditure, and that metabolic activities altered with changes in food availability. In the light of more recent work it was found food deprivation was accompanied by reduction in total metabolism to "basal" levels (Smith and Hasceneyer 1980; Smith, 1981; Lied, Lund and Von der Decken, 1982; Houlihan , 1991; Jobling, 1993) and reduced protein synthesis and degradation (Jobling, 1993). The decline in protein synthesis was attributed to reduction in both ribosomal activity and number under conditions of food deprivation (Houlihan, 1991).

Physical and chemical metabolic activity constitutes a high energy cost during fish early life stages dependent on limited yolk resources. During food limitations and starvation suc-fry probably have to adopt a "switching strategy" to save energy. It is well known that starvation induces differential readjustments of numerous enzyme activities (Jurss, Bittorf, Vokler and Wacke, 1984) to reduce energy expenditure. As deprivation of food resources and starvation induces an overall reduction in activity,

respiratory metabolism may be accompanied by a reduction in blood supply to, and the permeability of the gill lamellae (Jurss *et al.*, 1984). This could mean that the reduction in water flow and filtering rate across gills may be reduced. Therefore, in sac-fry with depleted yolk reserves undergoing starvation the reduced gill activity may reduce toxic metal uptake in its milieu, and thus, reduce metal stress. Therefore, it is hypothesised here that starvation induced reduction in activity and respiration rate may be beneficial under metal exposure.

To test this hypothesis different post-hatch ages of mouth-brooding yolk sac-fry, belonging to 2 genera, *Oreochromis* and *Sarotherodon*, were selected, and exposed to cadmium and copper lethal stress. The most sensitive species among tested candidates were selected to measure growth and metabolic traits under non-stressed conditions to test the hypothesis.

5.1.2 Intraspecies size specific tolerance of sac-fry

Intraspecific comparisons revealed no significant effect of fish egg size on rate of embryonic development. Mass hatch of *O. niloticus* and *O. mossambicus* eggs incubated at 27°C to 28°C occurred within 96 h of spawning and was independent of egg size (Rana, 1986b). Other studies reported neither the time to eyed stage nor the hatching time of fish embryos were influenced by the egg size (Blaxter and Hempel, 1963; Kamler and Kato, 1983; Escaffre and Bergot, 1984; Birge *et al.*, 1985; Marsh, 1986). It is well-documented, however that, egg size has a profound effect on the developmental events of yolk sac-fry. Maximum dry body weight, end of yolk-sac resorption and survival time of tilapia mouthbrooding yolk sac-fry were delayed in fry

derived from larger eggs than those from smaller eggs (Rana, 1990).

Under comparable hatchery conditions the growth performance of *O. niloticus* and *O.mossambicus* fry were found to be significantly dependent on the maternal age, and hence the egg size (Rana, 1985,1990). In the previous chapter a significant correlation between the interspecific growth performance and tolerance capabilities of tilapia yolk sac-fry to metal stress was revealed. A similar variation in the response to metal stress between yolk-sac fry emerging from different sizes of eggs of the same species may be expected due to their differences in growth characteristics. Onset of feeding capabilities (exogenous feeding) commenced at 5-6 days post-hatch in *Oreochromis* species, and was independent of maternal age, and hence, egg size (Rana, 1986b). However, delaying the initial feeding in smaller *Oreochromis* yolk sac-fry than larger resulted in higher early mortalities (Rana, 1990). This suggests that even though these smaller yolk sac-fry may be capable of ingesting some of the food during delayed initial feeding (Rana, 1986b) they may nevertheless die because of irreversible physiological damage to organs such as the liver and pancreas (Stroband and Dabrowski, 1979). Thus, within a species smaller sac-fry may physiologically differ from larger sac-fry during and after yolk resorption.

There are several hundred studies in the literature reporting the effects of body size on rates of oxygen consumption in fish (see review by Jobling, 1993). It is also believed that the contribution of maintenance metabolism to total metabolism differs between fish of different sizes (Houlihan, 1991; Jobling, 1993). All these factors suggest a physiological difference between large and small yolk sac-fry which may

influence the capability of an organism to tolerate metal stress. Moreover, these differences in sac-fry size may also be reflected in their growth efficiency. Sac-fry originating from small eggs have a higher growth efficiency than those originating from larger eggs (Jobling, 1993). Adaptation to stress involves several active processes and hence, requires energy. Therefore, it is hypothesised here that the non-starving yolk sac-fry with small body size has the better adaptive potential to metal stress as they have better growth performance and energy efficiency to meet the maintenance energy requirements under stress than large yolk sac-fry.

To test this hypothesis, yolk-sac fry from 0+ year class (small eggs) and 2+ year class (large eggs) *O. niloticus* females, the most sensitive species (Chapter 4), were selected and exposed to lethal metal stress using cadmium (the more toxic among the 2 metals tested) metal. The growth and metabolic traits were measured under non-stress condition of the two size groups to test the hypothesis.

5.1.3 Useful physiological and biochemical traits that predict fitness of yolk sac-fry under non-stress (control) conditions

Under non-stress conditions, measurements of physiological and biochemical parameters involved in the balanced energy equation (equation 1.1) will be a useful tool to understand and predict fitness of organisms. The regulation of the rate of the synthesis of tissue protein is of fundamental importance to the energetic cost for maintenance and growth of the whole animal. Protein is the largest component of dry body mass and minimal theoretical estimates of the cost of synthesising proteins indicate that they represent the most expensive molecules to produce (Kiorboe, Munk

and Richardson, 1987; Jorgensen, 1988; Houlihan, 1991). Total RNA concentration in tissues has been frequently used as a measure of the "capacity for protein synthesis" (Milward, Garlick, James, Nnanyelugo and Ryatt, 1973, Milward, Brown and Odedra, 1981; Preedy, Paska, Sugden, Schofield and Sugden, 1988) and as a measure of growth rate (Bulow, 1987; Busacker, Adelman and Goolish, 1990). Studies using a variety of ectotherms have shown that RNA concentrations in the tissues are directly related to protein synthesis rates (Houlihan, 1991) and protein synthetic efficiency, providing a measure of contribution to maintenance (protein turnover) cost. The RNA:DNA ratios have been utilized as an indicator and predictor of protein synthesis related to nutritional status (Mustafa and Mittal, 1982; Richard, Bergeron, Boulhic, Galois and Ruyet, 1991) or growth rates (Bulow, 1970; Haines, 1973). The ratios RNA:Protein and Protein:RNA have been used as indices of ribosomal capacity for protein synthesis and protein synthesis efficiency (Garlick, Burk and Swick, 1976; Goldspink and Kelly, 1984).

5.1.4 Aim of the study

The aims of this study were to explore the hypotheses that yolk sac-fry emerging from smaller eggs and starving sac-fry may have fitness advantages with regard to tolerance of metal stress compared with yolk sac-fry emerging from larger eggs and non starving sac-fry, respectively.

In order to test these hypothesises the following aspects were investigated.

1. The existence of intra-size and intra-age specific sensitivity of tilapia yolk-sac fry to metal stress using cadmium as the reference metal, and,

2. To explore the possible underlying mechanism for the variation in sensitivity by measuring early life history and metabolic parameters, such as growth traits and respiration rate, protein synthesis and activity.

5.2 Materials and methods

5.2.1 Lethal toxicity tests

Lethal toxicity tests were performed with cadmium and copper according to the procedure described in section 4.2.1, 4.2.2 and 4.2.3 Experiment 1 on tilapia yolk-sac fry.

5.2.2 Determination of metabolic traits

A. Determination of oxygen consumption of tilapia yolk sac-fry

All measurements of oxygen consumption were carried out on individual yolk-sac fry using the static respirometry technique. Although flow-through respiratory technique provides advantages over static respiratory technique and the present flow-through system allows measurement of oxygen consumption, the static respiratory technique was adopted because the 15 channel flow-through system limits channels to measure temporal oxygen consumption together with an on-going experiment without interrupting it. Further, Lampert, (1986) has shown that oxygen consumption is the same in both static and flow-through techniques using duphnids as test organisms. Individual yolk-sac fry were enclosed in 60 ml Nunclon tissue culture flasks (Nunc, Intermed Denmark), containing ASTM dilution water (see chapter 2). The tissue culture flasks were sealed at the mouth after removal of any air bubbles. For each set a control tissue culture flask was prepared in the same manner but with no sac-fry present. The sac-fry were then maintained at 27° ± 1°C for a period of 2-3 hours to obtain a measurable decline in oxygen content of the medium. After the incubation

period a sample of medium was injected into a sealed jacket surrounding a Radiometer oxygen electrode connected to a StrathKelvin Oxygen meter (model 781). An initial volume (100 µl) of medium was injected into the jacket to flush the chamber and then a further 100 µl was injected to measure the oxygen content. The amount of oxygen consumed was calculated by obtaining the difference of amount of oxygen between the 'without fry' (control) and 'with fry' treatments. After measurement of oxygen consumption all fry were frozen at -70° C within one hour. These fry were freeze dried (Micro Modulyo) and dry weight measured to 0.00001 g on a balance (Mettler H51).

B. Determination of RNA, DNA and protein content of tilapia yolk-sac fry.

1 ml of nanopure water was added to each microcentrifuge tube containing a single freeze dried sac-fry, and then sonicated and centrifuged at 5000 rpm for 5 minutes at 4° C. A 100 µl sample of the homogenate was use to measure RNA and DNA content fluorometrically (Prasad, DuMouchelle, Kontuch and Oberleas, 1972). All solutions were kept on ice due to the temperature dependence of fluorescence (Van Dyke and Sgustkiewicz, 1968). The sample was added to a solution of 1 ml ethidium bromide (20 µg l^{-1}) plus 0.9 ml Tris-sodium chloride buffer (pH adjusted to 7.5). Blank solutions consisted of 1 ml ethidium bromide plus 1ml Tris-sodium chloride buffer. A luminescence spectrometer (Perkin Elmer LS 50B) was nulled with the reagent blank using 365 nm excitation, 590 nm emission, maximum excitation and emission slit widths. Fluorescence was then measured for samples at same excitation, emission and slit widths. The initial sample fluorescence was due to both RNA and DNA. RNA from calf liver (Sigma) and DNA from salmon testes (Sigma) dissolved in Tris-EDTA

buffer (100 mM Tris and 1 mM EDTA pH adjusted to 7.5), were used as standards. Standard curves for RNA and DNA were prepared daily.

After the initial fluorescence reading, 20 ul of RNAase (type III A, Sigma, 20 mg ml^{-1}) stored at -70° C prior to use was added to each sample and incubated for 2 hours at 50° C. The solutions were then cooled on ice for 30 minutes and fluorescence was remeasured. Hence RNA content was estimated by the difference between fluorescence before and after RNAase treatment, DNA was estimated from the final fluorescence

Another 100 µl of homogenate was used to measure protein content. Protein content was measured using a modified version of that of Bradford (1976). The sample was prepared according to Mckee and Knowles (1987). 5 µl of 0.3 M perchloric acid and 45 µl of 3.2 M sodium hydroxide, were added to the homogenate and mixed thoroughly. A sample of 50 µl of this solution was then added to a 2.5 ml protein reagent (Sigma). Values for protein content for each fry were obtained by comparing the absorbence of each sample at 595 nm, using a spectrophotometer (Kontron Uvikon 810) against standard bovine serum albumin (Sigma) dissolved in phosphate buffer saline at pH 7.5.

5.2.3 Experimental protocol

Experiment 1: Determination of intra-age specific sensitivity of mouth brooding tilapia yolk sac-fry to lethal metal stress

The fry of *O. niloticus*, *O. mossambicus*, *O. aureus*, and *S. galilaeus* on 3, 6, 9, 12

days post- hatch obtained from +2 year class female, were subjected to 96h. cadmium lethal toxicity test according to the procedure described in section 4.2.1 and 4.2.3. Three and 6 day post-hatch fry were used to represent sac-fry with sufficient amount of yolk reserves while 9 and 12 day post-hatchs were used to represent sac-fry with limited and deprived yolk respectively for the study. The whole design was repeated using copper to determine whether the observed intra-age specific sensitivity to cadmium is consistent among the other metals.

Experiment 2: Determination of intra-size specific sensitivity of *O. niloticus* to lethal cadmium stress

Since post-hatch yolk sac-fry size was found to be highly correlated with egg size and hence maternal age (see Kamler, 1992), females from +0 year and +2 year classes were used as source for different yolk sac-fry sizes. The most sensitive *O. niloticus* was selected to evaluate the size specific sensitivity to metal stress using the most toxic of the two metals tested, cadmium. Yolk sac-fry at 3, 6, and 9 days post-hatch, were obtained from +0 year and +2 year classes *O. niloticus* females to determined the 96-hour lethal response cadmium stress. The procedure followed is same as above.

Experiment 3. Determination of size allometry and growth of *O. niloticus* eggs and yolk-sac fry under non-stressed (control) conditions

Eggs were collected from +0 year class and +2 year class individual females within 12 hours of spawning (hereafter referred to as small and large size groups respectively). The width (I) and height (II) of 50 eggs randomly sampled from each egg clutch were measured under a calibrated binocular microscope (Olympus). Egg

volumes (V mm^3) were calculated from the following formula:

$$V = (\pi/6)L.H^2.$$

In addition a random sample of 50 eggs was dried on absorbent paper, then oven dried at 50° C and the mean dry egg weight (±0.1 mg) determined. The remaining eggs were incubated in the hatchery. Since the CV (%) of eggs and yolk sac-fry sizes within clutches was low the mean egg and yolk sac-fry sizes of individual clutches was considered to be representative (Rana, 1986b). To estimate allometry of yolk sac-fry size and growth, one day after hatching, yolk sac-fry from an individual egg clutch were transferred to 15 exposure chambers at a stocking rate of 40 yolk sac-fry per chamber. Three randomly selected exposure chambers were removed at 3, 6, 9, 12 and 15 days after hatching and the 30 sac-fry per chamber was used to measure length, weight, growth rate and yolk utilization. Remaining sac-fry in exposure chambers were used in Experiment 4 and 5. The study with small yolk sac-fry from smaller eggs terminated at 14 day as high mortality occurred due to starvation. The growth and yolk utilization was measured as described previously (see section 4.2.3 Experiment 3). Prior to dissecting the yolk sacs from bodies, the standard lengths (±0.1 mm) were measured according to the method of May (1971). Initial measurements (day 1) were performed on randomly-sampled triplicated batches of 30 fry from the same clutch.

Experiment 4. Determination of oxygen consumption, RNA, DNA and protein contents of *O. niloticus* yolk sac-fry from the two size groups at different ages. Two sac-fry per replicate (ie, n = 6 per post-hatch age) were obtained from the above experiment at 3, 6, 9, 12 and 15 days post-hatch and enclosed in the static

respirometer to measure oxygen consumption. This parameter was performed on small sac-fry up to the day 12. After measuring oxygen consumption all fry were frozen at -70° C within 1 hour. At a latter stage the sac-fry were freeze dried (Micro Modulyo) and used for analysis of RNA,DNA and protein content.

Experiment 5. Determination of age specific-activity levels of *O. niloticus* sac-fry. The sac-fry were allowed to move voluntarily in this experiment. Single yolk sac-fry were held in a 10 ml capacity Leighton tube (Costar) at day 3, 6, 9, 12, and 15 post-hatch and allowed to be voluntarily active. Tubes were placed under a binocular microscope (Olympus) and opercular beat rates were counted with the help of a telecounter. The opercular beats were counted within the first three minutes of sac-fry enclosure in order to minimise stress. For each age five sac-fry from each triplicated exposure chamber (n = 15 per post-hatch age) were used to record the ventilation frequency.

5.2.4 Statistical analyses

A proportional mortality response based on measured actual rather than nominal concentrations was calculated using a standard probit procedure (Finney, 1971) to estimate the 50% lethal concentration (LC50). Comparisons of oxygen consumption rates, RNA concentrations, RNA:DNA and opercular frequencies for different ages of *O. niloticus* yolk sac-fry under non-stress conditions were carried out using One-way ANOVA and Tucky HSD multiple range technique (Zar, 1984). The RNA concentration, RNA:DNA and Protein RNA ratios between small and large yolk sac-fry at each age were tested by directional t-test (Zar, 1984). RNA, DNA, and protein

content of each yolk sac-fry was calculated from their respective standard curves using simple linear regression technique (Zar, 1984). The concordance of age-specific sensitivity of *O. niloticus* yolk sac-fry to cadmium and copper was tested by correlation coefficient (Zar, 1984).

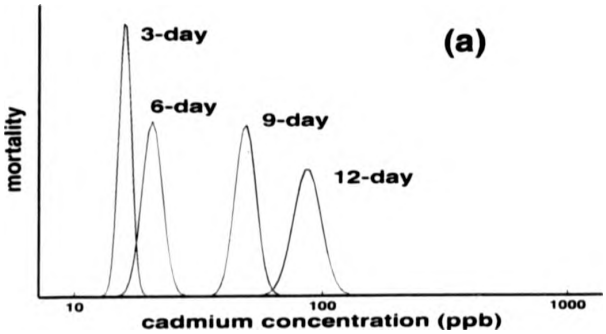
5.3 Results

5.3.1 Intra-age specific and intra-size specific tolerance of mouthbrooding tilapia yolk sac-fry to lethal metal stress

There was an intra-specific age and size variation in response of yolk sac-fry to both lethal cadmium and copper stress. In all cases, concentration response data fitted the probit model adequately ($P > 0.05$). The predicted LC50 values, together with its calculated 95% confidence intervals for each age and size of each species are shown in Figure 5.1 to 5.3 as a normal probability density function (midpoint = LC50, Kurtoses = intensity of the response). It is apparent from the density functions given in Figures 5.1 to 5.3 that there was a significant variation in the responses among both the ages and sizes of tilapia yolk sac-fry tested. This variation, however, is less than an order of magnitude (Table 5.1 to 5.3). The fry with depleted yolk reserves and smaller fry size, as predicted, were more tolerant than sac-fry with yolk reserves and larger fry size. The correlation coefficient showed that there was a strong concordance ($r = 0.996$, $P = 0.004$) in the rank order of age specific response of *O. niloticus* sac-fry to the two metals tested.

5.3.2 Size allometry and growth of *O. niloticus* yolk sac-fry under non-stressed (control) conditions

Mean egg volumes and mean dry egg weights between 0+ and 2+ year classes of *O. niloticus* are given in Table 5.4. Eggs from the 0+ females were 36 and 40 % smaller than eggs from 2+ females in terms of volume and weight, respectively



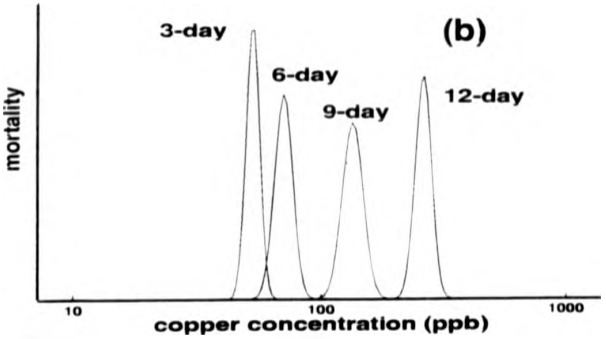


Fig 5.1 age-specific sensitivity of 3, 6, 9 and 12-day old yolk-sac fry of the tilapia *O. mossambicus* to (a) cadmium and (b) copper stress. Each curve is a probability density function representing the mean and 95% confidence limits of the 96hr LC50.

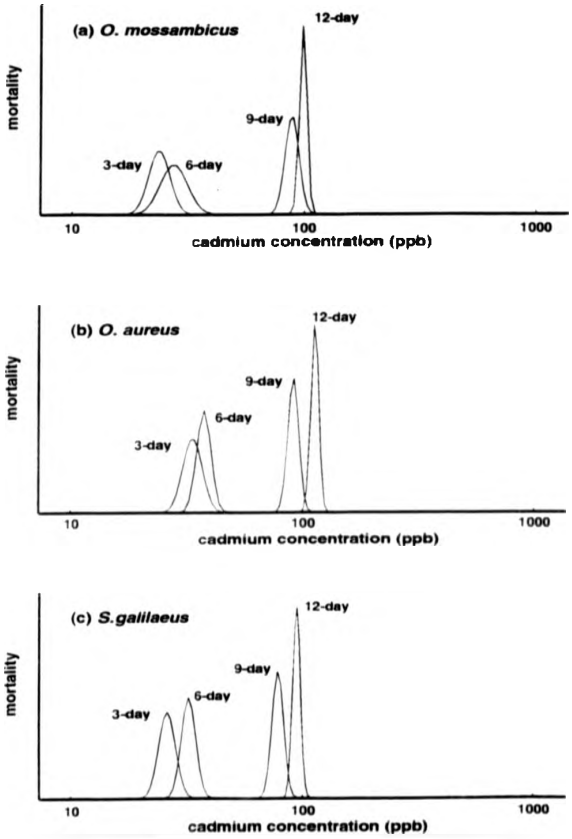


Fig 5.2 age-specific sensitivity of 3, 6, 9 and 12-day old yolk-sac fry of three species of tilapia (a) *O. mossambicus* (b) *O. aureus* and (c) *S. galilaeus* to cadmium stress. Each curve is a probability density function representing the mean and 95% confidence limits of the 96hr LC_{50}.

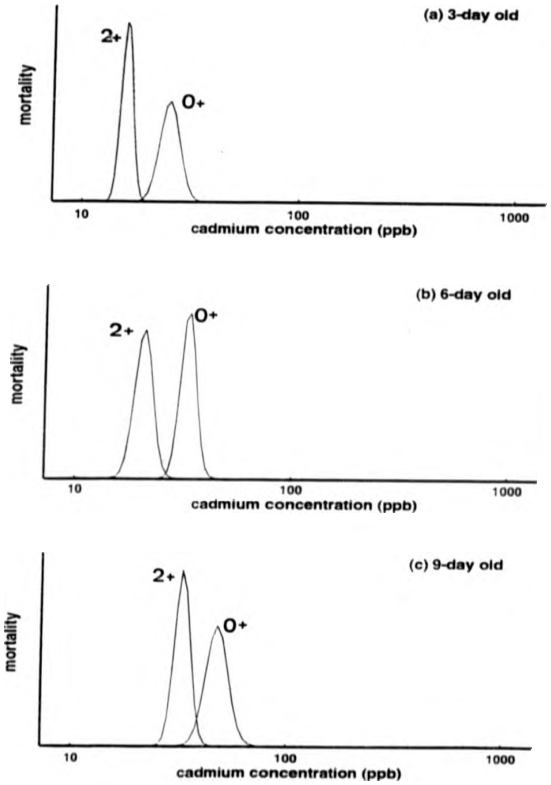


Fig 5.3 the effect of egg size (as a function of maternal age - 0+ = small eggs, 2+ = large eggs) on the response of *O. niloticus* yolk-sac fry of different ages ((a) 3-day old (b) 6-day old and (c) 9-day old) to cadmium. Each curve is a probability density function representing the mean and 95% confidence limits of the 96hr LC_{50}

Table 5.1 The rank order of 96h-LC50 of cadmium (μgl^{-1}) of different ages of yolk sac-fry of mouth brooding tilapia species (95% confidence interval: upper limit;lower limit given in parenthesis)

	Species			
Age (post-hatch days)	*O.niloticus*	*O. mossambicus*	*O. aureus*	*S. galilaeus*
3	16.00 (15.34;16.70)	23.26 (21.58; 25.10)	33.03 (30.83;35.40)	27.88 (26.20;29.72)
6	20.64 (22.06;19.31)	26.95 (24.50;29.67)	37.02 (35.21;38.92)	35.09 (37.04;33.24)
9	49.07 (45.01;53.50)	88.05 (82.75;93.68)	90.67 (86.37;95.20)	91.73 (86.80;96.95)
12	86.74 (77.22;97.43)	98.25 (95.14;101.46)	111.90 (108.16;115.77)	111.91 (107.8;116.10)

Table 5.2 The rank order of 96h-LC50 of cadmium and copper (μgl^{-1})of different ages of *O. niloticus* yolk sac-fry (95% confidence limits: upper limit;lower limit given in parenthesis)

Age (post-hatch days)	Cadmium	Copper
3	16.00 (15.34;16.70)	53.21 (50.35;56.24)
6	20.64 (22.06;19.31)	70.55 (66.57;74.76)
9	49.07 (45.01;53.50)	134.44 (125.68;143.80)
12	86.74 (77.22;97.43)	261.07 (247.48;275.41)

Table 5.3 The rank order of 96h-LC50 of cadmium (μgl^{-1}) to small and large *O. niloticus* yolk sac-fry (fry from 0+ and 2+ year class females respectively) at different ages (95% confidence limit: upper limit;lower limit given in parenthesis)

Age (post-hatch days)	0+ sac-fry	2+ sac-fry
3	25.46 (23.60;27.47)	16.00 (15.34;16.70)
6	33.36 (31.43;35.40)	20.64 (22.06;19.31)
9	57.28 (53.88;60.90)	49.07 (45.01;53.50)

Table. 5.4 Mean growth characteristics of *O. niloticus* yolk sac-fry: Comparison of fry from 0+ and 2+ female brood fish (means given with ± SD)

Growth characteristic	Age class of females (years)	
	0+	2+
Egg volume (mm^3)	6.70	10.52
	±0.58	±0.72
Mean dry egg weight (mg)	1.93	3.20
	±0.07	±0.06
% difference in egg size		
Volume	64.00	100.00
Dry weight	60.00	100.00
Fry growth at 1 day post-hatch		
Standard length (mm)	3.40	5.70
	±0.15	±0.15
Dry body weight (mg)	0.08	0.17
	±0.02	±0.02
% difference in fry growth at 1 day post-hatch		
Standard length	59.60	100.00
Dry body weight	47.00	100.00
Fry growth at maximum body weight		
Standard length (mm)	7.80	9.00
	±0.12	±0.21
Dry body weight (mg)	1.55	1.80
	±0.01	±0.02
% difference in fry growth at maximum body weight		
Standard length	86.60	100.00
Dry body weight	86.00	100.00
Age at maximum fry growth (days)		
Standard length	9	9
Dry body weight	9	9
End of yolk sac stage	9	12

The mean allometry of yolk sac-fry from 0+ and 2+ females in terms of growth traits are given in Table 5.4. The size of small yolk sac-fry in terms of length and dry weight were 40% and 53% less than of large yolk sac-fry, respectively, on day one after hatching and the difference reduced to 14% at maximum body dry weight attainment. The weight of fry from both year class females reached maximum body weight within 9 days of hatching, while the complete yolk absorbtion of fry from 0+ female occurred 3 days earlier than their 2+ conspecifics indicating a higher developmental rate in terms of growth than their conspecifics from 2+ females.

The specific growth rate and yolk utilization efficiency for the period from hatching to maximum body weight attainment of the yolk sac-fry from both year class females are shown in Table 5.5. There was a significant ($P<0.05$) intra-size variation with the mean values of specific growth rate for the period hatching to maximum body weight attainment. The *O. niloticus* smaller yolk sac-fry showed significantly higher specific growth rate ($t_{(0)}= 43.72$; d.f.=4; $P<0.05$). The yolk utilization efficiency for smaller and larger yolk sac-fry were not significantly different ($P>0.05$).

5.3.3 Oxygen consumption, RNA, DNA and protein contents of *O. niloticus* yolk sac-fry from the two size groups at different ages

Relationship between RNA and RNA:DNA with respiration rate

To investigate whether differences in oxygen consumption rate associated with both age and size of yolk sac-fry were related to differences in protein synthesis (measured as RNA concentration and RNA-DNA ratio), regression analysis of the pooled data

Table 5.5 The rank order of specific growth rate (SGR) and yolk utilization efficiency (YUE) of small and large *O. niloticus* yolk sac-fry (fry from 0+ and 2+ year class females respectively. Means given with ± SD)

A. 0-9 post-hatch days: period corresponding to hatching to maximum weight

Size group	Specific growth rate (% day^{-1})	Yolk utilization efficiency (%)
Large	25.86^{a} ±0.15	58.73^{a} ±2.88
Small	32.17^{b} ±0.20	60.00^{a} ±1.46

B. 3-7 post-hatch days: period corresponding to the cadmium exposure of 3 day post-hatch fry

Size group	Specific growth rate (% day^{-1})	Yolk utilization efficiency (%)
Large	24.00^{a} ±1.15	56.74^{a} ±2.14
Small	32.08^{b} ±1.08	58.00^{a} ±0.66

C. 6-10 post-hatch days: period corresponding to the cadmium exposure of 6 day post-hatch fry

Size group	Specific growth rate (% day^{-1})	Yolk utilization efficiency (%)
Large	7.34^{a} ±0.40	48.72^{a} ±2.00
Small	10.04^{b} ±0.50	51.10^{a} ±0.70

The means bearing different superscripts in a colomn are significantly ($P<0.05$) different.

was performed. There was a significant linear relationship between both RNA concentration (df= 1,64, F= 25.41, P<0.05) (Figure 5.4) and RNA:DNA (df= 1,64, F= 145.10, P<0.05) (Figure 5.7) with respiration rate (measured as oxygen consumption rate). Both the small and large yolk sac-fry showed significant linear relationships between both RNA concentration (df= 1,28, F= 32.18, P<0.05 and df= 1,34, F= 5.25, P<0.05, respectively) (Figures 5.5 and 5.8 respectively) and RNA:DNA (df= 1,28, F= 55.02, P<0.05 and df=1, 28, F= 377.87, P<0.05, respectively) (Figures 5.6 and 5.9 respectively) with respiration rate.

Age-specific differences in oxygen consumption rate, RNA and RNA:DNA

The rate of oxygen consumption, RNA, RNA-DNA and Protein:RNA of *O. niloticus* sac-fry at different ages for two size groups are given in figures 5.10 to 5.13. There was a significant difference (P<0.05) in oxygen consumption rate and biomolecule ratios between sac-fry with yolk and with depleted yolk. Oxygen consumption (d.f.=5,12; F=108.38; P<0.05), RNA (d.f.=5,12; F=19.86; P<0.05) and RNA-DNA (d.f=5,12; F=163.43; P<0.05) were significantly decreased in sac-fry with depleted yolk compared with those of fry with yolk reserves indicating reduced state of metabolic activity.

Size-specific differences in oxygen consumption rate, RNA, RNA:DNA and protein

The whole body oxygen consumption in yolk sac-fry originating from smaller eggs was less than whole body oxygen consumption of larger fry from larger eggs. The oxygen consumption mg^{-1} dry body weight gave opposite results. The present study

measured the protein synthetic capacity between the two size groups as RNA concentration and RNA:DNA. The protein synthesis expressed as whole body RNA concentration of *O. niloticus* small sac-fry were significantly lower at 3 day post-hatch (d.f.= 10; $t_{(1)}$=-6.7959; P< 0.05), 6 day post-hatch (d.f.=10; $t_{(1)}$=-12.7506; P< 0.05) and 9 day post-hatch (d.f.=10; $t_{(1)}$=-8.0496; P< 0.05) than the whole body RNA concentration of large fry at corresponding ages. A similar trend was observed for the bio-molecule ratio, RNA:DNA. The protein synthesis efficiency given as Protein:RNA ratio showed results opposite to whole body RNA concentrations. The Protein-RNA ratios were significantly (P< 0.05) higher in *O. niloticus* small yolk sac-fry at all three ages than Protein-RNA ratios of larger fry at corresponding ages (d.f.=10, $t_{(1)}$=2.38117, P< 0.05; d.f.=10, $t_{(1)}$=7.9805, P< 0.05); and d.f.=10, $t_{(1)}$=3.5506, P< 0.05) for 3,6 and 9 days post-hatch fry respectively).

5.3.4 Age-specific activity levels of *O. niloticus* yolk sac-fry

The age specific variation in physical activity measured as opercular frequency is shown in Figure 5.14. The opercular beat rate decreased significantly (d.f.= 4, 10, F= 158.52, P< 0.05) in fry with limited or deprived yolk than growing fry on yolk.

Overall, the oxygen consumption, RNA concentration, RNA-DNA, and opercular beat rate were low in *O. niloticus* fry during yolk depletion period and growth performance and protein synthesis efficiency were low in larger yolk sac-fry than in smaller yolk sac-fry while protein synthesis rate was higher in larger yolk sac-fry than in smaller yolk sac-fry.

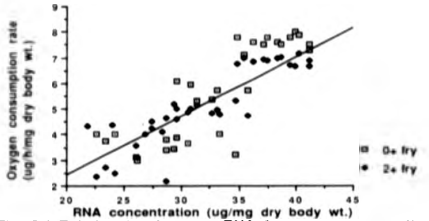


Fig. 5.4 Relationship between RNA & oxygen consumption in O. niloticus fry (pooled data, Oxygen= -2.18+0.23 X RNA. r2=0.769 ,n=66)

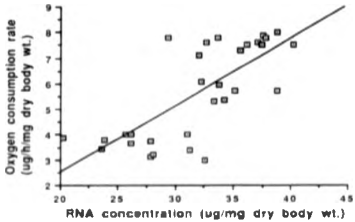


Fig.5.5 Relationship between RNA & oxygen consumption in O.niloticus fry from 0+ female (Oxygen=-2.76+0.26 X RNA. r2=0.545, n=30)

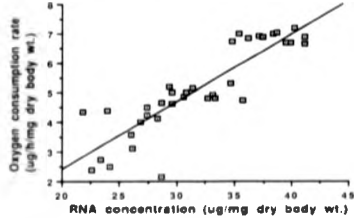


Fig5.6 Relationship between oxygen consumption & RNA in O.niloticus fry from 2+ female (Oxygen=-2.18+0.22 X RNA. r2=0.769, n=36)

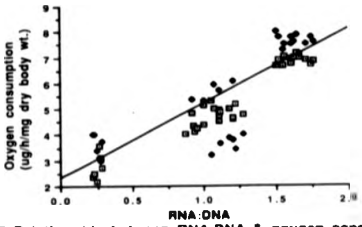


Fig. 5.7 Relationship between RNA:DNA & oxygen consumption in O. niloticus fry (pooled data, Oxygen=2.32+2.88 X RNA, r2=0.650, n=66)

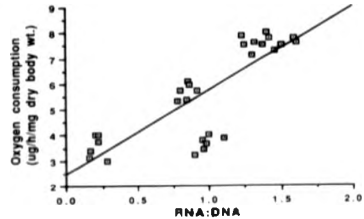


Fig. 5.8 Relationship between RNA:DNA & oxygen consumption in O. niloticus fry from 0+female (Oxygen=2.47 +3.27 X RNA, r2=0.655, n=30

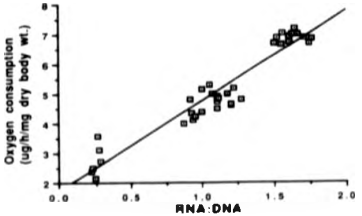


Fig. 5.9 Relationship between RNA:DNA & oxygen consumption in O. niloticus fry from 2+ female (Oxygen=1.74+ 3.01 X RNA, r2=0.917, n=3

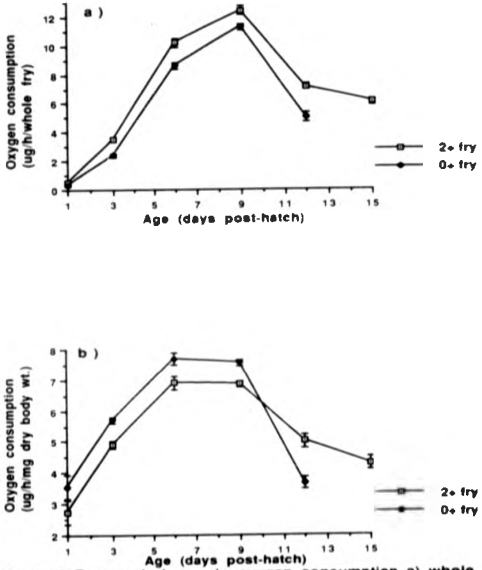


Fig. 5.10 Temporal change in oxygen consumption a) whole body b) per mg dry weight of O. niloticus fry from 0+ & 2+ females (means given with SE)

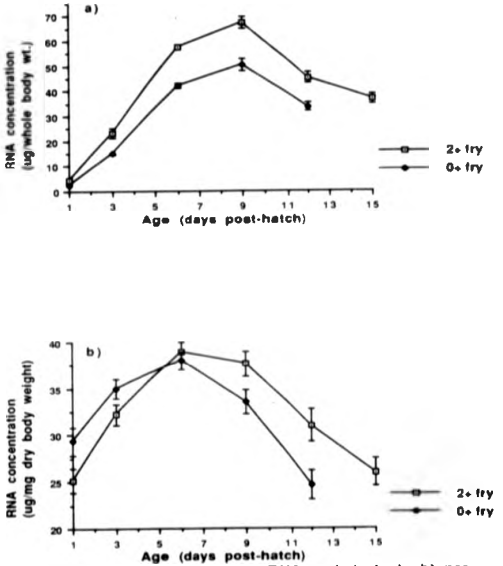


Fig. 5.11 Temporal changes in RNA a)whole body b) per mg dry body weight of O. niloticus fry from 0+ & 2+ females (means given with SE)

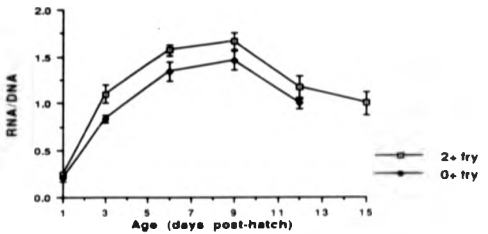


Fig. 5.12 Temporal change in RNA/DNA of Q. niloticus fry from 0+ & 2+ females (means given with SE)

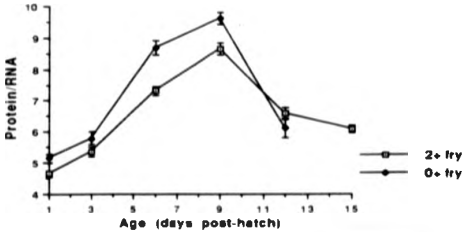


Fig. 5.13 Temporal change in Protein/RNA of Q. niloticus fry from 0+ & 2+ females (means given with SE)

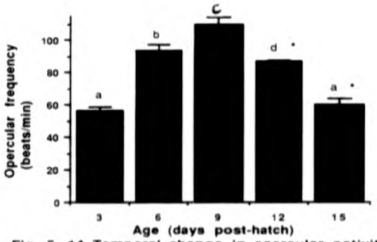


Fig. 5. 14 Temporal change in opercular activity of O. niloticus sac-fry (means given with SE and with different superscripts are significantly different. P<0.05) * with limited or no yolk

5.4. Discussion.

The present study investigated the tolerance of different post-hatch ages of *Oreochromis* and *Sarotherodon* yolk sac-fry and different sizes of yolk sac-fry from 0+ and 2+ females of *O. niloticus* to metal stress. Both the age and sizes of yolk sac-fry showed varying tolerance capabilities to the tested metal stress.

5.4.1 Age-specific tolerance of mouth brooding tilapia yolk sac-fry to metal stress.

The results indicated that sac-fry with adequate yolk reserves (3 and 6 days post-hatch) were more sensitive than sac-fry with depleted yolk reserves (9 and 12 day post-hatch) in terms of lethal response and these differences were significant for both cadmium and copper. Studies conducted to determine the age sensitivity of a particular early-life stage to metal stress in general and to cadmium in particular, are scarce. Michibata *et al.*, (1987) evaluated the sensitivity of embryonic developmental stages of *Oryzias latipes* to cadmium stress and found that earlier developmental stages were more sensitive than later stages. Decrease in sensitivity to cadmium, in terms of 48-hour lethal toxicity, was reported for mummichog yolk sac-fry, from 7 to 14 day post-hatch, (Middaugh and Dean, 1977). These results are in disagreement with those of Rombough and Garside (1982), who reported that salmon embryos were less sensitive during cleavage and that peak mortality rates occurred during gastrulation and axiation, during vitelline circulation, and shortly before hatching. Similar increase in sensitivity to cadmium has been observed near the completion of yolk absorption. Chapman (1978) found increasing sensitivity in terms of lethal response to cadmium from newly hatched yolk sac-fry to swim-up stage, in steelhead trout/rainbow trout

(*Oncorhyncus mykiss*) and chinook salmon (*Oncorhyncus tshawytscha*). Similar increases in sensitivity in terms of lethal response have been reported for sockeye salmon (*Oncorhyncus nerka*) hatch to fry stage (Servizi and Martens, 1978). Apart from the differences in test conditions in these studies, the duration of lethal toxicity tests were extended from 8 to 24 days. Since no food was offered during lethal toxicity tests, the studies with later yolk sac-fry had been subjected to 'deliberate' prolonged starvation. Thus, it is obvious prolonged starvation reduced fitness. However, no previous studies comparable to the test conditions of the present study can be found in the literature for lethal response of yolk-sac fry at different post hatch ages to cadmium and copper.

The variability in response and the rank order of tolerance between yolk sac-fry of different post-hatch ages were similar for cadmium and copper in the present study. This indicates a general response to lethal levels of the two metals tested and the observed difference in tolerance between sac-fry with yolk and depleted yolk may reflect a difference in the general response. This difference in general response may relate to differences in physiological status of the sac-fry with and without adequate yolk reserves.

The present study revealed that, as expected, the oxygen consumption in sac-fry with depleted yolk reserves was significantly lower than that of sac-fry with yolk reserves. This reduction in oxygen consumption may reflect the adjustment in physiological status during depleted yolk conditions in order to reduce the total metabolic cost, and hence, energy expenditure. The decrease in total metabolic cost may be due to either

a decrease in maintenance metabolism or/and activity metabolism. Maintenance metabolism involves costs for supplying oxygen to various body tissues, and hence, cost involved in circulatory function, and cellular maintenance functions. Even though the maintenance cost has been recognised as involving two types of costs, i.e. supplying oxygen to cells and maintaining cellular functions, the biochemical events contributing to both functions may not be mutually exclusive (Jobling, 1993). Thus, it is reasonable to expect a reduction in both circulatory and cellular maintenance functions during depleted yolk stages.

In maintenance metabolism several energy demanding processes are involved. They have been recognised as protein turnover, Na^+, K^+-ATPase activity, substrate recycling and nucleic acids turnover and urea biosynthesis (Jobling, 1993). The two most important processes contributing to energy demand are protein turnover which involves protein synthesis and degradation (Hawkins, 1991; Houlihan, 1991) and Na^+, K^+-ATPase activity which is responsible for the active transport of substances, the maintenance of cellular homeostasis and is involved in the generation and maintenance of membrane potentials (Milligan and McBride, 1985; Kelly and McBride, 1990).

The decreased oxygen consumption rates during yolk depletion period may be accompanied by a decrease in protein synthesis in yolk sac-fry, since there is evidence from a whole range of animals that oxygen consumption increases with increasing rate of protein synthesis (Lydon, Houlihan and Hall, 1989; Waterlow and Milward, 1989; Houlihan, 1991). In the present study this is confirmed by the decrease in RNA concentration and RNA/DNA ratio during the yolk depletion period and is in

agreement with those in the literature for other species (Buckley, 1981; Raae, Opstad, Kvenseth and Walthger, 1988; Richard *et al*., 1991). A number of studies with a variety of ectotherms have found that protein synthesis rates are directly related to RNA concentrations in the tissues (Houlihan, 1991). Therefore a fall in protein synthesis could be attributed to a reduction in ribosomal activity and/or a decrease in ribosomal numbers. Reduction in ribosomal number and/or activity could be expected in *O. niloticus* sac-fry during the yolk depletion period, as changes in the nutritional status of an animal lead to changes in tissue RNA concentration. A reduction in RNA concentration may take place within several hours to days during starvation (Houlihan, 1991). Activity levels of fish have been estimated by monitoring changes in a range of physiological parameters, such as ventilation frequency, heart rate and muscular contraction in the swimming muscle (Weatherley and Gill, 1987). Ventilation rate is the number of opercular cycles per unit of time and it indicates respiratory responses of fish (Jobling, 1993). In the present study yolk sac-fry were allowed to move voluntarily, since at high swimming speed, some species of fish may switch from the usual form of gill ventilation to ram ventilation, whereupon ventilation frequency becomes uncoupled from their overall activity level (Jobling, 1993). In addition to the lower levels of maintenance metabolic cost the ventilation frequency of *O. niloticus* fry in the present study showed a significant reduction during yolk depletion period. Thus, activity energy cost could be expected to be reduced. The energy costs of activity under normal conditions can account for approximately 10% to 20% of the total energy expenditure or greater in highly active pelagic fish (Jobling, 1993). Increased oxygen demands during activity have been confirmed in studies carried out on a range of fish species (Beamish, 1978; Brett and

Groves, 1979). Decreased oxygen consumption in *O. niloticus* sac-fry during yolk depletion period may be partly due to a reduction in maintenance metabolism and partly due to a reduction in activity level.

As observed in the present study, the reduced metabolic rate in *O. niloticus* sac-fry with depleted yolk reserves may be an adaptation to reduce energy expenditure when energy intake is reduced or absent. In order to adapt to the reduced energy intake level they reduced their rate of protein synthesis and mechanical activity to reduce their total metabolic energy. As a result the respiratory water flow passing through the gills may reduce in yolk sac-fry during yolk depletion. Therefore, the main uptake route of water born metals may be reduced. Clearly, in order to test this hypothesis further, it will be necessary to demonstrate differences in the rates of uptake and partitioned body burdens of cadmium between the yolk sac-fry of *O. niloticus* with sufficient yolk reserves and with depleting yolk reserves. This is considered in chapter 6.

5.4.2 Intra-size specific tolerance of *O. niloticus* yolk sac-fry to metal stress.

The present study revealed that egg size, and hence, size of the yolk sac-fry can significantly influence the tolerance capability to lethal cadmium stress. *O. niloticus* yolk sac-fry originated from small eggs were more tolerant to lethal cadmium stress than yolk sac-fry of the same age from larger eggs. This disparity in tolerance capability to lethal cadmium stress, however, was not prominent between the small and large yolk sac-fry groups at their maximum weight attainment (9 days post-hatch) as the initial size advantages conferred upon yolk sac-fry hatched from large eggs tend to diminish with the subsequent development. The persistency of initial size advantage

conferred upon yolk sac-fry at hatching from large and small eggs could vary between species. It could persist into the juvenile phase (Thorpe, Miles and Keay, 1984), be obscured during subsequent development or even decrease (Springate and Bromage, 1985). Information on the effects of metal stress to yolk sac-fry from varying egg sizes is lacking and therefore comparison of this study with the literature is not possible.

Clearly the total energy content of larger yolk sac-fry is higher than that of smaller yolk sac-fry at hatching (Kamler and Kato, 1983). ATP is generally considered to be the most important metabolically available energy source in organisms, generating from the catabolism of food, requiring oxygen. In the present study, even though the larger yolk sac-fry consumed more total oxygen than smaller yolk sac-fry, smaller yolk sac-fry consumed more oxygen on a unit weight basis than larger yolk sac-fry, suggesting that smaller sac-fry could generate more ATP in weight specific terms than their larger conspecifics. Because of the greater metabolic expenditure of larger yolk sac-fry the energy efficiency falls as development proceeds (Lasker, 1962; Hansen and Moller, 1985). This may mean small sac-fry utilise meagre energy resources that they have at their disposal more efficiently or economically. A possible bioenergetical regulatory mechanism for variation in tolerance capabilities to metal stress between small and large yolk sac-fry, may be the increased energy utilization efficiency of the smaller yolk sac-fry, which allows enhanced adaptive capability to metal stress in smaller yolk sac-fry than larger conspecifics. To gain insight into this predicted regulatory mechanism, early life history growth traits and metabolic traits were monitored under non-stressed (control) conditions for yolk sac-fry from small and

large *O. niloticus* eggs.

Significant variations in early life history traits in terms of growth were found among the two size groups studied. The measured growth traits in small yolk sac-fry were significantly higher than those of large *O. niloticus* yolk sac-fry. Hitherto, somatic growth has been defined as an increase in the energy content of the fish body, and it has been assumed that an increase in body weight is synonymous with an increase in energy content (Jobling, 1993). As growth is the resultant energy between assimilated energy and expended energy (see equation 1.3.) higher growth may indicate less energy expended on metabolic activities and more energy deposited as growth. Therefore, under stress conditions to meet the higher metabolic cost due to elevated maintenance cost a trade-off could take place from growth energy to maintenance energy. This is in agreement with data in that faster growing small *O. niloticus* yolk sac-fry were more tolerant than large conspecifics which had lower growth rates. However, change in body weight will accurately reflect changes in the energy content of the body if it is assumed that the composition of fish tissue is constant (Jobling, 1993). As the somatic composition of sac-fry between different size groups of a species may not be necessarily constant, growth in weight specific terms may not always give an accurate reflection of growth in terms of energy gain. Therefore, in the present study metabolic and biochemical traits were also explored to gain an insight to the mechanism underlying differences in tolerance capabilities between the two size groups.

Slower growth rate of larger yolk sac-fry was marked with higher rate of protein

synthesis as measured by whole body RNA and RNA:DNA indicating a high rate of maintenance metabolism, and a low synthesis efficiency, relative to the faster growing smaller yolk sac-fry. Hawkins, Bayne and Day (1986) found similar results for protein synthesis and maintenance metabolism in two groups of mussels (*Mytilus edulis*) of the same age, one slow growing and the other fast growing. They concluded that protein synthesis comprised a major element of maintenance metabolism, which carried a high metabolic cost. Protein synthesis in weight specific terms, however, showed the opposite results. Protein synthesis represents protein needed for both maintenance as well as growth. Therefore, protein synthesis alone would not be a good indicator to predict energy performance of a fish. It has to be considered together with protein synthesis efficiency, which is indicative of energy performance. Individuals with reduced efficiency of protein synthesis therefore incur a higher metabolic expenditure on maintenance and a low net rate of somatic growth. Therefore, in the present study, differences in growth rates between the two size groups may derive from differences in energy balance, leaving proportionally more energy for growth in smaller fry. Sacrificing protein synthetic efficiency allowing a trade-off to take place from growth energy to maintenance energy could be expected in smaller yolk sac-fry to meet the elevated maintenance cost under metal stress. Thus, smaller yolk sac-fry have the better ability to tolerate metal stress than their larger conspecifics. Similarly, Hawkins *et al.*, (1987) reported that in mussels, having a low rate of protein synthesis and higher protein synthesis efficiency can confer a fitness advantage (under high temperature stress) over those having higher rates of protein synthesis and low protein synthesis efficiency. Therefore, it can be suggested that stress factors such as metals and temperature which elevate metabolic rate, act

differently on small and large individuals. The lower the maintenance metabolism, the more surplus energy will be available for growth and for diverting to tolerate metal stress. When energy resources in the environment are ample genotype-dependent variability in maintenance efficiency may be a quantitatively minor factor in affecting individual differences in the rate of growth (Koehn and Bayne, 1989). But, when the energy source is limited, as in the case of mouth brooding yolk sac-fry, differences in maintenance efficiency may play a determining role in the variability between individual in stress tolerance.

To conclude, there was a variation in tolerance capability to lethal cadmium and copper stress between mouth brooding tilapia yolk sac-fry with adequate yolk reserves and with depleted yolk, the latter being more tolerant. Similar variations were observed for lethal cadmium stress between smaller and larger *O. niloticus* yolk sac-fry of the same age originating from 0+ and 2+ females. In the case of the former, their energy saving strategy reduced the total metabolic cost. During yolk depletion period the reduced ventilation rate may have caused a reduction in respiratory water flow and as a result a reduction may have occurred in metal uptake via gills. The higher protein synthesis efficiency of 0+ yolk sac-fry compared with the 2+ yolk sac-fry may have enabled a trade-off from growth energy to maintenance energy to meet the elevated maintenance cost under cadmium stress. The partitioned cadmium body burden levels among yolk sac-fry of varying sizes and ages may give an insight into the mechanism by which they reduce the body burden to increase tolerance capability.

CHAPTER 6

UPTAKE AND PARTITIONED BODY BURDEN OF CADMIUM IN TILAPIA YOLK SAC-FRY

6.1 Introduction

In the previous chapters the differences observed in metal tolerance between species and age and size of tilapia yolk sac-fry were attributed to their mode of life and physiological differences. These differences in the general response may be related to the varying degrees of metal uptake levels and partitioned body burden. One may speculate that more tolerant species, ages and size tend to reduce uptake and partitioned body burden than sensitive species, ages and size. In this study uptake and body burden refers to the amount of metal absorbed into the whole body while partitioned body burden and actual body burden refer how this amount is distributed among organs or various tissues and the amount in the toxic form, respectively.

High metal body burden levels in tolerant species have been reported for organisms at different taxonomic levels (Bryan and Hummerstone, 1971; Brown, 1977; Klerks and Levinton, 1987). These species are likely to have an increased capacity to sequester metals in less toxic forms, therefore, the varying tolerance capabilities between aquatic organisms may suggest the presence of mechanisms which can decrease actual body burden of the metal through reduced uptake or one which can prevent the toxic action of metals from affecting cellular or metabolic functions or damage sensitive intracellular structures.

Studies on the kinetics of metal uptake by aquatic organisms indicate that this is a two

step process consisting of rapid adsorption or binding to the surface followed by slow, transport into the cell interior (Crist, Oberholser, Schwartz, Maczoff, Ryder and Crist, 1988; Xue, Stumm and Sigg, 1988). Transport of metals to the cell interior may occur either by diffusion of metal ions across the cell membrane or by active transport by a carrier protein. Once in the cell, the metal ions interact and disrupt cellular proteins and functions (Brezonik, King and Mach, 1991). Moreover, the impact of metals on cellular functions may vary among tissue types and depend on the amount of intracellular metal accumulation. In order to link the observed interspecies, intra-sac-fry age-specific and intra-sac-fry size-specific sensitivities of tilapia yolk sac-fry to cadmium and copper stress in the present study (Chapters 4 and 5) to the amount of metal uptake, it is necessary to determine the amount absorbed into the body, rather than the total adsorbed and absorbed. Then the question arises, how much is adsorbed and how much is absorbed?.

Most of the studies on metal accumulation in general and cadmium accumulation in particular, pay little attention to quantifying or removing adsorbed cadmium from absorbed cadmium. The attempts made to remove adsorbed cadmium from the surface of tissues are a few. Bodar (1989) placed test animals (*D. magna*) in cadmium free test solution for a period of 10 minutes after the exposure while Michibata (1981) and Somasundaram, King and Shackley (1984) rinsed (*Oryzias latipes* and *Clupea harengus* eggs respectively, in distilled water and glycine buffer pH 2 in order to eliminate adherent cadmium. Exley (1989) washed Atlantic salmon (*Salmo salar*) briefly in 5% (v/v) nitric acid to remove surface adsorbed aluminium. It seems that Bodar (1989) and Exley (1989) assumed that all adsorbed cadmium and aluminium

were removed from *D. magna* or fish body surfaces within 10 minutes by placing them in distilled water and by a brief rinse with 5% nitric acid respectively. Michibata (1981) and Somasundaram, *et al.*, (1984) did not include a specific time period for rinsing organisms in distilled water and glycine buffer in their studies. It is unclear from these studies whether these methodologies for removing adsorbed cadmium from tissue surface removed 'all' or 'only' adsorbed cadmium from the tissues. Therefore, it was necessary to develop a method to remove adsorbed cadmium prior to determining the body burden in the present study.

The form of complexation of metals is an important aspect of bioavailability as complexed metal ions will behave differently in terms of transport, toxic effects and bioaccumulation from free ions. This principle may be used to remove adsorbed cadmium from sac-fry body surface. Unlike inorganic metal complexes, metal ions complexed with natural macromolecular organic matter, such as humic acids, or strong synthetic cheating agents that are generally unavailable to aquatic organisms (Brezonik, *et al.*, 1991). Among synthetic organic compounds, polyaminocarboxylates, such as ethylenediamine tetra acetic acid (EDTA) and nitrilo triacetic acid (NTA) may complex with metal ions (Brezonik, *et al.*, 1991). Among chelating agents EDTA and NTA have been reported to reduce accumulation and toxicity of heavy metals in a wide range of organisms (Muramato, 1980, 1981; Holwerda, Hemelraad, Veenholf and Zandee, 1988; Maeda, Mizoguchi, Ohki, Inanaga and Takeshita, 1990). Therefore it was intended to evaluate the use of EDTA to remove adsorbed cadmium from the surface of sac-fry to ascertain more accurately the levels of absorbed cadmium.

The mode of cadmium uptake and accumulation in fish are two important processes that must be considered in order to explore the mechanisms by which they tolerate cadmium stress. The mode of uptake in fish is ascribed to dietary or water sources (Lucas, Edgington and Colby, 1970; Atchinson, Murphy, Bishop, McIntosh and Mayers, 1977; Murphy, Atchinson and McIntosh, 1978). Some workers consider gills of fresh water fish the most important site not only for uptake but also for accumulation of cadmium from water (Mount and Stephen, 1967; Varanasi and Markey, 1978), while others consider the liver, kidney and gastrointestinal tract tissues of fish are the main organs of greatest importance in cadmium accumulation (Mount and Stephan, 1967; McFarlane and Franzin, 1980; Ney and Van Hassel, 1983; Bendell-Young, Harvey, and Young, 1986). The two major pathways of incorporation of cadmium in fish are absorption across the gill surface and across the intestinal mucosa (Sorensen, 1991). In yolk sac-fry, the developing gills may be an important site for cadmium accumulation as they form the interface organ between the external environment and the interior body. The gastrointestinal route of cadmium uptake in yolk sac-fry may be more important than the gill route, if yolk becomes progressively more contaminated with cadmium and there is no exogenous feeding. In the case of unfed sac-fry during yolk depletion, gills may be the main target site for cadmium uptake. In view of the differences in organ response, and reactions to cadmium stress, estimation of total cadmium body burden levels alone will limit the understanding of tolerance mechanisms which prevent accumulation, detoxification and removal of cadmium from yolk sac-fry. Hence, estimation of the partitioned body burden of cadmium would be a more useful approach in understanding the transport as well as the barrier functions of the organs with respect to cadmium, and would help to

elucidate the inter and intraspecific age- and size-specific tolerance differences to metal stress in yolk sac-fry observed in previous chapters.

6.2 Aim of the study

The aim of this study is to relate the body burden and partitioned body burden levels of cadmium in *O. niloticus* and *T. zillii* yolk sac-fry to the previously observed interspecific and intra age and size specific tolerance to lethal cadmium stress.

6.2 Materials and methods.

6.2.1 Test stock solutions

All chemicals used were Analar grade.

cadmium stock solution

A 10 mg l^{-1} (0.089 M) cadmium stock solution was prepared by dissolving 16.32 mg anhydrous cadmium chloride in one litre of ASTM soft dilution water (see Chapter 2). A 1.5 ml aliquot of the stock solution was mixed in one litre of dilution water to provide cadmium exposure medium of 15 $\mu g l^{-1}$ cadmium (nominal) concentration.

Cadmium-EDTA stock solution

331.30 mg dihydrated disodium EDTA and 4.0 g of sodium hydroxide was dissolved in 400 ml of ASTM dilution water (see chapter 2) and adjusted the pH to 7.5 with 11.4 M hydrochloric acid. To this solution, a solution of 16.32 mg anhydrous cadmium chloride dissolved in 25 ml of ASTM dilution water was added. The combined solution made up to one litre. The ratio of complexing agent to cadmium (0.89M:0.089M EDTA:cadmium) in the stock solution was maintained at 10:1 to provide the equilibrium in favour of the complex. A 1.5 ml aliquot of this stock solution mixed in one litre of dilution water was used to prepare the cadmium-EDTA exposure medium with a nominal concentration of 15 $\mu g l^{-1}$.

EDTA stock solution.

This solution was used to remove adsorbed cadmium from the surface of sac-fry. 331.30 mg of dihydrated disodium EDTA and 1078 mg of Tris buffer was dissolved

in 400 ml of ASTM soft dilution water (see Chapter 2) and the pH adjusted to 7.5 with 11.4 M hydrochloric acid and the solution was made up to one litre.

6.2.2 Cadmium determination in water and tissue samples

Water sample analyses

Test solution samples were taken from each exposure chamber at the beginning and at the termination of each experiment given below. Samples were collected in acid-soaked polythene bottles from the outflow of each exposure chamber to avoid unnecessary disturbance of tilapia sac-fry. Test solution samples were then stored in a matrix of 1% (V/V) concentrated nitric acid (Aristar: BDH Ltd.) at 4° C. The acid matrix was successful in reducing sample losses through adsorption. Total cadmium was measured by graphite furnace atomic absorption spectroscopy (Golterman *et al.*, 1978).

Tissue digestion and cadmium analyses

The sac-fry were sacrificed by means of quick freezing at -70° C and dried slowly to minimise cadmium loss due to inorganic and/or organic volatiles, at 50° C to constant weight. The dry mass, of known weight, was then digested in sealed teflon tubes using 1 ml of concentrated nitric acid (Aristar: BDH Ltd.) and moderate heating (50° C) in a water bath for 30 minutes. The sealed teflon tube greatly reduced sample loss due to acid fuming and prevented sample contamination. The digestant was allowed to cool at room temperature, and diluted with 1 ml of nanopure water. The samples were then stored for analyses at a later stage at 4° C.

6.2.3 Histological preparation

The yolk sac-fry were sacrificed by means of quick freezing at - 70° C and immediately fixed in appropriate fixative solutions (see below).

6.2.3.1 Electron microscopy

Tissue fixation

The whole yolk sac-fry were fixed immediately after sacrifice in cold 2.5% gluteraldehyde in 0.2m cacodylate buffer. This fixation lasted for 2 hours and was followed by overnight washing in the same buffer at 4° C. The gills of sac-fry were then dissected under a binocular microscope (Olympus) in a fume cupboard, while in the same buffer medium. The gills were then post-fixed in 1% osmium tetroxide in cacodylate buffer for 1.5 hours. The gills were then subjected to two washes, 15 minutes each, in the above buffer and passed through 50% and 70% alcohol. At this stage of dehydration gill tissue samples were subdivided into two groups; one for scanning electron microscopy (SEM) and the other for transmission electron microscopy (TEM) studies. Tissues were stored (not more than 3 to 4 days) in case further processing had to be delayed as recommended (Hyat, 1978).

Scanning Electron Microscopy

Gill tissue samples designated for SEM studies were transferred from 70% alcohol to 70% acetone and were further passed through 90% and 100% acetone (2 changes in each grade for 30 minutes each). The tissue samples were then critical point dried (Polaron E-300 critical point dryer), mounted on aluminium stubs and coated with gold-palladium in a sputter coater (Edwards S-150) and examined under a scanning

electron microscope (ISI-60 A)

Transmission Electron microscopy (TEM)

Gill tissue to be processed for TEM were passed through an alcohol series and propylene oxide mixtures for complete dehydration. Tissue samples were then taken into Epon medium hard resin (TAAB) through different grades of propylene oxide and resin mixtures. After overnight impregnation in fresh resin at room temperature and then at 37° C for 2 hours, tissues were embedded in the moulds using fresh resin and allowed to harden at 60° C for 16-20 hours. Ultra-thin sections of selected tissue areas were then cut on a LKB III ultratome in the gold colour region using glass knives. Sections were mounted on coated copper grids and were double stained with uranyl acetate and lead citrate (Glauert, 1975; Hyat 1978). Stained sections were then examined under JEOL electron microscope. Representative areas were then photographed.

6.2.4 Experimental protocol

Experiment 1 Investigation into the effects of EDTA on cadmium uptake of *O. niloticus* yolk sac-fry

A random sample of 40 five-day old *O. niloticus* yolk sac-fry was introduced into each of 15 exposure chambers attached to the flow-through system filled with ASTM soft dilution water (see Chapters 2 and 3). After a 24 h acclimation period yolk sac-fry in exposure chambers were randomly allocated to one of the following three treatment levels in five replicates

1. Cadmium test solution containing 15 $\mu g\ l^{-1}$ (nominal concentration) of cadmium.

2. Cadmium-EDTA complex containing 15 $\mu g\ l^{-1}$ (nominal concentration) of cadmium.

3. ASTM soft dilution water (control).

To estimate the cadmium and cadmium-EDTA uptake levels of *O. niloticus* yolk sac-fry, five fry from each exposure chamber were sampled after 6, 12, 24, 36, 48, and 72 h of exposure and rinsed in ASTM dilution water before being sacrificed by means of quick freeze at -70° C. Samples were dried and known dry weight was digested in Aritar (BDH Ltd.) nitric acid and stored in 4° C for cadmium analysis at a later stage.

Experiment: 2. Investigation into the use of EDTA to remove adsorbed cadmium from *O. niloticus* yolk sac-fry

In a second experiment, six day post-hatch *O. niloticus* fry in exposure chambers were exposed to cadmium test solutions containing 15 $\mu g\ l^{-1}$ (0.089M) (nominal concentration) cadmium for 72 h. At the end of 72 h sac-fry in each exposure chamber were divided into six groups containing five yolk sac-fry in a group. Five groups from randomly selected five exposure chambers (5 replicates) were subjected to the following treatments

1. Sacrificed, dried and known dry weight digested and stored in 4° C for cadmium analysis at a later stage.

2. Rinsed in distilled water, by using a distilled water bottle, for 5, 10, 15, 20, and 25 minutes and were sacrificed, dried and known dry weight digested and stored

in 4° C for cadmium analysis at a later stage. This procedure was repeated with 5% (v/v) nitric acid and EDTA solution prepared by mixing two ml of EDTA stock solution in one litre of ASTM soft dilution water (0.89M) (this solution contained 10-fold EDTA over cadmium in the test medium to maintain the equilibrium in favour of the complex for efficient removal of adsorbed cadmium from the surface of tissues).

Experiment 3. Investigation into cadmium uptake and partitioned body burden of tilapia yolk sac-fry

Four hundred and eighty *O. niloticus* sac-fry, derived from a single egg clutch from a 2+ year class female, were divided among 12, 260 ml exposure chambers, of the flow-through system (see chapter 3), filled with ASTM soft dilution water (see Chapter 2). After a 24 h acclimation period fry in exposure chambers were randomly allocated to two treatment levels. 9 exposure chambers containing 15 $\mu g l^{-1}$ cadmium test solution (nominal concentration) and 3 exposure chambers containing ASTM soft dilution water (control) (see chapter 2). To estimate cadmium uptake and partitioned body burden, 5 fry from each exposure chamber subjected to cadmium treatment were sampled at 6, 12, 24, 36, 48 and 72 h exposure. Triplicated groups containing five yolk sac-fry in each group were treated as below.

The fry were rinsed in EDTA solution (prepared by mixing 2 ml of EDTA stock solution in one litre of ASTM soft dilution water) for 10 minutes to remove adsorbed cadmium from the surface of yolk sac-fry, sacrificed by means of quick freeze at -70° C and subjected to one of the following:

1) dried and weighed and digested for total whole body cadmium analyses.

2) viscera was dissected out, dried, weighed and digested for cadmium analyses of 'body without viscera', and

3) gills were dissected out, dried weighed and digested for cadmium analyses in 'body without gills'.

The yolk sac-fry in control exposure were treated in the same manner, but only after 72h. of exposure.

An indirect method was followed to determine gill and viscera cadmium burden, since large numbers of fry would have been required to obtained a sufficiently large sample to analyse gill and viscera cadmium separately and directly. The difference in cadmium contents between whole body and 'body without viscera,' and, whole body and 'body without gills' will represent cadmium content in viscera and gills respectively.

The whole experimental design was repeated using,

1) 3-, 6-, 9- and 12-day post-hatch fry from the same 2+ year class *O. niloticus* female,

2) 3-,6- and 9-day post-hatch fry from 0+ year class *O niloticus* female, and

3) 2-day post-hatch fry from *T. zillii*. This design covers determination of whole body and partition body burden between different ages, sizes and species of tilapia yolk sac-fry.

Experiment 4. The morphological and cytological effects of cadmium exposure on tilapia yolk sac-fry gills

One hundred and eighty, five-day post-hatch *O. niloticus* sac-fry derived from a single egg clutch were randomly divided among six 260 ml exposure chambers linked to the flow-through system (see Chapter 3), filled with ASTM dilution water (see Chapter 2). Similarly one day old *T. zillii* sac-fry were allocated to another six exposure chambers linked to the flow-through system. After 24 h of acclimation, three exposure chambers containing *O. niloticus* and three exposure chambers containing *T. zillii* sac-fry were randomly selected and exposed to cadmium test solutions containing 20 μg^{-1} of (nominal concentration) cadmium (equivalent to the 96-h EC50 cadmium concentration of six day old *O. niloticus*) for 96 hours. Three sac-fry were obtained from each exposure chamber at 24 hour intervals and sacrificed for electron microscopic examination. Gills from three sac-fry were examined under scanning and transmission electron microscope. For consistency, the second, third and fourth holobranchs were used for examinations.

6.2.5 Statistical analyses

Comparison of cadmium uptake and burden levels in tilapia sac-fry were performed using either one-way ANOVA or directional t-test (Zar, 1984).

6.3 Results

6.3.1 Effects of EDTA on cadmium uptake and use of EDTA to remove adsorbed cadmium from *O. niloticus* yolk sac-fry

Effect of EDTA on cadmium uptake by yolk sac-fry

The temporal changes in whole body cadmium uptake by *O. niloticus* sac-fry exposed to cadmium and cadmium-EDTA mediums were shown in figure 6.1. The whole body cadmium uptake under cadmium and cadmium-EDTA exposure increased with exposure time. The whole body cadmium uptake in yolk sac-fry exposed to cadmium-EDTA medium, however, showed little variation with exposure time. The cadmium uptake levels in yolk sac-fry exposed to cadmium alone increased significantly (d.f= 5,24; F= 254.06; P<0.05) with time after 24 h and continued to rise to 72 h. of exposure. The yolk sac-fry exposed to cadmium-EDTA medium showed a significant increase (d.f= 5,24; F= 53.94; P<0.05) only after 24 hours and reached a more or less steady state. At the end of the 72 h exposure period cadmium levels in yolk sac-fry exposed to cadmium-EDTA medium were less than 40% of those in yolk sac-fry exposed to cadmium alone.

Effectiveness of EDTA of removing adsorbed cadmium from yolk sac-fry

The temporal changes in whole body cadmium uptake by *O. niloticus* yolk sac-fry subjected to different washing processes are shown in Figure 6.2. Distilled water washes up to 25 minutes were ineffective in removing adsorbed cadmium from yolk sac-fry as the whole body cadmium levels between yolk sac-fry rinsed with distilled

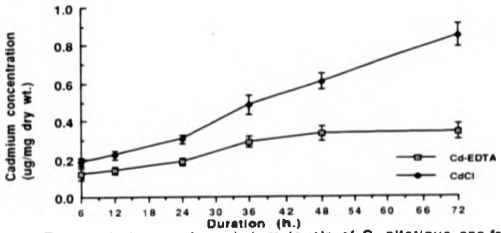


Fig.6.1 Temporal changes in cadmium levels of O. niloticus sac-fry when exposed to cadmium and Cd-EDTA mediums (means given with SD)

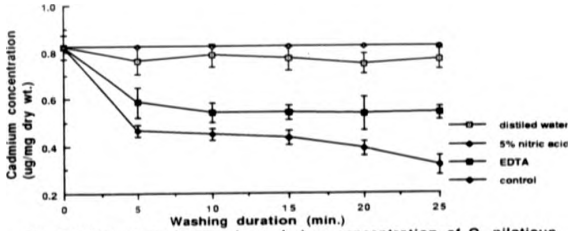


Fig. 6.2 Temporal changes in cadmium concentration of O. niloticus sac-fry subjected to different washing processes (means given with SD)

water and control were not significantly different ($P>0.05$). Whole body cadmium level in yolk sac-fry rinsed in EDTA solution for 5 minutes was significantly lower (d.f= 6, $t_{(1)}$= -5.7877, $P<0.05$) from control cadmium value, and, thereafter the cadmium level did not vary significantly (d.f= 4,15; F= 0.70; $P>0.05$) with the increased rinsing time. Rinsing yolk sac-fry in EDTA and nitric acid for 5 minutes removed 30% and 44% of total cadmium uptake, respectively. The whole body cadmium levels in *O. niloticus* yolk sac-fry rinsed for 5 minutes in 5% (v/v) nitric acid showed a significant decrease from the control value (d.f= 6, $t_{(1)}$=-12.816, $P<0.05$). The cadmium level after EDTA rinse for 5 minutes was significantly higher (d.f= 6, $t_{(1)}$= 3.4514, $P<0.05$) than the corresponding value after 5% nitric acid wash. The cadmium level in yolk sac-fry continued to decrease significantly (d.f=4,15; F= 16.12; $P<0.05$) with increasing 5% nitric acid rinsing time.

6.3.2 Cadmium uptake and partitioned body burden in tilapia yolk sac-fry

Interspecific cadmium body burden

The control cadmium values measured at the termination of each experiment was below detectable levels. Difference in cadmium body burden between *O. niloticus* and *T. zillii* yolk sac-fry is shown in Figure 6.3. For both species body burden of cadmium was highest in the gills. The weight-specific cadmium burden in *O. niloticus* gills was notably higher than the weight-specific cadmium burden in *T. zillii* gills. The cadmium burden in whole body (d.f= 4, $t_{(1)}$= -6.577, $P<0.05$) and viscera (d.f= 4, $t_{(1)}$= -5.716, $P<0.05$) in *T. zillii* were significantly lower than in *O. niloticus*.

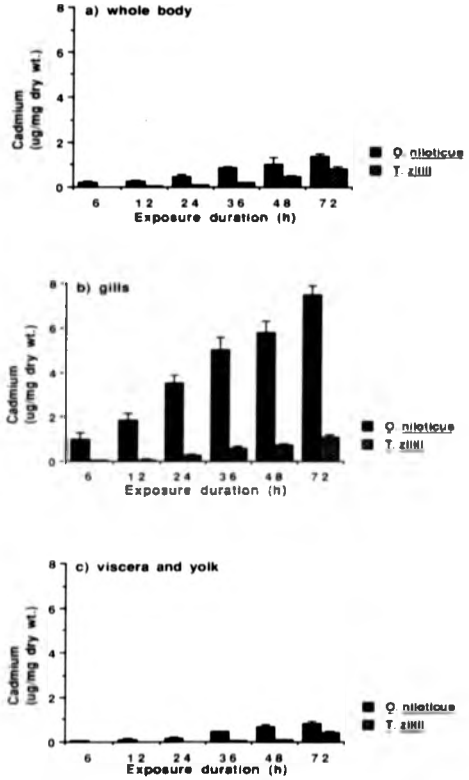


Fig. 6.3 Cadmium partitioned body burden, a) whole body b) gills c) viscera and yolk, in O. niloticus & T. zilli sac-fry (means given with SD)

Intra-age specific cadmium body burden of *O. niloticus* yolk sac-fry

The age-specific cadmium body burden levels in *O. niloticus* yolk sac-fry of varying ages is shown in Figure 6.4. At each yolk sac-fry age cadmium burden levels in gills were significantly ($P < 0.05$) higher than the cadmium burden levels in viscera and whole body. The whole body burden (d.f= 3,8; $F = 28.48$; $P < 0.05$) and viscera burden (d.f= 3,8; $F = 21.86$; $P < 0.05$) in 9- and 12-day post-hatch sac-fry were significantly higher than that in younger yolk sac-fry (3 and 6 day post-hatch sac-fry), while the gill cadmium burdens (d.f= 3,8; $F = 84.78$; $P < 0.05$) were significantly lower than the younger yolk sac-fry with yolk reserves.

Intra-size specific cadmium body burden of *O. niloticus* yolk sac-fry

Whole body gill and viscera cadmium burden levels between yolk sac-fry originating from 0+ (small yolk sac-fry) and 2+ (larger yolk sac-fry) *O. niloticus* females at different ages shown in Figure 6.5 to 6.7. Gill cadmium levels were significantly ($P < 0.05$) higher in yolk sac-fry of both sizes at all ages than the corresponding whole body or viscera cadmium levels. Three and 6-day post-hatch fry from the 2+ female showed significantly higher cadmium burden levels in both the gills (d.f= 4, $t_{(1)} = 6.2519$, $P < 0.05$; d.f= 4, $t_{(1)} = 4.8342$, $P < 0.05$ respectively) and whole body (d.f= 4, $t_{(1)} = 8.9565$, $P < 0.05$; d.f= 4, $t_{(1)} = 5.278$, $P < 0.05$ respectively) than yolk sac-fry from 0+ female at the same ages. In contrast, the 9 day post-hatch sac-fry from the 2+ female did not show any significant ($P > 0.05$) difference either in whole body, gill or viscera cadmium burden levels. Viscera cadmium burden levels between 0+ and 2+ yolk sac-fry behaved differently at post-hatch ages 3 and 6. The viscera cadmium burden in 3-day post-hatch sac-fry from 2+ female was significantly higher (d.f= 4, $t_{(1)} = 4.9973$,

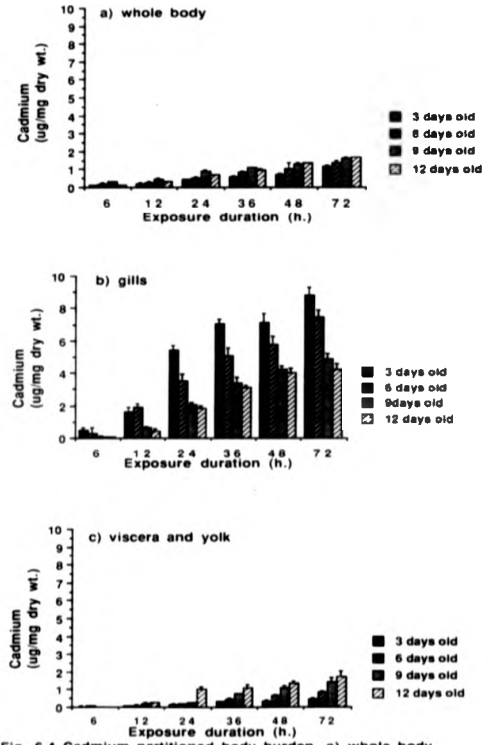


Fig. 6.4 Cadmium partitioned body burden, a) whole body b) gills c) viscera and yolk, in O. niloticus sac-fry at different ages (means given with SD)

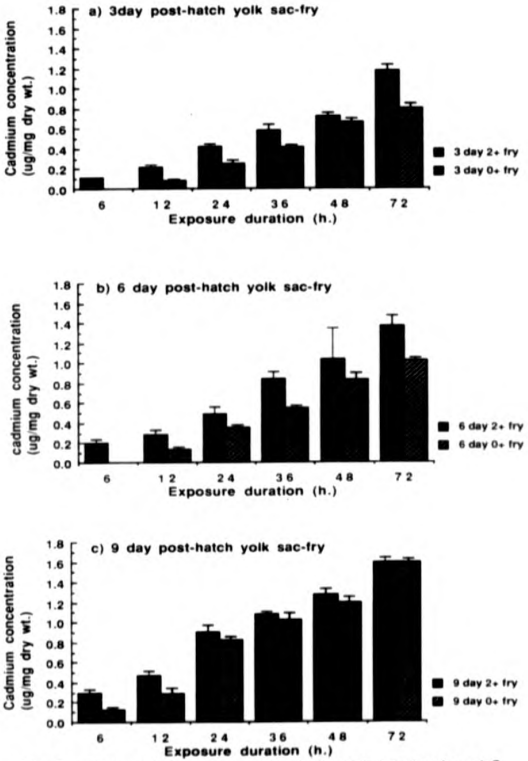


Fig. 6.5 Temporal changes in whole body cadmium burden of O. niloticus sac-fry from 0+ and 2+ females (means given with SD)

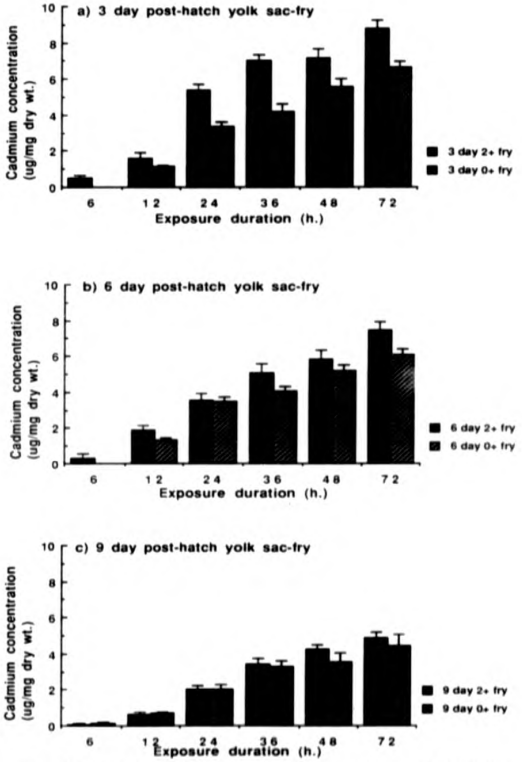


Fig 6.6 Temporal changes in gill cadmium burden of O. niloticus sac-fry from 0+ and 2+ females (means given with SD)

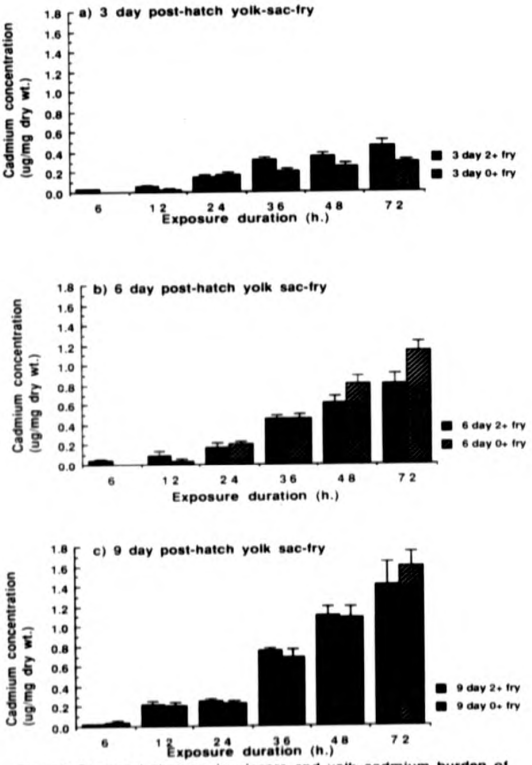


Fig. 6.7 Temporal changes in viscera and yolk cadmium burden of O. niloticus sac-fry from 0+ and 2+ females (means given with SD)

P< 0.05) than that in 0+ 3-day post-hatch sac-fry, while 6-day post-hatch sac-fry from 2+ female showed significantly lower (d.f= 4, $t_{(1)}$= -4.176, P< 0.05) viscera cadmium burdens than that in 0+ 6 day post-hatch sac-fry.

6.3.3 Effects of cadmium on gill morphology and ultrastructure

Typical examples of morphological and cytological aberrations of gill tissue are given in Plates 1 to 8. Cadmium exposure had destructive effects on gill tissue morphology and cytology in general and indicated increasing deterioration with increased duration of exposure. Frequency of changes in gill tissue morphology and ultrastructure, however, varied considerably between filaments and between fry exposed to the same treatment.

Each gill arch bears two rows of gill filaments (primary lamellae) with equally spaced lamellae (secondary lamellae) and no fusion between adjacent lamellae (Plate 1). The apical plasma membrane of the epithelial cells is folded into elevated structures, called microridges. Microridges are well defined on the filaments and are arranged in a concentric manner (Plate 2).

Considerable changes in the above morphology and ultrastructure of gill filaments and lamellae were noted in *O. niloticus* yolk sac-fry under the latter part of the exposure to lethal cadmium stress. The major changes observed in *T. zillii* exposed to the 96h lethal cadmium level of 6 day old *O. niloticus* sac-fry for 96 h were the proliferation and hypertrophy of mucous cells (Plate 3). After 24 to 48 h of exposure, the gill morphology of *O. niloticus* sac-fry under lethal exposure showed no major

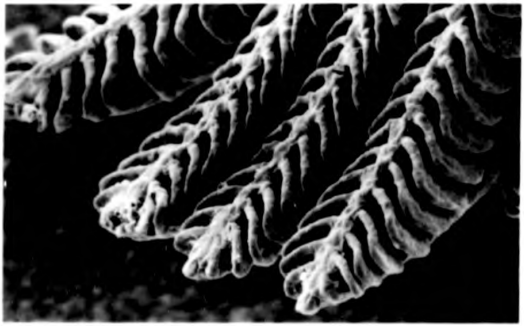
degenerative changes. The epithelial lamellae showed slight swelling and distortion and mucous cell proliferation and hypertrophy was clearly noticeable (Plates 4a to 4b). Obvious pathological changes on gill structure were observed after 72 to 96 h. of exposure. The major morphological changes were the appearance of hyperplastic and hypertrophic swellings on the gill arch and gill filament epithelium. The epithelial cells on these swellings had either totally or partially lost their microridges on filaments and gill arch epithelium and intercellular vacuolation were evident (Plate 5a and 5b).

An increased number of hypertrophic chloride (Plate 6a) and mucous cells was uniformly distributed on most of the lamellae. The hypertrophic chloride cells were characterised by swollen mitochondria with mitochondrial cristae and extended tubular system. Fully transformed chloride cells consisted of apical membranes and apical crypts. With increase duration of exposure many chloride cells showed necrotic areas and contained swollen mitochondria the cristae of which ultimately burst to form cytoplasmic vacuolation, tend to loose tubular system (Plate 6b and 6c) and rupture of apical membrane and exposing to exterior (Plates 6d).

Increasing mucous cell proliferation and increasing mucus cell activity are typical of prolonged exposure. The mucous cells showed necrotic changes. The integrity of the individual electronlucent mucus vesicles of the mucous cells were broken with dominated electron dark mucus vesicles (Plate 7). Necrotic areas were also observed within the lamellae epithelium (Plate 8).

Plate 1. Photomicrograph showing the arrangement of lamellae on gill filaments of six day post-hatch *O. niloticus* sac-fry exposed to control medium for 96 h as resolved by SEM (Note the lamellae are equally spaced and no sign of distortion 580 X)

Plate 2. Photomicrograph showing the gill filament surface epithelium of 6 day post-hatch *O. niloticus* yolk sac-fry exposed to control medium for 96 h as resolved by SEM
Note the characteristic microridge pattern (arrow) (3180 x)



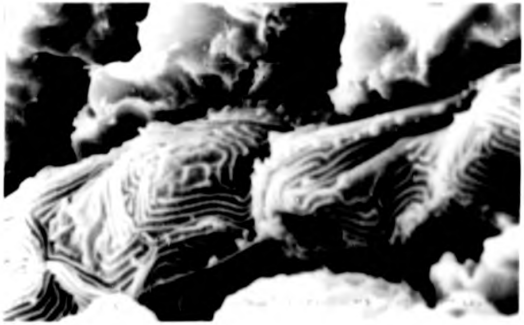
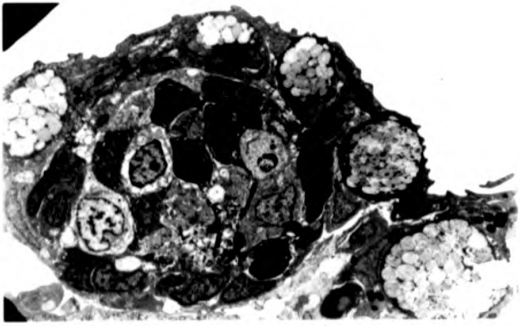
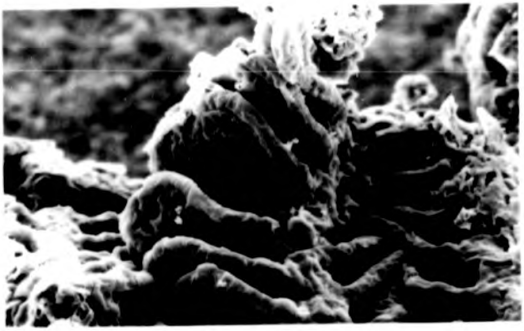


Plate 3. Photomicrograph showing mucous cell proliferation in 2 day post-hatch *T. zillii* yolk sac-fry gill after exposing for 96 hours to the lethal cadmium level of *O. niloticus* yolk sac-fry as resolved by TEM
Note the high density of electron lucent mucous vesicles (4,600 X)

Plate 4. Photomicrographs showing the pathological changes in gill lamellae epithelium and mucous cells of 6 day post-hatch *O. niloticus* yolk sac-fry exposed to lethal cadmium stress for 48 h
4a) Note the distortions and slightly swollen lamellae as resolved by SEM (1164 X)

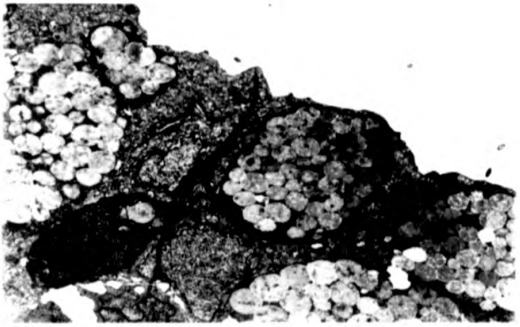




4b) Note the extensive presence of mucous cells (MC) and the predominance of electron lucent mucus vesicles within the mucous cells as resolved by TEM (7,000 X)

Plate 5. Photomicrographs showing the pathological changes in both gill filaments and lamellae epithelium of 6 day post-hatch *O. niloticus* yolk sac-fry exposed to lethal cadmium stress for 72-96 h

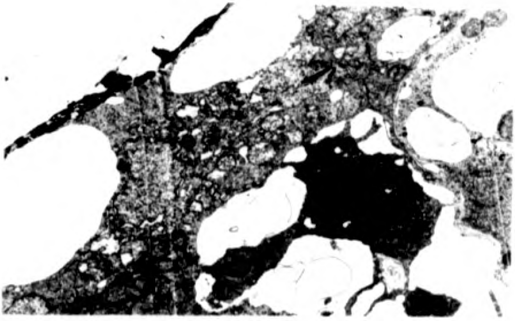
5a) Note a thin film of mucus on lamellae surface (arrow). Partial loss of microridges and change in microridge pattern. Nodules on filament epithelium (arrowhead) indicating obvious damage to the epithelial cells as resolved by SEM (2284 X)

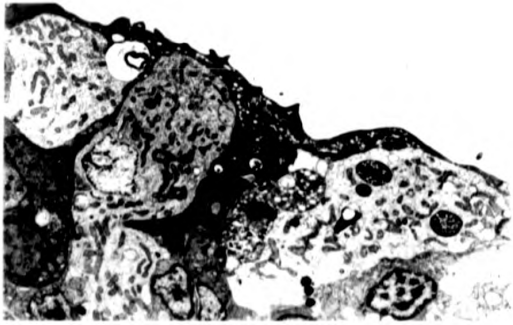


5b. Note the extensive intercellular vacuolation in gill filament epithelium as resolved by TEM (7,100 X)

Plate 6. Photomicrographs showing the pathological changes in chloride cells in 6 day post-hatch *O.niloticus* yolk sac-fry gills exposed to lethal cadmium stress for 72-96 h as resolved by TEM

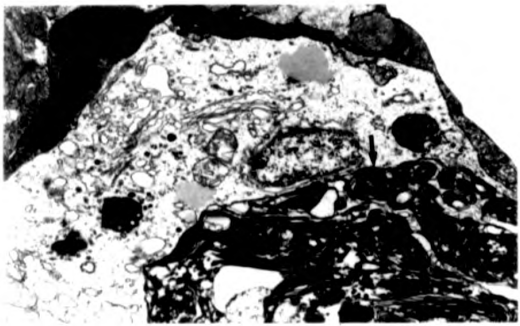
6a) Note the proliferation of chloride cells (6,000 X)

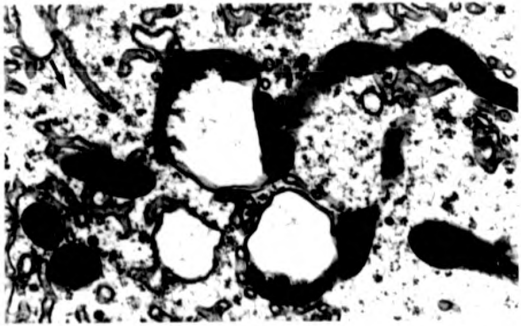




6b) Chloride cell with necrotic area (arrow) (16,000 X)

6c) Chloride cell with loosing tubular system (arrow) and containing swollen mitochondria with loosing mitochondrial cristae forming intracellular vacuoles (46,000 X)

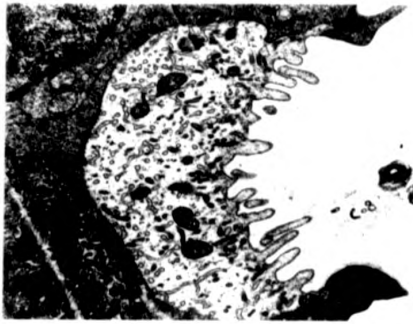




6d) Chloride cell opening to the exterior. Note cellular organelle are still in intact (16,400 X)

Plate 7. Photomicrographs showing pathological changes in mucous cells of 6 day post-hatch *O niloticus* yolk sac-fry exposed to cadmium lethal stress for 72-96 h as resolved by TEM

Note the more electron dense (arrow) and less electron lucent (arrowhead) mucus vesicles (12,600 x)



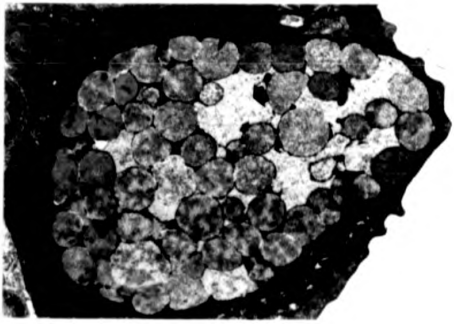
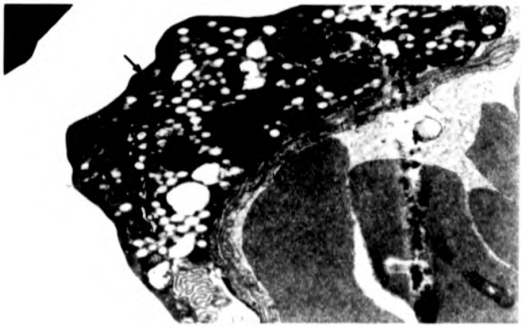


Plate 8. Photomicrograph showing nectrotic area (arrow) within the lamellar epithelium of 6 day Post-hatch *O. niloticus* yolk sac-fry exposed to cadmium lethal stress for 72-96 h as resolved by TEM (15,800 X)



6.4 Discussion

The bioavailability of metal ions may be reduced by chelation by natural substances like humic acids or by synthetic compounds like EDTA. Hence, in the presence of EDTA, the toxicity of cadmium to *O. niloticus* yolk sac-fry was greatly reduced by reducing the bioavailability of cadmium through the formation of cadmium-EDTA complex. Similar results have been observed for a range of organisms including marine molluses (Hung, 1982; Holwerda, *et al*., 1988), fish (Muramato, 1980, 1981) and unicellular algae (Maeda *et al*., 1990).

The mechanism by which chelating agents reduce the cadmium uptake in fish is not clear. Several possibilities have been proposed: either a lower uptake of the complexed metal, or a more rapid excretion of absorbed metal complexes (Part and Wikmark, 1984). The low bioavailability of metal may be due to the inability of metal-EDTA complexes to cross biological membranes (Part and Wikmark, 1984). Muramoto (1980) has suggested that cadmium-EDTA may be taken up, but will not be retained, as the chelator prevents the metal from being bound to tissue proteins. However, it is more likely that EDTA can not pass through the cell membrane and enter the cytoplasm as has been experimentally shown by using cadmium with C^{14} labelled EDTA (see Coombs, 1979). Moreover, Part and Wikmark (1984) showed that the transfer of the cadmium-EDTA complex in trout gill was 1000 times less than free cadmium ion. This observation is supported by the present study that bioavailability of cadmium-EDTA complex is greatly reduced. The adsorbed cadmium found on *O. niloticus* yolk sac-fry approximated the amount of cadmium found in yolk sac-fry

when exposed to

cadmium-EDTA medium. This suggests that, possibly, the cadmium found in *O. niloticus* yolk sac-fry exposed to cadmium-EDTA was on the surface.

In larger fish, the skin and gills are in direct contact with water, however, the uptake route via the skin is probably negligible as it is almost impermeable to dissolved substances (Part and Lock, 1983). The gills, however, with their large surface area and short diffusion distance between water and blood (Hughes, 1972) serve as the main uptake site for metals (Part and Lock, 1983). In the present study, when the weight specific cadmium accumulation of tilapia yolk sac-fry gills was compared with whole body and viscera accumulation, cadmium accumulation in gills was significantly greater and yolk did not act as a cadmium sink to contribute to cadmium accumulation via the dietary source.

In the present study, the age- or size-dependent variation in cadmium accumulation in *O. niloticus* yolk sac-fry, depended upon the type of tissue studied. The cadmium levels in *O. niloticus* yolk sac-fry whole body and viscera increased with increasing age, but the gill cadmium levels decreased with increasing age. In contrast, increased cadmium levels in gills and decreased cadmium levels in viscera were observed with increasing size. However, the whole body cadmium levels increased with increasing size.

The studies that were reported in the literature determining cadmium uptake and accumulation levels in yolk sac-fry of other species involved exposure of newly

hatched yolk sac stage of herring (von Westernhagen, *et al.*, 1979) and Atlantic salmon (Rombough and Garside, 1982) to cadmium for 16 and 46 days respectively. They found that newly hatched yolk sac-fry increased cadmium accumulation with exposure time. Similar patterns of cadmium uptake by rainbow trout alevins were reported by Beattie and Pascoe (1978). However, these studies can not be compared with the present study as the yolk sac-fry were continuously exposed throughout the yolk sac-fry stage and not subjected at different ages or sizes. The other studies reported in the literature of size and age effect on cadmium accumulation were mainly done on adults obtained from the wild. Increased cadmium levels were reported in the viscera (Pentreath, 1977) and in the whole body (Cutshall, Naidu and Pearcy, 1977) with increasing age of wild-collected adult fish species. In contrast, no changes in whole body cadmium contents of fish have been found either with age (Lovette, Gutenmann, Pakkala, Youngs and Lisk, 1972; Kelso and Frank, 1974) or size (Bohn and McElroy, 1976).

The observed interspecific and intra-age and -size specific differences in physiological and biochemical factors in the present study (chapters 4 and 5) of tilapia may play a role in determining the level of cadmium accumulation. To provide an insight into the question, how do the more tolerant tilapia yolk sac-fry avoid cadmium accumulation, morphological and cytological changes in the target organ (the gills) were examined.

Mallatt (1985) reviewed comprehensively the morphological and cytological effects of metal exposure on fish gills and categorised these into two groups. Firstly, the accumulated aberrations due to direct toxic effects of the metal and second, the

compensatory responses, which appear to be associated with the repair of gill damage. The first group includes, separation of epithelial layers, tissue oedema and clubbing of lamellae at moderate toxic levels. Tissue necrosis and rupture and fusion of secondary lamellae under more severe conditions. Hypertrophy and hyperplasia of mucous and chloride cells, and a general thickening of the filamental and lamellae epithelia are common in the second group. Morphological effects belonging to both the groups were observed in gills of *O. niloticus* yolk sac-fry subjected to the lethal cadmium level in the present study. The characteristic histopathological changes that were observed with *O. niloticus* yolk sac-fry gill tissue following lethal cadmium exposure included: cell necrosis, epithelial lifting, loss of microridges, intracellular and intercellular vacuolation and chloride and mucous cell proliferation and hypertrophy.

Most distinctive pathological changes in gills were observed in mucous and chloride cells. Both increased in number (proliferation), cell size (hypertrophy) suggesting increased activity. These changes were interpreted as a possible compensatory response to metal stress. Under non-stress conditions, mucus secretion is involved with osmoregulation and defence mechanism of fish (Pottinger, Pickering and Blackstock, 1984; Mallat, 1985; Handy, 1989; Handy and Eddy, 1989). Proliferation , hypertrophy and increased activity of mucous cells may be a defence against cadmium exposure. There is a general agreement (e.g. Varanasi and Markey, 1978; Lock and Van Overbeeke, 1981; Eddy and Fraser, 1982; Miller and Mackay, 1982; Handy and Eddy, 1989) that excess production of mucus which keeps metals away from the epithelial surface is one of the first lines of defence against metal exposure (McDonald and Wood, 1993).

Mucus is a mixture of glycoproteins, mucopolysaccharides, low molecular weight compounds and water (Fletcher, Jones and Reid 1976; Wold and Selset, 1977). Although no metal binding protein in mucus has been isolated, it is known that the glycoproteins have sufficient binding capacity to trap metals (McKone, Young, Bache and Lisk, 1971; Coombs, Fletcher and White, 1972; Varanasi and Maekey, 1978; Lock and Van Overbeeke, 1981). Cadmium is known to form strong covalent bonds with sulphydryl (SH-) groups of proteins, S-containing amino acids and wide range of biomolecules (Friberg, Piscator, Nordberg and Kjellstrom, 1974; Webb, 1979). It is therefore, probable that mucus traps cadmium by complexing it in mucus proteins, and thus prevent them from being absorbed into gill epithelial cells. Varying degree of trapping capabilities of cadmium in mucus may be a possible reason for interspecific as well as intraspecific differences in tolerance capabilities to cadmium stress.

In the present study increased tolerance of *T. zillii* and 9- and 12-day post-hatch and smaller yolk sac-fry *O. niloticus* was accompanied by less branchial accumulation of cadmium (Figures 6.3b, 6.4b and 6.6). The development of cadmium tolerance, may involve adaptations that reduce the toxic impact of cadmium on branchial morphology, ultrastructure as well as physiology. In the present study, less branchial accumulation in more tolerant tilapia may indicate that the increased metal tolerance was achieved through decreased net metal accumulation by the gills. The question then could be asked as to how these decreased cadmium accumulation occurred to varying degrees in different ages, sizes and species of tilapia yolk sac-fry. Two possible explanations are offered. Either the 'mucus turnover' rate or 'complexing capacity' of mucus or

both could vary among tolerant and non-tolerant tilapia. These two possibilities are illustrated in Figures 6.8 and 6.9. The 'mucus turnover' rate is continuous sloughing off and replacing the mucus layer. This is particularly activated when heavy metals are present in the water (Varanasi and Markey, 1978; Lock and Van Overbeeke, 1981; Eddy and Fraser, 1982). Under cadmium stress the elevated 'mucus turnover' rate from the normal level may be higher in tolerant tilapia forms than in the less tolerant tilapia forms (Figure 6.8a). Alternatively the increased level of 'mucus turnover' rate may be the same in both the forms but in less tolerant form the elevated 'mucus turnover' rate may be maintained to a lesser duration than in the tolerant form (Figure 6.8b). Due to increased availability of binding sites at higher 'mucus turnover' rate the cadmium ion complexing in mucus layer will be efficient, thus, reducing the cadmium being absorbed. Activation of mucus cells and the secretion of mucus which contains proteins, may involve an energy cost leading to an elevation of maintenance cost. The extent to which the 'mucus turnover' rate can be increased and/or maintained at the increased level may be limited by the ability to expend energy to meet the mucus cell activation and mucus secretion cost. Therefore, among tested tilapia yolk sac-fry, those forms and species with higher growth performance and energy efficiencies may be able to meet the elevated maintenance cost, and hence, show higher tolerance capability to cadmium stress.

An increase in 'mucus turnover' rate may not be the expected mechanism of tolerating cadmium stress in *O. niloticus* yolk sac-fry during the yolk depletion period, since they are expected to adopt an energy saving strategy and be unable to meet the elevated maintenance cost due to high 'mucus turnover'. The possible mechanism in

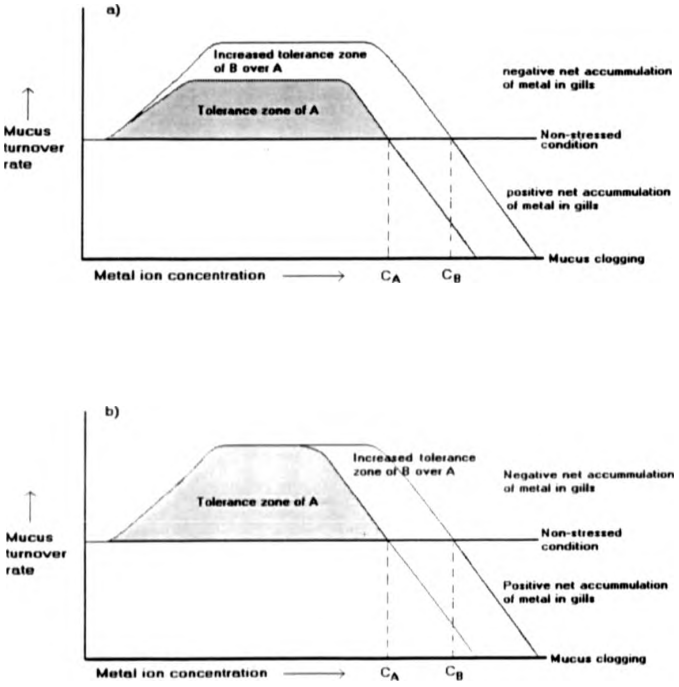


Fig. 6.8 Schematic diagram illustrating increase mucus turnover rate and increase metal tolerance (A and B represent a less tolerant and a more tolerant form respectively. a) Two levels of increase mucus turnover rate. b) Different maintenance durations of mucus turnover rates. C_A and C_B represent minimum effective concentrations of metal for A and B).

increased tolerance to cadmium stress in *O. niloticus* yolk sac-fry during yolk depletion would be the increase in 'complexing capacity' of the mucus layer. Metal 'complexing capability' within the mucus layer will be governed by the number and species of ions present and by the 'charged density' (valence and hydrated ionic radius) associated with each ion (Robinson and Stokes, 1965), as well as by the number and type of metal-binding ligands within the mucus (Campbell and Stokes, 1985). Therefore, metal 'complexing capacity' could be increased by increasing the availability of metal binding ligands. A reduction in the ionic content of mucus will lead to an increase in the availability of metal binding ligands. Handy and Eddy (1990) showed that sea water adaptation clearly increases mucus ion content in fish, whereas starvation in fresh water fish reduces mucus ion content. Handy and Eddy (1990) illustrated this observation by measuring ionic content of mucus in starved and fed adult rainbow trout. They found that starved fish decreased their mucus ion content by 587% against 173% increase in fed fish. Thus, mucus from starved fish has a lower ionic content and may have more metal binding ligands to trap metal ions. Therefore, although the starved sac-fry may not be able to increase the 'mucus turnover' rate to the same extent as fed fry, the increased 'complexing capacity' of the former may compensate for the lower turnover rates to increase tolerance (Figure 6.9).

Changes were also observed in chloride cells. The ultrastructural changes in chloride cells seen in the present study was also reported by Oronsaye and Brafield (1984), for sticklebacks (*Gasterosteus aculeatus*) exposed to cadmium. Similar changes in chloride cell number, their distribution and ultrastructure have frequently been

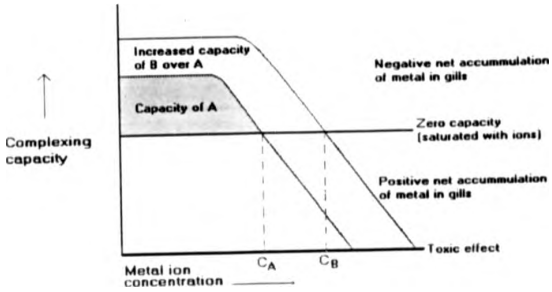


Fig. 6.9 Schematic diagram illustrating increase 'complexing capacity' of mucus and increase metal tolerance (A and B represent a less tolerant and more tolerant forms respectively. C_A and C_B represent minimum effective concentrations of metal for A and B).

reported in studies under a variety of circumstances other than exposure to cadmium (Matthiessen and Brafield, 1973; Pottinger *et al.*, 1984; Wendelaar Bonga and Dederen, 1986). Chloride cells are known to be involved in ion transport across the gill epithelium both in fresh and salt water (Evans, 1982; Pang, 1983; Pery and Wood, 1985). In fresh water fish, chloride cells perform an absorptive function, where both Na^+ and Cl^- are actively transported from its milieu to blood, while in salt water they perform the opposite function (Kirschner, 1979). It is well documented that when fresh water fish are transferred to salt water dramatic morphological and ultrastructural changes occur in chloride cells in order to alter the ionic movement (Loretz, Collie, Richman III and Bern, 1982; Wickes, Smith and Meade, 1983; Langdon and Thorpe, 1985; Lubin, Rourke and Bradly, 1989). Most of the ultrastructural alterations in branchial chloride cells of Atlantic salmon (*Salmo salar*) during fresh to sea water transformation (parr-smolt transformation), recorded by Lubin *et al.*, (1989), were observed in *O. niloticus* sac-fry during lethal cadmium exposure in the present study. These changes include, cell proliferation, hypertrophy, apical crypt formation, electron dense appearance of mitochondria and cristae degeneration, reduction in endoplasmic reticulum and cell rupture and expose to the external milieu. If these morphological and ultrastructural changes in chloride cells are exclusive to the ion transportation function, it could be indicative of an loss of essential ions from branchial cells creating an ionic imbalance under cadmium exposure rather than a compensatory mechanism to increase tolerance.

If this ionic imbalance is more likely, how could it possibly happen? Cadmium may compete for binding sites and replace essential ions such as Na^+ and Ca^{+2} in mucus

to form complexes and bind to negatively charged ions such as Cl in mucus, thus, creating an ionic gradient allowing essential ions to move from body fluid to the exterior. Such increases in Na^+ and Cl efflux, resulting in net ionic loss and eventual disruption of gill epithelium was observed in goldfish after treatment with the metal lanthanum (Eddy and Bath, 1979). Higher diffusion rates were also reported for essential ions such as Na^+, Cl (Marshall, 1978) and Ca^{+2} (Part and Lock, 1983) in fish mucus, indicating that these ions are rather loosely bound to the mucus and lend further support to the hypothesis of Kirschner (1979), who suggested that the mucus layer could act as a matrix from which essential ions are rapidly mobilised for uptake via gill epithelium. The lower diffusion rate (Part and Lock, 1983) and higher complexing ability (see Brezonick, *et al.*, 1991) of cadmium than essential ions in mucus facilitates the competition and replacement of essential ions from the mucus layer by cadmium. This replacement may take place rapidly, once the available free binding sites and negatively charged ions in mucus are saturated with cadmium. Increased efflux of essential ions under metal stress may be the reason for the observed increased synthesis in both Na,K-ATPase (Lauren and McDonald, 1987) and Ca^{+2}-ATPase (Shephard and Simkiss, 1978; Watson and Beamish, 1981) in fish gills. In the present study the observed changes in chloride cells in gill epithelium could be closely related to this ion loss. Therefore, ultrastructural changes in chloride cells, indicative of an ionic imbalance, may be a result of toxic effects of cadmium. Matthiessen and Brafield (1973) and Oransaye and Brafield (1984) attributed the proliferation and hypertrophy of chloride cells to the direct or indirect response to zinc and cadmium respectively in sticklebacks and suggested that the subsequent degeneration of chloride cells caused an excretion of metals. In addition to the

trapping of cadmium by mucus secretion and excretion of cadmium by chloride cells, proliferation and hypertrophy of both the cells types may cause a substantial thickening of the gill epithelium (Matthiessen and Brafield, 1973). In addition to the mucus layer offering protection to the gill epithelium, the thickening may cause a further increase in the distance between the cadmium source and interior blood. However, due to the action of both cell types the gaseous exchange may be impaired making diffusion difficult. Therefore, it is most likely that, changes in both cell types, under cadmium lethal stress, carry a compensatory response followed by a toxic response, which may lead to death.

However, the most frequently stated reason for increased cadmium tolerance in fish is the increased synthesis of metallothioneins (MT) or MT-like proteins (Benson and Birge, 1985; Stone and Overnell, 1985; Klaverkamp and Duncan, 1987; Fu, Steinebach, Van den Hamer, Balm and Lock, 1991). Increased tolerance via increased metal storage and detoxification through binding to MT or MT-like proteins was not supported by the present study, since gills of tolerant tilapia sac-fry consistently exhibited significantly lower cadmium levels than the sensitive sac-fry. The present study supports the hypothesis that tolerance capability in tilapia sac-fry to cadmium is due to decreased burden levels rather than storage and detoxifying through binding to MT or MT-like proteins in the gills. Wicklund-Glynn and Olsson (1991) found no effect of cadmium on MT or MT like protein levels in the gills of minnows, even though gill cadmium increased 20-fold compared with controls. This level was well above the cadmium levels that are needed to induction of MT or MT-like proteins (Mc Donald and Wood, 1993). The sites of greatest induction of MT or MT-like

proteins are the liver and kidneys, but they are also found in the gills of fish (see review by Hamilton and Mehrle, 1986). The increased weight specific levels of viscera cadmium in more tolerant older yolk sac-fry with yolk depletion, when compared to their younger conspecifics with yolk reserves, could be attributed to the decreased weight in viscera due to lack of yolk when compared with younger sac-fry with yolk rather attributing to the sequester cadmium by MT or MT-like proteins produced by the developed yolk sac-fry liver. However, the latter is more unlikely as the cadmium in viscera was not present in sufficiently high levels to indicate production of such proteins.

Another striking gill lesion associated with acute cadmium exposure was the increased occurrence of intercellular vacuolation in the lamellar epithelium. An implication of this may be the increased ion efflux by disruption of the sealing of intercellular diffusion pathways. Similar observations have been made with metals other than cadmium (Matthiessen and Brafield, 1973; Dietge, Jonsson and Bergman, 1988; Evans, Brown and Hara, 1988). Infiltration of cells into the intercellular spaces to suggest a protective osmoregulatory manifestation of the gills (Hughes and Gray, 1972) was not observed in the present study.

A partial or total loss of surface microridges in gills, was observed as a consequence of cadmium exposure. Possible causes of disappearance or breakdown of microridge patterns include oedematous changes in gill tissue and/or increases in cell size (Karlsson-Norrgren, Runn, Haux and Forlin, 1985). The partial or total loss of surface microridges contributes to the impairment of gill functions, such as, suggested by

Hughes (1980). Therefore, a reduction in microridges may cause a reduction in both the capacity for gaseous exchange and metal trapping in mucus. Therefore, the observed vacuolation and loss of microridges were toxic responses to lethal cadmium stress rather than compensatory responses.

To conclude, the cadmium burden in gills, when compared to viscera and whole body cadmium burden, in *O. niloticus* and *T. zillii* sac-fry, was significantly ($P<0.05$) higher in all species, ages and sizes tested. This suggested gills were the main target organ. The more tolerant older and smaller *O. niloticus* yolk sac-fry had less gill cadmium burden than their conspecifics and also the more tolerant *T. zillii* had less gill cadmium burden than more sensitive *O. niloticus* yolk sac-fry. This confirmed the association between less gill cadmium burden and tolerant capability to cadmium lethal stress.

The possible mechanism by which the tolerant tilapia forms reduced their cadmium burden was dependent upon the species, age and size of sac-fry. The faster-developing species such as *T. zillii* and small fry from smaller eggs had higher growth efficiency than slow-developing species such as *O. niloticus* and large fry from larger eggs. This increased efficiency may have made it possible for growth energy to be diverted to meet the increased maintenance energy cost, possibly due to increased mucus turnover rate, under cadmium stress. Increased tolerance of older sac-fry under starvation conditions may be due to less energy demanding higher cadmium binding capacity of mucus.

CHAPTER 7

NON-LETHAL EFFECTS OF CADMIUM ON SOME PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF TILAPIA YOLK SAC-FRY

7.1 Introduction

Lethal toxicity tests have been acclaimed as the most convenient and useful tool for both first screening of chemicals for their toxic effects and sensitivity of species to toxic stress (Forbes and Forbes, 1993). A general problem in using lethal toxicity tests alone in ecotoxicology to assess ecological 'harm' is that, the 'harm' is irreversible since the lethal response is a condition involving a stimulus severe enough to rapidly induce a biological response resulting in death. To be able to understand, explain and predict the effects of stress on individuals, populations and communities, it is necessary to estimate whether responses to stress occur to resist and increase stability under stress. Lethal responses alone would restrict such understandings. Therefore, lethal responses alone may not be useful in linking individual effects to effects at higher levels, such as populations, communities and ecosystems. It is often assumed that there is a functional link between responses in individuals to short-term lethal and long-term non-lethal stress (Giesy and Graney, 1989). However, potential existence of time lags in the lethal responses of an organism may provide misleading individual responses to lethal stress (Alabaster and Lloyd, 1980; Haux, 1985) ie. the organisms may not die during acute exposure, but die a few days after the exposure. On the other hand non-lethal toxic impacts on the population level may show a time lag between the impact and response due to various compensatory mechanisms (Forbes and Forbes, 1993). If there is a functional link between lethal and non-lethal responses, it must depend on general underlying mechanisms of lethal and non-lethal toxic actions and tolerance. If the observed responses to lethal cadmium stress in

tilapia yolk sac-fry are functionally linked to non-lethal cadmium stress, the predicted fitness advantages to lethal stress should come into action under non-lethal stress. To investigate the existence of such a relationship, the equivalent stages of the most sensitive *O. niloticus* and the most resistant *T. zillii* yolk sac-fry were subjected to non-lethal cadmium stress.

As the measured response, death in lethal tests will not provide an early warning of 'harm' in individuals and populations. Indices of stress relating to the aspects of 'fitness' of an organism may be indicative of an early warning under non-lethal stress.

7.1.1 The use of physiological and biochemical indices in stress tolerance

The 'fitness' or 'health' of an organism is dependent on the balance between energy gains and losses to the organism. It may be represented in the 'balanced energy equation' given in Chapter 1 (equation 1.3) for tilapia yolk sac-fry. If an environmental stress reduces energy assimilation or increases metabolic cost (maintenance metabolic cost or/and routine metabolic cost) the somatic growth rate will reduce, potentially reducing fitness. Therefore, monitoring ecophysiological condition indices of a fish could be a useful tool for evaluating 'fitness' or physiological activity under stress. These ecophysiological condition indices that include physiological and biochemical variables indicative of metabolic and energy state of the fish will act as sensitive indices to environmental stress. These indices are of two types, static and dynamic.

Static indices

This includes both physiological and biochemical indices. The widely used static condition indices are the dry weight wet weight, RNA concentration, RNA DNA, Protein RNA or Protein RNA DNA ratios and adenylate energy charge (AEC). Dry weight wet weight has been used on the basis that high proportion of water in tissue signifies a state of depleted energy resources, as observed in starvation stress (Johnston and Goldspink, 1973) or in winter conditions (Ansell, 1975, Beninger and Lucas, 1984). This index may be calculated for a whole animal or for the body tissues in the case of yolk sac-fry. AEC, RNA and RNA DNA ratio have been proposed for use as biochemical indices of environmental stress (Ivanovici and Wiebe, 1981) and have been discussed in previous chapters (Chapter 1 and 5 respectively). AEC is a direct measure of ATP and ADP in the body and probably indicates the energy performance of an organism. While very sensitive, this index poses two major problems. The first is that measurements of adenylate levels are quite time consuming and difficult and the second is one of sample collection (Lucas and Beninger, 1985).

RNA concentration and RNA DNA ratio which represent the protein synthetic capacity will provide information regarding maintenance metabolism of the fish. Hence, they can be used under environmental stress as an indicator of changes in maintenance metabolism. Protein RNA or Protein RNA DNA represent protein synthetic efficiency (Houlihan, 1991). Therefore, the measures of protein synthesis and efficiency under environmental stress indicate the changes in growth efficiency. The balance between net protein gain and synthesis will indicate protein degradation. Therefore measuring RNA, DNA and protein under stressed and non-stressed

conditions and using their ratios in different combinations will provide information regarding changes in metabolic cost and availability of energy for growth

Dynamic indices

Net growth efficiency (NGE), availability of energy for growth or scope for growth (SFG) and oxygen nitrogen (O N) ratio are the important dynamic indices that provide information regarding the energy status of the fish under stress NGE is the efficiency with which assimilated energy (AE) is utilized for somatic growth (Pg)

A net growth efficiency (NGE) value of 0 indicates that metabolic requirements are balanced by consumed energy and no tissue growth takes place A greater value than 0 for NGE indicates a positive energy balance, and hence positive tissue growth Conversely values less than 0 reflect a negative energy balance indicating the organism has to meet higher metabolic demand using its stored energy resources Thus a negative growth takes place Therefore, determining NGE under both stress and non-stress conditions, will be indicative of an energy trade-off from growth to maintenance metabolism

Scope for growth (SFG) has been widely used by a number of workers as a 'fitness' index (Bayne, Widdow and Thompson, 1975, Bayne and Widdows, 1978, Widdows, 1978) In practice, the term SFG is synonymous with the bioenergetic term production since,

$$SFG = AE - TM$$

where, TM is total metabolic cost (Worrall, Widdows and Lowe, 1983)

A general response by an organism to stress is the utilization of nutrient reserves to meet a metabolic requirement that may have been enhanced above normal values (Widdows, 1985). If one assumes the energy is generated aerobically then oxygen consumption rate can provide a rapid method for estimating metabolic rate (Lampert, 1983). The measurement of oxygen consumption as an indicator of metabolic status has been used in many aquatic studies (Philippova and Postnov, 1988). Depletion of nutrients under stress can be measured in terms of nutrient substrate and its amount, when the stress is most extreme, but generally it is necessary to use a more sensitive index which reflects the alterations in the balance between catabolism of nutrient substrates (Widdows, 1985). Oxygen nitrogen (O:N) ratio provides an index of the relative utilization of protein in energy metabolism and assesses the physiological state of fish under the given set of environmental conditions. If the whole animal O:N ratio is used, it is possible to follow the physiological changes in a given group of organisms under varying environmental conditions, as this index does not require the sacrifice of the animals. It provides meaningful data on the metabolic state of an organism and it may thus be used to complement the NGE, although the O:N does not sum up the physiological conditions of an animal as well as does the NGE (Lucas and Beninger, 1985).

7.1.2 Aims of the study

The aim of this study was to explore whether there is a functional link between lethal and non-lethal responses of tilapia yolk sac-fry to cadmium stress. To achieve this aim the most sensitive (*O. mloticus*) and most resistant (*T. zilli*) tilapia species were employed to perform the following: 1) Physiological and biochemical correlates of

previously predicted fitness advantage to lethal cadmium stress (chapter 4 and 5) were monitored under non-lethal stress. These included changes in physiological and biochemical correlates of protein turnover and associated energy metabolism under non-stress and stress conditions.

2. The responses with respect to theses physiological and biochemical correlates of the two species were compared.

7.2 Materials and methods

7.2.1 Test solutions

All chemicals used were Analar grade. A 10 mgl^{-1} cadmium stock solution was prepared as described previously. Four nominal test concentrations of cadmium, 0.5, 1.5, 3.0, and 6.0 μgl^{-1} for stress and ASTM dilution water (see chapter 2) for non-stressed conditions were selected.

7.2.2 Method of sacrifice of yolk sac-fry and cadmium determination in water samples

Methods of sacrifice of yolk sac-fry and cadmium determination in water samples were the same as previously described in Chapter 6.

7.2.3 Physiological and biochemical measurements of yolk sac-fry

Oxygen consumption, RNA, DNA and protein determination in yolk sac-fry were the same as previously described in Chapter 5. Ammonia concentrations were measured on a technicon-Sampler IV Autoanalyser.

7.2.4 Experimental protocol

Experiment 1. Determination of growth performance of tilapia yolk sac-fry under non-lethal cadmium stress and non-stressed conditions

A total of 600 two-day post-hatch yolk sac-fry of *O. niloticus* from an individual egg clutch were transferred to 15 exposure chambers at a stocking rate of 40 sac-fry per

chamber. After 24 h acclimation yolk sac-fry in exposure chambers were randomly allocated to one of the triplicated four cadmium concentration levels and ASTM dilution water. Yolk sac-fry were exposed up to the maximum body weight attainment (9-day post-hatch) and for each treatment 30 sac-fry per replicate were sampled for the estimation of growth rates and yolk utilization efficiencies. The growth and yolk utilization were estimated as described previously (see Chapter 4). This design was repeated exposing newly hatched *I. zillii* yolk sac-fry after acclimatising for 24 h up to the maximum body weight attainment (6-day post-hatch).

Experiment 2. Determination of physiological and biochemical metabolic traits of tilapia yolk sac-fry under non-lethal cadmium stress and non-stressed conditions

The remaining sac-fry in each treatment of the above experiment were used for the physiological and biochemical measurements. Two yolk sac-fry from each replicate of each treatment were individually used to measure the oxygen consumption rate. Individual yolk sac-fry was placed in the static respirometer filled with freshly prepared relevant test solution. At the end of the oxygen consumption measurement experiment a sample of 50 ml test solution was transferred into a clean plastic bottle, stoppered and frozen for analyses of ammonia at a later stage. Each yolk sac-fry used in the oxygen consumption measurement was quick frozen, freeze dried and subsequently weighed on a balance (Mettler H51). The freeze dried sac-fry were later sonicated in one ml nanopure water. The homogenate was used for the determination of RNA, DNA, and protein contents.

Prior to the exposure triplicated samples consisting of two yolk sac-fry per replicate from the same yolk sac-fry clutch of each species were sampled. The bodies of yolk sac-fry were separated from the yolk and used for the determination of initial protein content. This was used to calculate the net protein gain. To investigate whether protein synthesis in terms of RNA/DNA and protein synthesis efficiency in terms of Protein/RNA is related to protein growth, the relative protein growth rate of control yolk sac-fry of both species were calculated as

$$\frac{\text{rate of net protein gain } (\mu g \text{ day}^{-1})}{\text{protein content at the termination of exposure } (\mu g)} \times 100$$

7.2.5 Statistical analyses

One way ANOVA (Zar, 1984) were performed to compare the effect of non-lethal cadmium stress on all the parameters tested for each species. Correlation coefficients (Zar, 1984) were calculated to investigate the relationship between RNA/DNA and Protein/RNA with relative protein growth for pooled data of control tilapia yolk sac-fry.

7.3 Results

The mean actual concentration recorded for each nominal concentration is shown in Table 7.1. It was found that *O. niloticus* and *T. zillii* yolk sac-fry significantly ($P < 0.05$) vary in their specific growth rates and yolk utilization efficiencies (chapter 4). There were obvious differences between *O. niloticus* and *T. zillii* yolk sac-fry in all tested parameters under all treatment and control conditions.

7.3.1 Growth performance of tilapia yolk sac-fry under non-lethal cadmium stress when compared to non-stressed conditions

The effect of non-lethal cadmium stress on the mean values of specific growth rate and yolk utilization efficiency for both *O. niloticus* and *T. zillii* yolk sac-fry are shown in Figures 7.1 and 7.2. There was a significant variation in mean specific growth rate with increasing cadmium concentration in *O. niloticus* ($df = 4,10$, $F = 12.393$, $P < 0.05$) and the lowest effective concentration was 3.0 $\mu g l^{-1}$ cadmium while it was significantly ($df = 4,10$, $F = 4.606$, $P < 0.05$) decreased in *T. zillii* yolk sac-fry at the highest concentration tested (6.0 $\mu g l^{-1}$) when compared to the corresponding control values. Similarly, the yolk utilization efficiency showed significant variations in both *O. niloticus* ($df = 4,10$, $F = 14.53$, $P < 0.05$) and *T. zillii* ($df = 4,10$, $F = 4.256$, $P < 0.05$) yolk sac-fry. The lowest cadmium concentration (3.0 $\mu g l^{-1}$) that reduced yolk utilization efficiency significantly in *O. niloticus* when compared to the control value was lower than that in *T. zillii* (6.0 $\mu g l^{-1}$). Even though the specific growth rates and yolk utilization efficiencies of both species

Table 7.1 The observed actual mean cadmium concentration during the exposure period (SD given in parenthesis)

Nominal concentration ($\mu g l^{-1}$)	Actual concentration ($\mu g l^{-1}$)
0.5	0.831 (± 0.04)
1.5	1.640 (± 0.10)
3.0	3.760 (± 0.15)
6.0	6.440 (± 0.20)

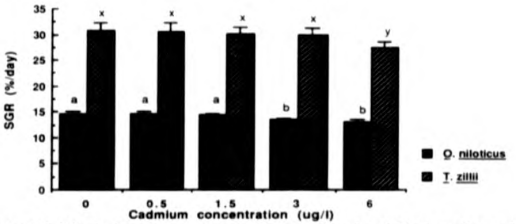


Fig. 7.1 Effect of cadmium on specific growth rate of tilapia yolk sac-fry (means given with SD and with different superscripts are significantly different, P<0.05))

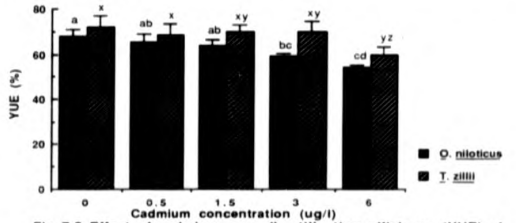


Fig 7.2 Effect of cadmium on yolk utilization efficiency (YUE) of tilapia yolk sac-fry (means given with SD and with different superscript are significantly different, P<0.05))

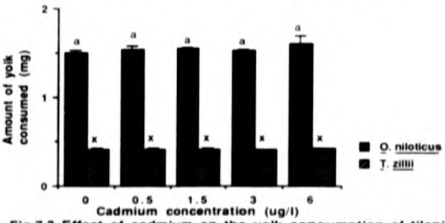


Fig.7.3 Effect of cadmium on the yolk consumption of tilapia yolk sac-fry (means given with SD and with same superscripts are not significantly different, P>0.05)

were significantly reduced under cadmium stress the amount of yolk consumed was not significantly ($P > 0.05$) affected (Figure 7.3).

The variation in mean values of protein growth rate under stress and non-stress conditions for both species is shown in Figure 7.4. Protein growth rate did not follow a similar pattern for *O. niloticus* as in the case of specific growth rate. The protein growth rate was significantly ($df = 4,10$, $F = 26.05$, $P < 0.05$) reduced at all cadmium concentrations tested for *O. niloticus* yolk sac-fry when compared to the control value. For *T. zillii* yolk sac-fry protein growth was significantly ($df = 4,10$, $F = 8.334$, $P < 0.05$) reduced only at the highest concentration (6.0 $\mu g l^{-1}$ cadmium) tested.

7.3.2 Physiological and biochemical metabolic traits under non-lethal cadmium stress when compared to non-stressed conditions

Oxygen consumption and ammonia excretion rates and O:N ratio

The variations in oxygen consumption and ammonia excretion rates of yolk sac-fry of both species with increasing cadmium stress intensity are shown in Figures 7.5 and 7.6. Both oxygen consumption and ammonia excretion were significantly affected by cadmium stress in *O. niloticus* ($df = 4,10$, $F = 26.88$, $P < 0.05$ and $df = 4,10$, $F = 35.97$, $P < 0.05$, respectively) and in *T. zillii* ($df = 4,10$, $F = 4.75$, $P < 0.05$ and $df = 4,10$, $F = 12.66$, $P < 0.05$, respectively) yolk sac-fry. In *O. niloticus* yolk sac-fry both oxygen consumption and ammonia excretion rates were significantly increased at 3.0 and 1.5 $\mu g l^{-1}$ cadmium.

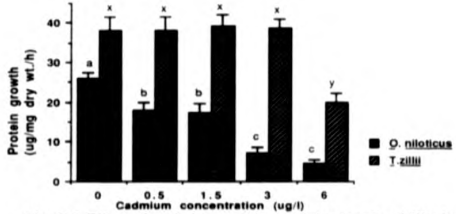


Fig. 7.4 Effect of cadmium on the protein growth of tilapia yolk sac-fry (means given with SE and with different superscripts are significantly different, P<0.05)

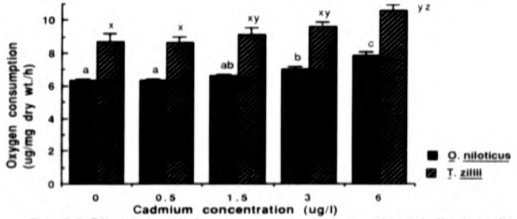


Fig. 7.5 Effect of cadmium on oxygen consumption of tilapia yolk sac-fry (means given with SE and with different superscripts are significantly different, P<0.05)

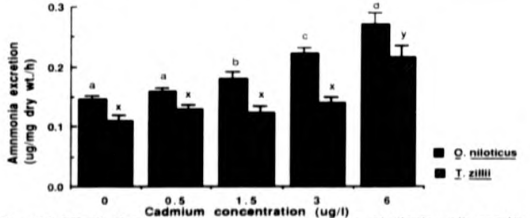


Fig. 7.6 Effect of cadmium on ammonia excretion of tilapia yolk sac-fry (means given with SE and with different superscripts are significantly different, P<0.05)

respectively, when compared with the corresponding control values. These parameters were significantly greater than the control values only at the highest cadmium concentration tested (6.0 μgl^{-1}) for *T. zillii* yolk sac-fry.

The variations in mean values under stress and non-stress conditions of O:N ratios for both species is shown in Figure 7.7. Variation in O:N ratios in both species showed an opposite pattern to both oxygen consumption and ammonia excretion with increasing cadmium concentration. The O:N ratio was significantly ($df=4,10$, $F=10.14$, $P<0.05$) affected at the highest cadmium concentration (6.0μgl^{-1}) tested in *T. zillii* while the O:N ratios in *O. niloticus* yolk sac-fry was significantly ($df=4,10$, $F=39.45$, $P<0.05$) reduced at all cadmium concentrations tested.

RNA:DNA and Protein:RNA

Protein synthesis expressed as RNA:DNA ratios and protein synthesis efficiency expressed as Protein:RNA ratios under both stress and non-stress conditions are shown in Figures 7.8 and 7.9. In *O. niloticus* yolk sac-fry RNA:DNA ratio was significantly ($df=4,10$, $F=11.712$, $P<0.05$) increased at 3.0 μgl^{-1} cadmium when compared to the control value. The Protein:RNA ratio in *O. niloticus* yolk sac-fry, however, was significantly ($df=4,10$, $F=24.618$, $P<0.05$) reduced at all cadmium concentrations tested when compared to the control value. In *T. zillii* yolk sac-fry, RNA:DNA ratios ($df=4,10$, $F=10.976$, $P<0.05$) and Protein:RNA ratios ($df=4,10$, $P<0.05$) were significantly increased and decreased, respectively, only at the highest cadmium concentration (6.0 μgl^{-1}) tested.

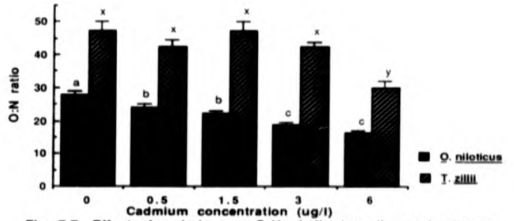


Fig. 7.7 Effect of cadmium on O:N of tilapia yolk sac-fry (means given with SE and with different superscripts are significantly different, P<0.05)

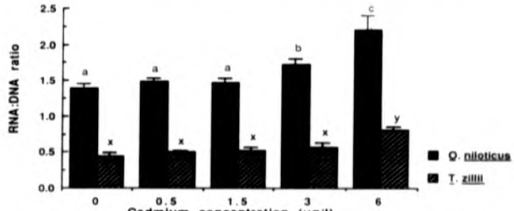


Fig. 7.8 Effect of cadmium on RNA:DNA of tilapia yolk sac-fry (means given with SE and with differentsuperscripts are significantly different, P<0.05)

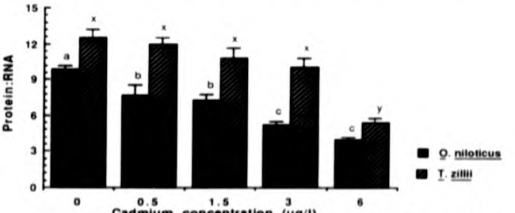


Fig. 7.9 Effect of cadmium on Protein:RNA of tilapia yolk sac-fry (means given with SE and with different superscripts are significantly different, P<0.05)

Overall, all the parameters tested in *T. zillii* yolk sac-fry were significantly (P< 0.05) affected only at the highest cadmium concentration (6.0 μgl^{-1}) tested when compared to the corresponding control values. In *O. niloticus* yolk sac-fry protein growth, O:N ratio and Protein:RNA (protein synthesis efficiency) were significantly (P< 0.05) reduced at all cadmium concentrations tested while specific growth rate, yolk utilization efficiency, oxygen and ammonia excretion rates and RNA:DNA (protein synthesis) were significantly (P< 0.05) affected either at 1.5 or 3.0 μgl^{-1} cadmium.

7.3.3 Relationship between RNA:DNA ratio and Protein:RNA ratio with relative protein growth

To investigate whether protein synthesis in terms of RNA:DNA and protein synthesis efficiency in terms of Protein: RNA ratio is related to protein growth, regression analysis of the pooled data of control tilapia yolk sac-fry was performed. There was a significant negative correlation (df=10, r=0.952, P=0.001) between RNA:DNA and a significant positive correlation (df=10, r=0.777, P=0.01) between Protein:RNA and relative protein growth (Figures 7.10 and 7.11).

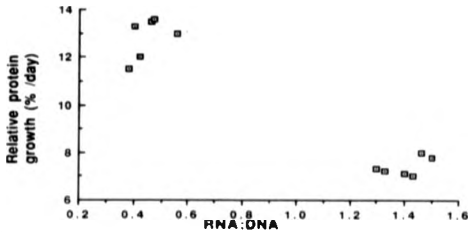


Fig. 7.10 Relationship between RNA:DNA and relative protein growth of tilapia yolk sac-fry under control conditions (pooled data. Relative protein growth (r = 0.952)

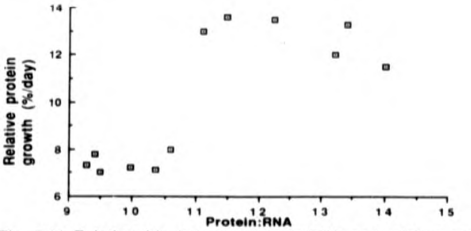


Fig. 7.11 Relationship between Protein:RNA and relative protein growth of tilapia yolk sac-fry under control conditions (pooled data. Relative protein growth (r = 0.777)

7.4 Discussion

The two tilapia species, *O. niloticus* and *T. zillii*, used to determine the non-lethal stress responses showed significantly different responses, in terms of effective concentrations to cadmium stress. As the two species markedly differ in their developmental rate, the exposure was commenced and terminated at the equivalent developmental stages. One day post-hatch *T. zillii* and three day post-hatch *O. niloticus* can be considered as equivalent developmental stages (Rana, 1986b; Galman and Avtalion, 1989; Mutsekwa, 1989). The exposure was terminated on the previously determined maximum weight attainment day (see Chapter 4) in order to prevent stress due to starvation.

The relative non-lethal sensitivities of *O. niloticus* and *T. zillii* yolk sac-fry to cadmium were concordant with their relative lethal sensitivities to cadmium. The differences in non-lethal sensitivities for the parameters tested, except for growth in terms of weight and protein synthetic capacity in terms of RNA/DNA, like their lethal sensitivities, were within an order of magnitude. Therefore, lethal toxicity tests may provide an indication of relative interspecies tolerance to non-lethal stress. This may suggest that the route of uptake and mode of action under lethal as well as non-lethal stress could be the same and that a general mechanism may be underlying the tolerance for both the lethal and non-lethal stresses.

The growth responses of both species to cadmium, in terms of dry weight gain and in terms of protein growth indicated a general reduction in energy supply and/or

reduction in energy deposition under cadmium exposure. In yolk sac-fry the growth rate is determined by the amount of yolk consumed and efficiency by which ingested yolk energy is assimilated into the body. The present study revealed that both species showed no significant variation in the amount of yolk consumed under cadmium exposure when compared with the control values. This suggests that cadmium did not affect yolk consumption. In contrast, reduced yolk consumption was observed for sac-fry of *Salmo salar* at 2.0 $\mu g l^{-1}$ under lower hardness and temperature conditions than in the present study (Peterson *et al*., 1983). Since, no part of the yolk is lost as faecal matter and the composition of yolk of sac-fry of the same age were similar (Craig and Baksi, 1977), the yolk assimilation efficiency may not vary between sac-fry of control and different treatments. Therefore, the observed increased oxygen consumption rate with increasing non-lethal cadmium stress in the present study, may be associated with other metabolic costs. To compare the observations of the present study no other work was found in the literature.

In the present study the nitrogenous excretion was increased with increasing cadmium concentration. Nitrogenous excretion is the end result of deamination of amino acids which releases amino groups that can not be recycled through other metabolic processes and must therefore be excreted. Nitrogenous excretion could originate from two sources, endogenous and exogenous (Jobling, 1993). Endogenous nitrogenous excretion resulting from breakdown of tissue proteins (protein degradation), while exogenous nitrogenous excretion result from direct deamination of amino acids during assimilation of ingested food. Under non-stressed conditions, endogenous nitrogenous excretion is generally quite low (Jobling, 1993), but can be considerably high under

stress conditions, if tissue protein breakdown is elevated (Hawkins *et al*., 1987). The relative contribution of these two sources towards nitrogenous excretion is hard to determine in endogenously feeding yolk sac-fry as in practice, it is measured under nitrogen free diet. However, assimilation efficiency was not apparently affected in the present study (see above). Therefore, the increase in nitrogenous excretion with increasing stress intensity was most likely due to protein degradation.

The cadmium concentration affecting growth in terms of dry weight and in terms of protein growth in *O. niloticus* was not similar. Indeed, overall dry weight gain was not necessarily related to protein deposition. Protein growth was significantly affected at the lowest nominal concentration (0.5 $\mu g l^{-1}$ cadmium) while growth in terms of dry weight was affected relatively at higher concentration (3.0 $\mu g l^{-1}$ cadmium). This indicates the protein growth may be a more sensitive index to stress than the growth in overall weight since protein is the major component of the dry body tissues.

The significant elevation in protein synthesis at higher stress intensities may be due to increased protein degradation and this increase may have stimulated protein synthesis. A proportion of the synthesised protein will be retained as growth and the rest will be mainly invested in protein turnover (protein turnover = degradation of protein + replacement of protein = maintenance metabolism).

$$\text{Net protein growth} = \text{protein synthesis} - \text{protein degradation} \qquad 7.1$$

An increase in synthesised protein and a corresponding decrease in protein growth

suggests the protein turnover has increased. In order to understand the relationships between the rates of the energy demanding costs of protein synthesis and protein growth, it is clearly necessary to have accurate measures of both processes (Houlihan, 1991). In the present study, the methods utilized by other workers (eg. Garlick, McNurlan and Preedy, 1980; Hawkins, 1985) to determine protein synthesis can not be utilized due to practical difficulties. Therefore the present study employed an indirect method to determine protein synthesis using RNA DNA. Unfortunately there is not yet a reliable method to determine protein degradation rates in the short-term (Haulihan, 1991) and degradation rates are generally determined from the difference between protein growth and synthesis rates (Millward, Garlick, Stewart, Nnanyelugo and Waterlowm, 1975).

The protein synthesis in terms of RNA DNA and protein growth was not affected at the same cadmium concentration for *O. niloticus* yolk sac-fry in the present study. A significant increase in RNA DNA was observed in *O. niloticus* at 3.0 μgl^{-1} and in *T. zillii* at 6.0 μgl^{-1} cadmium implying increased protein synthesis. However, protein growth in *O. niloticus* sac-fry significantly decreased at all cadmium concentrations, while it decreased only at the highest concentration (6.0 μgl^{-1}) for *T. zillii*. This may imply decreased protein deposition at all concentrations tested for *O. niloticus* and at the highest concentration for *T. zillii*. As shown above decreased protein growth rate could be attributed to the differences in protein synthesis and protein degradation rates. It is possible that decreased protein growth could occur through a decrease in protein synthetic rate with degradation rate remaining constant or alternatively protein degradation could increase and synthesis could remain constant. The other possibility

is both rates could increase but degradation rate increase more than synthetic rate Decreased protein synthesis rates were not observed in the present study for either species tested At effective non-lethal cadmium concentrations of both species where significant decrease in protein growth was observed, either the protein synthesis was significantly elevated or remained without significant change This implied elevated protein degradation rates at all effective concentrations for both species The unchanged protein synthesis together with significantly changed protein growth at low stress intensities may imply that elevated protein degradation may be replaced through a trade-off from growth protein The reason for this may be increase in protein synthesis is energetically costly as protein is the most energetically costly biomolecule to produce (eg Kiorboe *et al*., 1987, Jorgensen, 1988) In the present study at the higher stress intensities, the diversion of protein from growth alone may not be sufficient to meet the protein demand created through elevated protein degradation rates, and as a result protein synthesis rates increased

At all effective concentrations protein investment to replace elevated protein degradation

was in concordance with the O N ratios observed in the present study The O N ratio has been used in several studies as an index of nutrient utilization for energy production (Conover and Corner, 1968, Johns and Miller, 1982) and in interpretation of the balance in catabolism between nutrient reserves in the tissues (Correa, 1987) The decreasing O N ratios with increasing stress intensity in the present study suggests an increasing rate of protein catabolism relative to other nutrient, which is generally indicative of a stress condition (Widdows, 1978) A value of around 50 for O N has

been suggested for a healthy *Mytilus edulis* catabolizing relatively little protein and value under 30 has been suggested for a stressful condition (Bayne, 1973, Widdows, 1978). In the present study the non-stressed *O. niloticus* yolk sac-fry showed a O:N ratio value less than 30. However, the interpretation of O:N should be based on relative changes rather than absolute values as documented O:N values for many animals show inter- and intra-specific differences under unstressed conditions (Widdows, 1985).

Protein synthetic efficiency may indicate the relative proportion of protein being deposited as growth. The present study measured protein synthetic efficiency using Protein:RNA. The non-stressed yolk sac-fry showed a significant positive relationship between Protein:RNA and relative protein growth rate, while there was a significant negative relationship between RNA:DNA and relative protein growth rate. Therefore low protein synthesis and high protein synthetic efficiency was associated with high protein growth rates. This indicates low protein degradation and high protein deposition. Therefore faster growth is derived from decreased energy requirements for maintenance. Similar results have been obtained for *D. magna* using the same biochemical ratios for the components in protein dynamics (Barber *et al*, 1990). Hawkins *et al*., (1986) and Hawkins, Widdows and Bayne (1989) concluded similar results with direct measurements of protein synthesis, protein synthetic efficiency and protein growth for *Mytilus edulis*. Unlike RNA:DNA, Protein:RNA was in concordance with the protein growth under non-lethal cadmium stress for both species tested. As the stress intensity increased both protein growth and protein synthetic efficiency decreased. This confirms that as stress intensity increases, protein

degradation increases and protein deposition decreases, and hence maintenance cost increases. As the effective concentration for significant increase in protein degradation rate for *O. niloticus* yolk sac-fry (0.5 $\mu g l^{-1}$ cadmium) was significantly lower than the effective concentration of *T. zillii* for protein degradation rate (6.0 $\mu g l^{-1}$ cadmium) it can be inferred *T. zillii* yolk sac-fry were more tolerant than *O. niloticus* yolk sac-fry under non-lethal cadmium stress. Protein synthesis alone will not provide clear information on the protein dynamics as it does not provide the relative proportions being invested in growth and maintenance.

To conclude, a concordance was observed between non-lethal sensitivities of *O. niloticus* and *T. zillii* yolk sac-fry with their relative lethal sensitivities to cadmium suggesting a common route of uptake and mode of action for both types of cadmium stress. At all effective concentrations of cadmium, the protein growth and synthesis efficiency were reduced. Protein synthesis in both species, however, was either unchanged or increased. Therefore, protein degradation was increased at all effective cadmium concentrations suggesting a general response to non-lethal cadmium stress between the two species. The higher tolerance capability of *T. zillii* yolk sac-fry to cadmium stress than that of *O. niloticus* yolk sac-fry imply faster growing *T. zillii* may sacrifice growth to meet the elevated maintenance costs under stress more efficiently than that of *O. niloticus*. Therefore, the difference in tolerance capabilities of the two species may be a difference in the degree of a general response to cadmium stress. Further, the predicted mechanism for fitness advantage under lethal stress was observed under non-lethal stress.

CHAPTER 8

GENERAL DISCUSSION

The present study designed and developed a flow-through system and used it to investigate the tolerance capability of substrate spawning and mouth brooding tilapia species to cadmium stress. Some physiological and biochemical parameters of protein turnover and associated metabolic costs were measured to elucidate whether there is a general mechanism underlying the stress tolerance.

The observed results of the present study have several implications for the generalization of toxic responses based on laboratory bioassays for natural populations. Single-species toxicity tests have been employed to generate LC50 values, no observed effect concentrations (NOEC), maximum acceptable toxic concentrations (MATC) and to establish the damage caused by the toxicant for a large number of aquatic organisms under a variety of conditions. Because of their simplicity, low cost and potential for standardization, single-species toxicity tests play a major role in establishment of water quality criteria for metals (Clements, 1991). It is not known for certain to what extent the information based on the single-species toxicity tests can be reliably extrapolate to the higher levels of biological organization such as populations, communities and ecosystems. The communities receiving a toxicant will contain species that have not been tested for their response to that toxicant. The results of the present study revealed that tilapia as a group of fish exhibited an order of magnitude of difference in tolerance to cadmium and copper. This implies that if one fails to recognise the most sensitive species in single-species toxicity tests, the information based on such tests may not provide any protective value for the higher biological organization in the natural environment. The

description and prediction of toxic responses from single-species tests have become more difficult due to standardization. During standardization of single-species tests use of one genotype (Soares and Calow, 1993) and eliminating allometric aspects, such as size, and age (Newman and Heagler, 1991) is a common practice to increase precision and repeatability. This implies that standardization is focused on narrowing the gap between toxicity data generated from within as well as between laboratories rather than widening the extrapolation of such results to the higher biological organisation in the natural environment. Typically, predictions should be expected to decline in reliability as the extrapolation distance increases (Levin, Harwell, Kelly and Kimball, 1989). The observed differences in tolerance capability between different sizes and ages of the same life stage of the same species in the present study indicate that eliminating allometric aspects and age from toxicity tests limits their ability of prediction of toxic responses to the natural populations. However, unless we work with systems that are controlled experimentally, we can not know how to interpret the results that derive from them (Soares and Calow, 1993). Standardized community level ecotoxicological properties more acceptable for regulatory decisions concerning the potential safety of chemical releases in to the environment (Harrass and Sayer, 1989). Taub (1993) reported encouraging reproducibility of such a standardized microcosom test. Therefore, there is a need for research into multispecies tests under controlled conditions which will enhance extrapolation of experimental data to the field. One of the major unaccounted source of variance in the extrapolation of toxicity from the laboratory to the field relates to the condition of the fish under starvation or low ration (Suter,Barnthouse, Bereck, Gardner and O'Neill, 1985). The present study observed a variation in the tolerance capability among yolk sac-fry with adequate and

depleted yolk reserves further complicating predictions.

The sensitivity recorded for tilapia in terms of lethal and non-lethal responses are comparable to widely used test organisms such as salmonids (Calamari *et al.*, 1980) and *Daphnia* (Baird *et al.*, 1991). The sensitivity of tilapia to metal stress, as observed in the present study, suggests it may be used as an indicator organism in the tropics. The simplest ecotoxicity test endpoints concern direct effects on particular species that have a direct interest to humans, including species valued for economic, food, recreation or aesthetic reasons (Harwell, Harwell, Weinstein and Kelly, 1987). The economic value together with the sensitivity of tilapia to metal stress has two implications in using them as an indicator organisms. One way of using tilapia species is as indicators of metal stress. In this respect a more appropriate way to use tilapia species is to examine the structure and composition of the populations. Reduced species diversity and richness are the most frequently reported consequences of metal stress, however these may not be the most sensitive indicators (Clements, 1991). Species composition has been recorded as a more evident response to metal stress than the number of species as observed in periphyton (Say and Witton, 1981) and in macro-invertebrates (Leland, 1985). Moreover the taxonomic knowledge established for Tilapiini fish (Trewavas, 1983) is in favour of using them as indicator organisms as difficulties have been experienced in taxonomic identification in species composition of some indicator organisms with respect to the community responses to metal stress (Patrick, 1978).

The second way is to use tilapia species as the ecological endpoint. The observed

variation in both lethal and non-lethal responses to cadmium and copper stress in the present study suggests that mouth brooding tilapia are more sensitive than substrate spawning tilapia. Therefore, among tilapia, mouth brooders may play an important role to detect potential changes in the ecological endpoints. Mouth brooding tilapia such as *O. niloticus* may have an 'intrinsic importance', ie, the indicator organism is the ecological end point (Kelly and Harwell, 1989). Demonstrating changes through population levels, age structures, recruitment rates and mortality rates in mouth brooders as indicators would constitute ecological impact. Striped bass populations of the Hudson river have been used as indicators with 'intrinsic importance' for major anthropogenic disturbances (Kelly and Harwell, 1989).

Concentrations as low as 0.5 to 6.0 $\mu g l^{-1}$ cadmium (actual values 0.83 to 6.44 $\mu g l^{-1}$ respectively) had non-lethal effects on tilapia yolk sac-fry, affecting the growth rate. Cushing (1976) has hypothesised that growth rate, by changing the time of exposure to predation, may be the most important factor controlling recruitment. Thus, non-lethal concentrations of cadmium may decrease recruitment of tilapia by slowing the development of yolk sac-fry. This in turn may affect the tilapia fishery.

Those aquatic organisms accumulating metals to concentrations much greater than those found in the surrounding environment have been suggested as indicators of metal pollution (Prosi, 1979). In the present study the accumulated cadmium levels at near lethal response in tilapia yolk sac-fry were less than the exposure medium. Occurrence of lethal responses at lower burden levels than the exposure medium indicates that tilapia yolk sac-fry lack mechanism/s to detoxify accumulated metals.

The tolerance capability to metal stress has been attributed either to a pre-adapted or post-adapted mechanisms (see chapter 4). If a post adapted tolerance is not passed on to the offspring, it may not be genetically based (Klerks and Levinton, 1989). The tolerance capability to metals can be achieved either by decreasing the net rate of metal entry or by increase in the storage and detoxification of the metal through sequestering by metallothionien or metallothionein-like proteins (see chapter 6). An induction of these proteins coincides with increased tolerance when exposed to the metals (Benson and Birge, 1985) and therefore, may be considered as post-adaptive. The present study did not support such a specific detoxifying mechanism underlying the tolerance capability as the more-tolerant substrate-spawning *T. zillii* had a lower cadmium body burden than the less-tolerant mouth-brooding *O. niloticus*. The same pattern was seen for age- and size-specific effects in *O. niloticus* yolk sac-fry. Therefore, the tolerance capability in tilapia yolk sac-fry to lethal metal stress may be a pre-adaptation to reduce the net rate of metal uptake.

The rank order of sensitivity between mouth brooders and substrate spawners is the same for both cadmium and copper. Similar concordance was observed between younger and older *O. niloticus* yolk sac-fry. This suggests a general response to lethal metal stress and a similar mode of action in tilapia yolk sac-fry. Moreover, a correlation was observed between the tolerance capabilities of mouth brooders and substrate spawners, and, their early life history growth performances. Similar correlation was observed for smaller and larger *O. niloticus* yolk sac-fry between their tolerance capabilities and early life history growth performances. The differences in growth derive from differences in energy balance and energy balance reflects, in part,

differing costs of metabolic maintenance, caused by variability in the efficiency of protein synthesis (Koehn and Bayne, 1989). There was a significant variation between the less tolerant mouth brooding *O. niloticus* and more tolerant *T. zillii* yolk sac-fry for the measured physiological and biochemical parameters of protein turnover and associated metabolic costs under non-stressed (control) conditions (Chapter 7). Slow growth of *O. niloticus* was marked by low protein growth, low rate of protein synthesis efficiency and high rate of protein synthesis, and hence, higher maintenance metabolic cost. The same was true for the less tolerant larger rather than smaller *O. niloticus* yolk sac-fry. Mechanisms that increase stress tolerance may divert energy from growth under optimal conditions (Hoffmann and Parsons, 1989). Therefore, the growth performance and level of maintenance cost may be predictive of the tolerance capability to metal stress. Thus, the phenotypic variations for early life-history growth traits may have contributed to the variations in tolerance capability between mouth-brooding and substrate-spawning tilapia and between smaller and larger *O. niloticus* yolk sac-fry to metal stress. The differences between growth performances of mouth-brooding and substrate-spawning tilapia yolk sac-fry are related to their developmental rates. Even though this differences in developmental rates may not have been evolved due to stress, it may have resulted in a difference in the ability to tolerate metal lethal stress. Since stress tolerance may involve processes requiring energy expenditure (Hoffman and Parsons, 1989; Sibly and Calow, 1989) the increased resistance to cadmium noticed in the smaller yolk sac-fry in the present study compared with their larger conspecifics may possibly be due to the availability of energy to meet the required energy requirement under stress. The increased availability of metabolic energy in smaller yolk sac-fry may have been brought about by utilizing more

efficient energy production pathways. It is also known that animals utilise both oxidative and glycolytic pathways for metabolic energy production. The oxidative pathway is more efficient than the glycolysis pathway and it was recorded that with increasing body size energetically more efficient oxidative pathway decreases, as noticed in *O. mossambicus* (Bashamohideen and Parvatheswararao, 1976). Moreover, a negative relationship between oxidative enzyme activity and body weight appears to be a general rule (Houlihan *et al.*, 1993). Therefore, the difference in tolerance capability to metal lethal stress in tilapia yolk sac-fry may have been brought about by the genetically pre-determined differences in the early life history, in terms of developmental rates and growth rates.

The cadmium body burden differences between different post-hatch ages also failed to support the existence of the storage of cadmium through specific detoxification mechanisms, as more tolerant older post-hatch yolk sac-fry under cadmium lethal stress had lower body burden levels than younger post-hatch yolk sac-fry. This may be attributed to an adjustment in metabolism under depleted nutritional status to switch over to an energy saving strategy. Depleted yolk reserves in older yolk sac-fry were marked with a reduced overall metabolic activity (Chapter 5). The entry of cadmium may have reduced through the resultant decrease in respiratory flow rate and blood circulation in the gills due to general reduction in the metabolic activity. Therefore, the adjustments made in the metabolic rate to tolerate nutritional stress may have brought about the tolerance capability to metal stress.

Mouth brooding *O. niloticus* and substrate spawning *T. zillii*, which demonstrated the

most sensitive and most tolerant responses respectively, to both cadmium and copper lethal stress, were used to investigate the physiological and biochemical responses to non-lethal cadmium stress. It was assumed that, because these two differed in their lethal tolerances to cadmium and copper stress due to predicted differences in fitness advantages, under non-stress conditions (control), based on their differences in developmental rates they would also differ in their relative tolerance to non-lethal stress.

The measured physiological and biochemical parameters related to protein turnover and associated metabolic costs under non-lethal cadmium stress suggests (chapter 7), responses involving energy expenditure occurred with a consequent reduction in growth in both species. *O. niloticus* yolk sac-fry were affected at lower cadmium concentrations than *T. zillii* yolk sac-fry, confirming their lower tolerance to non-lethal cadmium exposure, as observed for lethal cadmium stress. The range over which differences in response between the two species for parameters such as growth in terms of dry weight, oxygen consumption, ammonia excretion, RNA:DNA under non-lethal cadmium stress was less than for their relative tolerance capability to lethal cadmium stress, while parameters such as protein synthesis efficiency, protein growth and O:N were more or less similar for their tolerance capability to lethal cadmium stress. Therefore, the assumption of a constant factor to relate acute to chronic effects that is made in establishing environmental standards is questionable in tilapia yolk sac-fry as it varies with the stress index. The parameters such as protein growth, protein synthesis efficiency and O:N which measure specific components of the balanced energy equation were more sensitive stress indices than parameters such as

oxygen consumption, dry weight gain and protein synthesis which reflect overall changes in several components in the balanced energy equation.

To conclude, the observed inter and intraspecific variations in stress tolerance to metals indicates just how far single species toxicity data are from ecological realism. The sensitivity of tilapia to metal stress may enable them to be used as indicator organisms for ecological endpoints or as the ecological endpoint. The present study supports a general response mechanism for tolerance to both 96 h lethal and non-lethal cadmium stress. The variation in tolerance response to metal stress in the present study was related to the variations in body burden levels and to the predicted availability of metabolic energy under non-stressed conditions to meet the required increased maintenance cost under stressed conditions. These predictions were based on genetically determined phenotypic variations for early life history traits such as growth and developmental rates. Therefore, genetically pre-determined phenotypic variations for early life history traits may translate into variations in metal stress tolerance. There was a concordance between the range over which differences in responses of the two species occur under non-lethal cadmium stress, in terms of the more sensitive stress indices, and their tolerance capability to lethal cadmium stress. Thus, there may be a possible link between responses to lethal and non-lethal cadmium stress based on a general response. The nutritional status of an animal, however, may cause a different response, but achieve a similar tolerance. The body burden level, and hence, tolerance capability was related to the reduced overall activity as a consequence of the reduced metabolic rate under starvation. Therefore, post-adapted physiological acclimation to starvation stress carries a fitness advantage

for metal stress.

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Appendix 1 Fish species used in early life stage toxicity tests.

Fresh water fish

Common name	Species neme	Reference
Brook trout	*Salvelinus fontinalis*	Christensen, 1975; Trojnar, 1977b; Eaton *et al.*, 1978; Mckim *et al.*, 1978; Kwain and Rose, 1985.
Brown trout	*Salmo trutta*	Johanson *et al.*, 1977; Eaton *et al.*, 1978; McKim *etal.*, 1978.
Channel catfish	*Ictalurus punctatus*	Carlson *et al.*, 1974; Birge *et al.*, 1979.
Coho salmon	*Oncorhynchus kisutch*	Halter and Johnson, 1974; Eaton, 1978.
Common carp	*Cyprinus carpio.*	Vladimirov, 1969; Kapur and Yadav, 1982.
Fathead minnow	*Pimephales promelas*	Pickering and Vigor, 1965; Carlson, 1971; Mckim *et al.*, 1975; Call *et al.*,1987.
Garpike	*Esox lucius*	Hakkila and Niemi, 1973; Johansson and Kihlstrom, 1975; McKim *et al.*, 1975; von Westernhagen *et al.*, 1975; McKim *et al.*, 1978.
Guppy	*Poecilia reticulata.*	Uviovo and Beatty, 1979.
Goldfish	*Carassius auratus.*	Birge *et al.*, 1979.
Lake herring		McKim *et al.*, 1978.
Lake trout	*Salmo namaycush.*	Eaton *et al.*, 1978; McKim *et al.*, 1978.
Largemouth bass	*Micropterus salmonides*	Carlson and Seifert, 1974; Birge *et al.*, 1979.
Japanese medaka	*Oryzias latipes.*	Aoki, 1978; Dial, 1978; Michibata *et al.*, 1981; Soloman and Faustman. 1987.
Pink salmon	*Oncorhynchus gobuscha*	Servizi and Martens, 1978.
Rainbow trout,	*Oncorhynchus mykiss.*	Giles and Klaverkamp, 1971; McKim *et al.*, 1978; Birge *et al.*, 1979.
Smallmouth bass	*Micropterus dolomieu*	McKim *et al.*, 1975; Eaton *et al.*, 1978; McKim *et al.*, 1978.
Sockeye salmon	*Oncorhynchus nerka*	Servizi and Martens, 1978.

White sucker.	*Catostomus commersoni*	Mckim *et al.*, 1975; Trojnar, 1977a; Eaton *et al.*, 1978; McKim *et al.*, 1978.
Tilapia	*Oreochromis mossambicus*	Pandya and Rao, 1986.
Zebra fish*	*Brachydanio rerio.*	Johansson *et al.*, 1973; Speranza *et al.*, 1977; Ozoh, 1980; Dave, 1985.
Salt water fish.		
Atlantic salmon	*Salmo salar.*	Daye and Garside, 1980; Peterson *et al.*, 1980; Haya and Waiwood, 1981; Rombough and Garside, 1982; Peterson and Martin-Robichaud, 1983.
Baltic herring	*Clupea harengus membrass*	Rosenthal and Sperling, 1974; Ojaveer *et al.*, 1980.
Black Sea flounder	*Platichthys flesus luscus*	Mazmanidi and Bazhasvili, 1975.
California grunion	*Leuresthes tenuis*	Winkler *et al.*, 1983; Goodman *et al.*, 1985b.
Cod	*Gadus morhua.*	Dethlefsen, 1977; Swedmark and Granmo, 1981; Kijorsvik, *et al.*, 1982.
Flounder	*Platichthys flesus.*	von Westernhagen and Dethlefsen, 1975.
Herring	*Clupea harengus.*	Rosenthal and Sperling, 1974; von Westernhagen, *et al.*, 1974; Somasundaram *et al.*, 1984.
Killifish	*Fundulus heteroclitus*	Weis and Weis, 1977; Middaugh and Dean, 1977; Sharp and Neff, 1980, 1982.
Menidia species.		Voyer *et al.*, 1979; Goodman *et al.*, 1983; Goodman *et al.*, 1985a.
Pacific herring	*Clupea harengus pallasi*	Mounib *et al.*, 1976; Struhsaker, 1977; Rice and Harrison, 1978; Alderdice *etal.*, 1979 a,b; Smith and Cameron, 1979.
Perch	*Perca fluviatilis*	Runn *et al.*, 1977; Rask, 1983.
Sea bass	*Dicentrarchus labrax*	Cosson and Martin, 1981.
Sheepshead minnow	*Cyprinodon variegatus*	Schimmel *et al.*, 1974; Weis and Weis, 1976. McCulloch and Rue, 1989.
Walleye pollock	*Theragra chalcogramma*	Hulsman *et al.*, 1983; Carls and Rice, 1984.
Winter flounder	*Pseudopleuronectes americanus*	Smith and Cole, 1973; Middaugh and Dean, 1977; Voyer *et al.*, 1977, 1982.

* Used in both fresh and salt water.

APPENDIX: II Summary of sensitive responses in fish early life stage toxicity tests corresponding to non-lethal cadmium concentration

Species	Common name	Early life stage	Concentration ($\mu g l^{-1}$)	End point	REFERENCE
Clupea harengus pallasi	Pacific herring	egg	1000	chorion strength	Alderdice *et al.*, 1979a
Clupea harengus pallasi	Pacific herring	embryo	1000	morphology	Alderdice *et al.*, 1979b
Clupea harengus	herring	embryo	1500	embryonic activity hatching success	von Westernhagen *et al.*, 1974
Esox lucius	pike	embryo	>500	embryonic activity	von Westernhagen *et al.*, 1975
Clupea harengus	Pacific herring	embryo	10,000	biochemical hatching success	Mounib *et al.*, 1976
Cyprinus carpio	Common carp	embryo		hatching success	Vladimirov, 1969
Clupea harengus	herring	embryo	10,000	Hatching success	Rosenthal and Sperling, 1974
Oncorhynchus nerka	sockeye salmon	embryo	174	hatching success	Servizi and Martens, 1978
Oncorhynchus mykiss	rainbow trout	embryo	124	hatching success	Woodworth and Pascoe, 1982
Esox lucius	pike	embryo	2000	hatching success	von Westernhagen *et al.*, 1975
Clupea harengus	herring	embryo		hatching success	von Westernhagen *et al.*, 1979
Salmo salar	Atlantic salmon	embryo	>750	hatching success	Voyer *et al.*, 1979
Pimephales promelas	fathead minnow	embryo	>57	hatching success	Pickering and Gast, 1972
	flagfish	embryo	31	hatching success	Spehar, 1976
Salmo salar	Atlantic salmon	embryo	270	hatchability	Rombough and Garside, 1982

Clupea harengus membrass	Baltic herring	embryo	500	hatchability	Ojaveer *et al.*, 1980
Clupea harengus	herring	embryo	100-1000	hatchability	Rosenthal and Sperling, 1974
Platichthys flesus americanus	flounder	embryo	5000	hatchability	von Westernhagen and Dethlefsen, 1975
Pseudopleuronectes americanus	winter flounder	embryo	1000	hatchability	Voyer *et al.*, 1982
Pseudopleuronectes americanus	flounder	embryo	2100	hatchability	Voyer *et al.*, 1977
Orizias latipes	medakafish	embryo	60	hatchability	Aoki, 1978
Orizias latipes	medakafish	yolk sac-fry[c]	60	histopathology	Aoki, 1978
Clupea harengus	herring	yolk sac-fry[b]	1000	growth	Rosenthal and Sperling, 1974
Clupea harengus membrass	Baltic herring	yolk sc-fry[b]	3000	growth	Ojaveer *et al.*, 1980
Clupea harengus	herring	yolk sac-fry[b]	500-1000	growth	von Westernhagen *et al.*, 1974
Lepomis macrochirus	bluegill	yolk sac-fry[b]	800	growth	Eaton, 1974
Salvelinus fontinalis	brook trout	yolk sac-fry[b]	3-7	growth	Christensen, 1975
Salvelinus fontinalis	brook trout	yolk sac-fry[b]	1-4	growth	Eaton *et al.*, 1978
Salmo trutta	brown trout	yolk sac-fry[b]	4-12	growth	Eaton *et al.*, 1978
Oncorhyncus kisutch	coho salmon	yolk sac-fry[b]	4-12	growth	Eaton *et al.*, 1978
Esox lucius	pike	yolk sac-fry[b]	4-13	growth	Eaton *et al.*, 1978
Micropterus dolomieu	smallmouth bass	yolk sac-fry[b]	4-13	growth	Eaton *et al.*, 1978
Catostomus commersoni	white sucker	yolk sac-fry[b]	4-12	growth	Eaton *et al.*, 1978
Oncorhyncus kisutch	coho salmon	yolk sac-fry	1.3-3.4	growth	Eaton *et al.*, 1978
Salvelinus fontinalis	brook trout	yolk sac-fry[b]	3-6	growth	Sauter *et al.*, 1976

Oncorhyncus mykiss	rainbow trout	yolk sac-fry[b]	124	growth	Woodworth and Pascoe, 1982
Clupea harengus pallasi	Pacific herring	embryo		metabolism	Alderdice *et al.*, 1979b
Clupea harengus	herring	yolk sac-fry[b]	5000-10000	metabolism	Rosenthal and Sperling, 1974
Leiostomus xanturus	larval spot	yolk sac-fry[c]		metabolism	Middaugh *et al.*, 1975
Salmo salar	Atlantic salmon	yolk sac-fry[b]	5	growth, metabolism	Rombough and Garside, 1982
Salmo salar	Atlantic salmon	yolk sac-fry	2	growth, metabolism	Peterson *et al.*, 1983
Clupea harengus membrass	Baltic herring	yolk sac-fry[b]	50	morphology	Ojaveer *et al.*, 1980
Clupea harengus	herring	yolk sac-fry[b]		morpohology	Rosenthal and Sperling, 1974
Clupea harengus	herring	yolk sac-fry[b]	>1000	morphology	von Westernhagen *et al.*, 1974
Lepomis macrochirus	bluegill	yolk sac-fry[b]	80	morphology, behaviour	Eaton, 1974
Salmo salar	Atlantic salmon	yolk sac-fry[b]	300	morphology	Rombough and Garside, 1982
Pseudopleuronectes americanus	winter flounder	yolk sac-fry[b]	>340	morphology	Voyer *et al.*, 1977
Clupea harengus	herring	yolk sac-fry[b]	1000-2000	morphology	von Westernhagen *et al.*, 1974
Belone belone	garpike	yolk sac-fry[b]	1000-2000	morphology	von Westernhagen *et al.*, 1975
Pseudopleuronectes americanus	winter flounder	yolk sac-fry[b]	100	behaviour	Voyer *et al.*, 1982
Salmo salar	Atlantic salmon	yolk sac-fry[c]	>0.8	morphology	Rombough and Garside, 1984
Jordanella floridae	flagfish	yolk sac-fry[c*]	17-31	biochemical	Spehar, 1976
Salvelinus fontinalis	brook trout	yolk sac-fry[b]	3.4-0.7	biochemical	Christensen, 1975

a- From parents exposed to cadmium; b-From cadmium treated eggs; c- From untreaded eggs
*- Exposed with other metals

Appendix III The chemical composition used for reconstituted dilution water (ASTM 1980)

Water type (mgl^{-1})	Compound	Chemical formular	Required amount
Soft water	sodium bicarbonate	$NaHCO_3$	48.00
	calcium sulphate	$CaSO_4.2H_2O$	30.00
	magnisium sulphate	$MgSO_4$	30.00
	potassium chloride	KCl	2.00

Water quality	pH	Hardness (mg $CaCO_3$ l^{-1})	Alkalinity (mg $CaCO_3$ l^{-1})
	7.2-7.6	40.00-48.00	30.00-35.00