

SPERM ACTIVATION IN NILE TILAPIA *OREOCHROMIS*
NILOTICUS AND THE EFFECTS OF ENVIRONMENTALLY
RELEVANT POLLUTANTS ON SPERM FITNESS

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**SPERM ACTIVATION IN NILE TILAPIA *OREOCHROMIS NILOTICUS*
AND THE EFFECTS OF ENVIRONMENTALLY RELEVANT
POLLUTANTS ON SPERM FITNESS**

by

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**UNIVERSITY OF
STIRLING**

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Dedicated to my late father who passed
away on December 18, 2006.

Declaration

I declare that this thesis has been compiled by myself and is the result of my own scientific investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Signature

Signature of supervisor

Date

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CONTENTS

Contents	iii
List of Figures	vii
List of Tables	ix
Abstract	x

CHAPTER 1 INTRODUCTION, LITERATURE REVIEW AND AIMS OF STUDY

1.1	Introduction	1
1.2	Gamete quality and fertilization rate	2
1.2.1	Fish sperm quality	3
1.2.2	Spermatogenesis in fish	4
1.2.3	Morphology of fish spermatozoa	6
1.3	Behavior of fish spermatozoa: mechanism of activation	10
1.3.1	Sperm motility	12
1.3.2	Seminal fluid and osmolality	14
1.3.3	Seminal fluid and ionic composition	18
1.3.4	Seminal fluid and pH	21
1.4	Pollution in aquatic environment	23
1.4.1	Cadmium and toxicity	24
1.4.2	Pesticides and toxicity	28
1.4.2.1	Malathion	32
1.4.2.2	Rotenone	35
1.5	Nile tilapia	36
1.6	Problem statements	37
1.7	Aims of study	38

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1	Procurement of male fishes	39
2.1.1	Collection of seminal fluid	39
2.1.2	Assessment of seminal fluid quality	40

2.1.3	Estimation of sperm density	40
2.1.4	Measurement of sperm motility	41
2.1.4.1	Motility score	41
2.1.4.2	Computer-aided sperm analysis	41
2.1.5	Measurement of seminal plasma osmolality and pH	45
2.1.6	Determination of ionic composition in seminal plasma	45
2.1.7	Sperm morphology	45
2.2	Preparation of experimental solutions	46
2.2.1	Ion channel studies	46
2.2.1.1	Lanthanum	46
2.2.1.2	Flunarizine	46
2.2.1.3	Amiloride	47
2.2.1.4	Quinine	47
2.2.1.5	Ouabain	47
2.2.2	Pollutant studies	48
2.2.2.1	Cadmium	48
2.2.2.2	Malathion	48
2.2.2.3	Rotenone	48
2.3	General statistical analyses	49

CHAPTER 3 SPERM QUALITY OF NILE TILAPIA

3.1	Introduction	50
3.1.1	Sperm quality in fish	51
3.1.2	Sperm motility score	53
3.1.3	Computer-aided sperm analysis (CASA)	53
3.1.4	Mechanism of fish sperm activation	54
3.1.5	Morphology of fish spermatozoa	56
3.1.6	Aims of study	57
3.2	Materials and methods	59
3.2.1	Physiochemical characteristics of seminal fluid	59
3.2.1.1	Seminal plasma osmolality	59
3.2.1.2	Density of spermatozoa	59

3.2.1.3	Ionic composition	59
3.2.2	Effect of seminal fluid stripping frequency on osmolality and sperm density	60
3.2.3	Sperm motility assay	60
3.2.3.1	Effect of dilution rate on sperm motility	61
3.2.3.2	Effect of osmolality of non-electrolytes and electrolytes solution on sperm motility	62
3.2.3.3	Effect of pH on sperm motility	62
3.2.4	Effect of membrane ion channel inhibitors on sperm motility	62
3.2.5	Effect of activation on sperm morphology	62
3.2.6	Statistical analyses	64
3.3	Results	66
3.3.1	Physiochemical characteristics of seminal fluid	66
3.3.2	Effect of stripping frequency on seminal plasma osmolality and sperm density	68
3.3.3	Effect of dilution rate on sperm motility	70
3.3.4	Effect of osmolality of non-electrolytes and electrolytes solution on sperm motility	74
3.3.5	Effect of pH on sperm motility	78
3.3.6	Effect of membrane ion channel inhibitors on sperm motility	80
3.3.6.1	Effect of lanthanum on sperm motility	80
3.3.6.2	Effect of flunarizine on sperm motility	82
3.3.6.3	Effect of amiloride on sperm motility	84
3.3.6.4	Effect of ouabain on sperm motility	84
3.3.6.5	Effect of quinine sperm motility	84
3.3.7	Effect of activation on sperm morphology	84
3.4	Discussion	94
CHAPTER 4 POLLUTANTS AND THEIR EFFECT ON SPERM MOTION AND MORPHOLOGY		
4.1	Introduction	104
4.1.1	Heavy metals and pesticides	106

4.1.2	Aims of study	109
4.2	Materials and methods	111
4.2.1	Effect of cadmium on sperm motility	111
4.2.2	Effect of malathion on sperm motility	111
4.2.3	Effect of rotenone on sperm motility	112
4.2.4	Effect of cadmium, malathion and rotenone on sperm morphology	113
4.2.5	Hobson sperm tracker and sperm motility analysis	114
4.2.6	Statistical analyses	115
4.3	Results	116
4.3.1	Effect of cadmium on sperm motility	116
4.3.2	Effect of cadmium on sperm morphology	121
4.3.3	Effect of malathion on sperm motility	121
4.3.4	Effect of malathion on sperm morphology	125
4.3.5	Effect of rotenone on sperm motility	125
4.3.6	Effect of rotenone on sperm morphology	129
4.4	Discussion	130
CHAPTER 5 GENERAL CONCLUSIONS		137
Reference list		

List of Figures

Figure	Page
1.1 Diagrammatic representation of type 1 sperm with flagellum perpendicular to the nucleus and type 2 sperm with flagellum parallel to the nucleus	8
1.2 A diagram represents a longitudinal section of fish spermatozoa	8
1.3 A diagram illustrates sperm motility descriptors generated by sperm tracker (Boyers et al., 1989)	16
2.1 A schematic diagram of equipments used for computer-aided sperm analysis (CASA)	43
3.1 Effect of dilution rate on sperm motility score, determined at 10 sec after activation	71
3.2 Effect of tank water and deionised distilled water on sperm motility score and motility duration determined until 540 sec	71
3.3 Effect of dilution rate on sperm motility variables determined at 5 sec after activation	73
3.4 Effect of tank water and deionised distilled water on sperm motility variables determined at 5 sec after activation	73
3.5 Effect of osmolality of mannitol on sperm motility score, determined at 10 sec after activation	75
3.6 Effect of osmolality of modified fish ringer's on sperm motility score, determined at 10 sec after activation	75
3.7 Effect of osmolality of mannitol on sperm motility variables, determined at 5 sec after activation	77
3.8 Effect of osmolality of modified fish ringer's on sperm motility variables, determined at 5 sec after activation	77
3.9 Effect of pH on sperm motility score, determined at 5 sec after activation	79
3.10 Effect of pH on sperm motility variables, determined at 5 sec after activation	79
3.11 Frequency (percentage) of fish spermatozoa in data set classified as PATN group 1 in relation to lanthanum treatment	81
3.12 Frequency (percentage) of fish spermatozoa in data set classified as PATN group 1 in relation to flunarizine treatment	83
3.13 SEM imagery of <i>Oreochromis niloticus</i> spermatozoa	86
3.14 TEM imagery of <i>Oreochromis niloticus</i> spermatozoa. Longitudinal sagittal section of the spermatozoa showing sleeve, mitochondria, flagellar axis, nucleus and cytoplasmic membrane	86
3.15 TEM imagery of <i>Oreochromis niloticus</i> spermatozoa. Longitudinal sagittal section of the spermatozoa showing mitochondria, flagellum and cytoplasmic channel	87
3.16 TEM imagery of <i>Oreochromis niloticus</i> spermatozoa. Cross section of the flagellum showing lateral fins, peripheral and central microtubules	87
3.17 SEM imagery of <i>Oreochromis niloticus</i> spermatozoa at 30 sec of activation	89
3.18 TEM imagery of longitudinal sagittal section of <i>Oreochromis niloticus</i> spermatozoa at 30 sec of activation	89

3.19	SEM imagery of <i>Oreochromis niloticus</i> spermatozoa at 60 sec of activation	90
3.20	TEM imagery of longitudinal sagittal section of <i>Oreochromis niloticus</i> spermatozoa at 60 sec of activation	90
3.21	SEM imagery of <i>Oreochromis niloticus</i> spermatozoa at 120 sec of activation.	91
3.22	TEM imagery of <i>Oreochromis niloticus</i> spermatozoa at 120 sec of activation	91
3.23	SEM imagery of <i>Oreochromis niloticus</i> spermatozoa at 480 sec of activation	92
3.24	TEM imagery of longitudinal sagittal section of <i>Oreochromis niloticus</i> spermatozoa at 480 sec of activation	92
4.1	Interaction plot of frequency of spermatozoa classified as PATN group 1 and 2 in relation to cadmium treatment and pre exposure at 0, 5 and 15 mins	119
4.2	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to cadmium treatment	120
4.3	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to the period of pre exposure to cadmium	120
4.4	Interaction plot of frequency of spermatozoa classified as PATN group 1 and 2 in relation to malathion treatment and pre exposure at 0, 5 and 15 mins	122
4.5	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to malathion treatment	124
4.6	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to the period of pre exposure to malathion	124
4.7	Interaction plot of frequency of spermatozoa classified as PATN group 1 and 2 in relation to rotenone treatment and pre exposure at 0, 5 and 15 mins	127
4.8	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to rotenone treatment	128
4.9	Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to the period of pre exposure to rotenone	128

List of Tables

Table	Page
1.1 Definitions of sperm motility descriptors generated by Hobson sperm tracker	15
1.2 Summary of world pesticide markets in 2000 and 2001	29
1.3 Relative toxicities to various fish species exposed to malathion	34
2.1 Motility score for sperm motility measurement	41
2.2 Gate parameters of Hobson Sperm Tracker	44
2.3 Features of each element for PERKIN ELMER 2280 for flame spectrophotometry	45
3.1 Sperm quality biomarkers in fish species	52
3.2 Seminal fluid characteristic of <i>Oreochromis niloticus</i>	67
3.3 Mean (\pm s.d.) comparison of major ion composition (mM) in various types of seminal fluid compartment	67
3.4 Mean (\pm s.d.) values of seminal plasma osmolality and sperm density (per ml) during sequential stripping from tilapia	69
3.5 Mean (\pm s.d.) values of seminal plasma osmolality, sperm density (per ml) and fish size during sequential stripping of 8-10 days from tilapia sampled from January 2005 to June 2005	69
3.6 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of lanthanum	81
3.7 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of flunarizine	83
3.8 Mean (\pm s.d.) diameter of spermatozoa mitochondria and nucleus of <i>Oreochromis niloticus</i> as observed by transmission electron microscopy (TEM)	93
4.1 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of cadmium	119
4.2 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of malathion	122
4.3 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of rotenone	127

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Abstract

In externally fertilizing fishes, multiple factors of the spawning environment may affect the sperm viability, and thus the fertilization rate. In this thesis, the sperm activation effect of osmolality of non-electrolytes and electrolytes activation media, pH and ion channel inhibitors on Nile tilapia, *Oreochromis niloticus*, and the effect of environmentally relevant pollutants (cadmium, malathion and rotenone) on sperm fitness (motility and morphology) were investigated.

Seminal fluid samples collected from male fishes (200-250g) were subjected to activation treatments, then analyzed for sperm motility using motility score, and motility variables using Hobson sperm tracker for straight line velocity (VSL), beat cross frequency (BCF) and percentage of motile cells (MOT). For the ion channel inhibitors and pollutants, the effect on sperm motility variables of VSL, VCL (curvilinear velocity) and LIN (linearity) were determined. Multivariate analysis was also carried out to determine the effects of ion channel inhibitors and pollutants on sperm subpopulations. The effects of pollutants on sperm morphology were observed using microscopy techniques, namely, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Sperm motility was initiated when the sperm were exposed to hypoosmotic electrolytes and non-electrolytes solution. We also found that sperm show optimal activity at pH range of 6-8 which depicts that the effect of pH on sperm motility is negligible. Lanthanum (calcium channel blocker) and flunarizine (sodium-calcium exchanger pump blocker) were found to inhibit sperm motility at 25 and 5 μ M, respectively, suggesting that both ion channels play a significant role in sperm activation in *O. niloticus*. In contrast amiloride, ouabain and quinine showed no effects on activation, indicating that epithelial sodium channels, sodium-potassium ATPase and voltage gated potassium channels respectively are unlikely to have major roles in sperm activation or motility. The spermatozoa of *Oreochromis niloticus* were uniflagellate with clearly differentiated oval-shaped head, midpiece and flagellum. Sperm exposed to hypoosmotic shock showed swelling of the midpiece and sleeve structure.

The pollutants showed dose- and time-dependent effect on sperm motility of the fast linear sperm subpopulation. Sperm morphology was not affected. Sperm motility was

inhibited at 0.44, 0.03 and 0.063 μM , cadmium, malathion and rotenone respectively. Both cadmium and malathion exerted effects very quickly after exposure. The effect of cadmium, which can exert toxicity by calcium antagonism, is consistent with the effects of calcium channel blockers and further supports an important role for calcium in sperm activation and motility. Malathion had effects at relatively low, environmentally relevant concentrations, suggesting the presence of functionally important acetylcholinesterase activity in sperm, and also the presence of activation cytochrome P450 activity. Rotenone, a well known mitochondrial poison, affected motility only after 15 min of pretreatment. The alteration of sperm trajectories in fast linear spermatozoa subpopulation by pollutants at submicromolar concentrations as demonstrated in our study implies potentially serious consequences for fish populations in polluted environments. Furthermore the results indicate that fish sperm motility as assessed by CASA could be an ecologically relevant, sensitive, and ethically acceptable method for toxicity testing in environmental risk assessment.

CHAPTER 1 INTRODUCTION, LITERATURE REVIEW AND AIMS OF STUDY

1.1 Introduction

One of the major constraints on the future development of aquaculture is the availability of high quality gametes, both sperm and eggs (Bromage, 1995; Bromage and Roberts, 1995). As early as the 1950s, research on fish sperm had considerably expanded, dealing with a much wider range of species, with sperm cells biology, morphology, motility, energetic requirements, and the biochemistry of the seminal fluid. However, information on sperm cell biology on either freshwater fish species or marine species is still limited and many uncertainties exist, with critical deficiencies in our knowledge of the mechanisms of sperm activation and sperm-environment interactions.

Problems constantly encountered by fish culturists include variable and unpredictable levels of survival, and growth and quality of larvae and fry. Since greater emphasis is placed on the study of female broodstock, with an interest in fertilization and embryogenesis, these problems have been more or less explained by low quality of female gametes. In these respects, research efforts on male gamete quality for maximising sperm fitness and reproductive success should be given equal effort. Currently, many issues on biology and physiology of the fish sperm such as the mechanism of sperm cell activation remain unexplored. It is important to carry out such studies, as it will improve the quality and quantity of fry produced. Further, there is a need to concentrate studies on sperm quality in fish species of economic interest. The tilapias are one such group, where only limited studies have been carried out on the euryhaline species, *Oreochromis mossambicus* (Morita et al., 2004).

The production of viable fry depends upon the quality of the gametes produced by the broodstock and even if fertilization has occurred, arrest of embryo developmental can still be observed. Although embryos and larvae are the most sensitive stages in the life cycle of teleosts, there are differences in susceptibility among developmental stages particularly at earlier life stages. Whilst a full embryo-larval developmental test is conducted for assessment of reproductive toxicity or impairment, it would be extremely beneficial and cost effective to utilize a simpler short-term assessment procedure. In animal species, such as fish which are adapted to a highly variable environment, and unlike higher vertebrates, high fecundities are preferred. In such circumstances, the quality of gametes, survival of the early life stages and the presence of prey and predators are the key factors determining the reproductive success. Therefore, experimental laboratory based studies are essential for understanding the mechanistic basis of environmental effects on reproduction. The quality of gametes produced are commonly measured by determination of fertilization rate, although there are many other factors which may compromise the quality of gametes. Whilst both types of gametes contribute equally to fertilization success, the majority of studies have been confined to determination of egg quality rather than sperm quality, simply because the eggs are larger and easy to handle. In contrast, male gametes are produced in larger number, but have a very short life span, typically a few minutes, and thus sperm fitness can be a major consideration in reproductive success.

1.2 Gamete quality and fertilization rate

The reproductive success of animals is dependent upon their survival to reproductive age and the normal functioning of their reproductive pathways (Redding and Patino, 1993). Whilst understanding the requirements for development in early life stages will

lead to optimum reproductive efficiency, research efforts particularly on the production of gametes and seed stocks of good quality, are needed for sustainable aquaculture. Further improvement of gamete quality is essential for cost efficient and reliable production and genetic selection of seedstock. However, any breakthrough in achieving these objectives will be only possible if basic information on the reproductive physiology of cultivated species as well as laboratory fishes is available. It is also vitally important to realize that much of the existing information on the fundamental principles of reproductive physiology has emerged from studies on fishes which have no economic or commercial importance, such as in medaka, *Oryzias latipes* (Yamamoto, 1969) and zebrafish, *Danio rerio* (Laan et al., 2002).

1.2.1 Fish sperm quality

Research in the past has focussed on sperm count as the single measurement to determine male reproductive health. Although sperm count is an important measure, there is a need to examine semen quality as a whole. Sperm quality generally refers to the ability of sperm to successfully fertilise an egg (Rurangwa et al., 2004). Sperm quality can vary between individuals and within an individual of the same species (Rana, 1995). In fish, sperm quality may depend on several factors including the husbandry environment, feeding regime as well as quality of the feed, broodstock condition and genetic variability, and also the methods utilized for artificial spawning (Kime and Nash, 1999; Rurangwa et al., 2004). Most biomarkers for assessment of fish sperm quality are associated with fertilization rate and include sperm cell density, osmolality and pH of seminal plasma, chemical composition of seminal plasma, sperm motility, sperm morphology, fertilising capacity, enzyme content and energy reserves i.e adenosine triphosphate concentration, ATP (Plouidy and Billard, 1982; Billard and

Cosson, 1992; Billard et al., 1995; Perchec et al., 1996; Fauvel et al., 1998; Lahnsteiner et al., 1998).

In fish species with external fertilization modes, sperm cells which are immotile in the testis become instantly motile upon contact with the external medium in which fertilization takes place. Therefore, osmotic pressure, pH or ionic content of the fertilization medium may influence the motility of fish spermatozoa in either freshwater or marine fish species (Nayyar and Sundararaj, 1969, 1970; Hines and Yashouv, 1971; Billard and Cosson, 1992; Cosson et al., 1999).

Estimation of spermatozoa motility is the most common method used to assess sperm cells quality and viability (Stoss and Holtz, 1983). Motility estimates are defined as a measurement of the percentage of motile sperm cells (Stoss, 1983), the total duration of sperm cell movement (Baynes et al., 1981) or a combination of both parameters. In most cases, a motility score is determined, scored from 0-5 or 1-10 (Suquet et al., 1992; Fauvel et al., 1999). The duration of sperm cell movement varies among fish species, although it is generally no longer than a couple of minutes (Stoss, 1983). One major constraint is that this conventional motility estimate is somewhat subjective and the criterion used to define sperm motility varies between studies. Thus, the resulting scores show large variation, and are difficult to compare. Recently, the introduction of computer-aided sperm analysis (CASA) for sperm motility assessment has become popular due to its rapidity, simplicity, repeatability and objectivity.

1.2.2 Spermatogenesis in fish

In teleosts, the male reproductive organ, the testis, is an elongated structure in the peritoneal cavity which is prolonged posteriorly by a sperm duct, the vas deferens, which terminates the genital papilla that lie posterior to the rectum. The testes are paired in most teleosts, although in some species such as Poeciliids, testes of single sac are observed (Nagahama, 1983). The structure of the teleost testes may also vary from species to species, but two main types can be identified; the lobular and the tubular type. The lobular is the most common type found in teleosts, and consists of a number of separated lobules. Within the lobules, primary spermatogonia undergo numerous mitotic divisions to produce cysts, containing several spermatogonia cells. At maturation, all the germ cells within a cyst are at a similar stage of development. These cysts then rupture and release the sperm into the lobular lumen, connected to the sperm duct. In general, spermatogenesis in fish takes place in cysts within the seminiferous tubules. The spermatogenic cysts form when Sertoli cells enclose a single primary spermatogonium (Pudney, 1993). The germ cells derived from a single primary spermatogonium then divide synchronously to constitute an isogenic germ cell clone that is bordered by the cytoplasmic extensions of a single layer of Sertoli cells. Spermiation, which is the release of mature germ cells by Sertoli cells, is achieved by rupture of the cysts (Nagahama, 1983). Many fish species grow throughout life, which require Sertoli cell proliferation as a basis for allometric testis growth (Schulz et al., 2004). Thus, Sertoli cell proliferation is the primary factor which is responsible for the testis growth and the increase in sperm production in adult fish. In Nile tilapia, the meiotic phase of spermatogenesis is critically sensitive to temperature changes. A temperature of 20°C promoted spermatogenic arrest at late pachytene spermatocytes, whereas at 30°C spermatogenesis was dramatically accelerated (Vilela et al., 2003). After mitotic divisions during spermatocytogenesis, the spermatogonia are transformed into primary

spermatocytes and undergo a meiotic division producing two daughter cells called secondary spermatocytes. The secondary spermatocytes transform into haploid spermatids through a second meiotic division. The spermatids then undergo a maturation process before they can serve as functional male gametes, the spermatozoa. Each of these processes during a spermatogenic cycle has their own regulatory mechanisms.

Two types of spermatogenesis in teleosts have been identified (Mattei et al., 1993). Within the cystic type, the breakdown of the cytoplasmic bridges of germ cells occurs within cysts. This cystic type of synchronous spermatogenesis can be observed in most teleosts. Another type is rarely found and has been called semicystic where the breakdown of cytoplasmic bridges is completed outside the cysts that apparently leads to asynchronous spermatogenesis. The occurrence of semicystic spermatogenesis has been reported in Scorpaenidae (Munoz et al., 2002) and Ophidiidae (Hernandez et al., 2005).

The principal control over these events is exerted by the androgens released by the testes and stimulated by pituitary gonadotropins (Exley and Philips, 1988). In addition, the duration of spermatogenesis has been determined in a few teleost species and found to vary between species. For instance, the duration of spermatogenesis in *Oreochromis niloticus* and *Danio rerio* was 10-11 days (Vilela et al., 2003) and 6 days (Leal et al., 2009), respectively.

1.2.3 Morphology of fish spermatozoa

Spermatozoa of fish show a more diverse morphology than those of other vertebrates, and their ultrastructure can thus provide parameters for phylogenetic analysis (Jamieson and Leung, 1991; Mattei, 1991). Both light and electron microscopy have illustrated

that morphological differences are found among spermatozoa of teleost species (Jamieson, 1991; Gwo et al., 2004). Perciformes, for instance, displays a great variety of spermatozoon types, structures and spermiogenesis processes (Jamieson, 1991). Two types of fish spermatozoa have been classified according to the position of the flagellar axis due to nuclear rotation (Mattei, 1970; Jamieson, 1991). Type I consist of spermatozoa where the flagellar axis is perpendicular to the nucleus and in type II, the flagellar axis is parallel to the nucleus (Figure 1.1). Both types are present in Perciformes (Jamieson, 1991).

Most spermatozoa form and function is influenced by reproductive anatomy of the female, reproductive strategies and/or the fertilization environment (Jamieson, 1991; Gomendio and Roldan, 1993; Pitnick et al., 1999; Miller and Pitnick, 2002) and should therefore be subject to natural selection. In externally fertilising fishes, where egg and sperm are shed into ambient water, spermatozoa are generally produced in great profusion with a simple morphology and a brief period of motility. In contrast, sperm of internally fertilizing fishes, being transferred into the female tract, are generally produced in smaller quantities but with a more elaborate morphology and greater energy stores that allow for a longer period of motility (Billard and Cosson, 1990). Spermatozoa size of internally fertilizing species are generally longer than those of externally fertilizing species and among the external fertilizers, the duration of sperm motility is related to egg size (Stockley et al., 1996).

Generally spermatozoa comprise three major compartments (Figure 1.2): (i) the sperm head containing the nucleus, involved in sperm-oocyte interaction; (ii) the midpiece

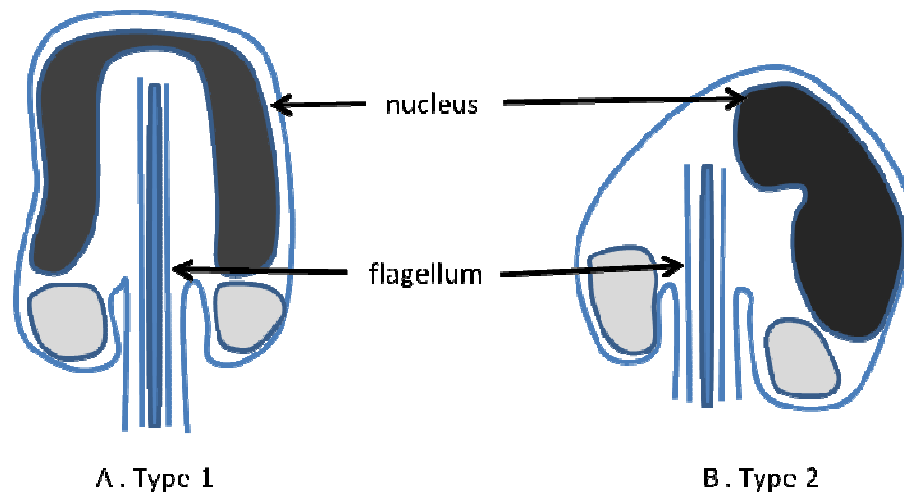


Figure 1.1. Diagrammatic representation of (A) type 1 sperm with flagellum perpendicular to the nucleus and; (B) type 2 sperm with flagellum parallel to the nucleus

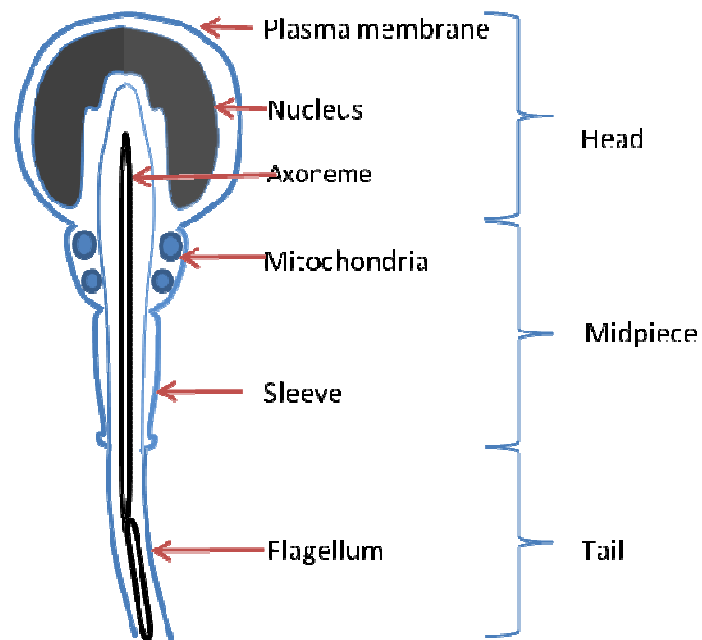


Figure 1.2 A diagram represents a longitudinal section of fish spermatozoa

with mitochondria, involved in energy production; and (iii) the tail or flagellum, involved in the cell motility. All these components are surrounded by a continuous plasma membrane, composed of proteins and lipids, mainly phospholipids and cholesterol. In contrast with mammals, the absence of acrosome can be explained by the presence of micropyle, a funnel shaped structure on the animal pole of the egg that allows the entry of sperm cell to fuse with the egg (Bromage and Roberts, 1995). However, some authors have argued that the absence of acrosome is not associated with the presence of micropyle, since in less derived fishes (Cruz-Landim et al., 2003) and lower phylogenetic groups, for instance, in insects, both an acrosome and a micropyle are present (Chawanji et al., 2005; Alves et al., 2006).

In teleosts the structure of spermatozoa also varies with mode of fertilization. In many fish species with external fertilization spermatozoa morphology is uncomplicated in comparison with those that employ internal fertilization for their reproductive strategy. The sperm head of Perciformes, is round or ovoid with a maximum diameter of 1.5–2.0 μm (Jamieson, 1991). Elongated sperm heads are observed in sturgeon, paddlefish and eel (Marco-Jiminez et al., 2006). Along the tail, the plasma layer often forms one or two fin ridges (Suquet et al., 1993) which help to improve propulsion efficiency (Cruz-Landim et al., 2003).

Spermatozoa of fish with internal fertilization, comprise of an elongated head, no acrosome, and a midpiece containing developed mitochondrial structures plus intercentriolar materials. Still, the midpiece remains separated from the flagellum by the cytoplasmic canal. Biflagellated sperm has also been reported in *Synbranchus marmoratus* (Lo Nostro et al., 2003).

The number and size of sperm mitochondria also vary between species (Gwo et al., 2004). In Perciformes, there are 5 to 10 mitochondria placed near the nucleus, located around the initial segment of the flagellum (Jamieson, 1991; Mattei, 1991). In Pomacentridae, the midpiece contains 6 spherical mitochondria located close to the initial flagellum segment (Mattei, 1991; Lahnsteiner and Patzner, 1998). In *Cichla intermedia* spermatozoa, the mitochondria (about 10 in number) are round or slightly elongated, and their location is very similar to that observed in Pomacentridae and Perciformes. The mitochondria may accumulate laterally and thus make the midpiece asymmetrical. In Labridae (Mattei, 1991), the mitochondria, about 3 to 5 in number, resemble in shape and organization, those found in Pomacentridae and Cichlidae. They are located at the base of the midpiece, around the flagellum and separated by the cytoplasmic channel. Mitochondria size and numbers differ even among relatively closely related fish species. For example 1 spherical mitochondrion of 1.0 μm diameter is present in *Pagrus major* (Gwo et al., 2004), 2 mitochondria with 0.71 μm diameter in *Rhabdosargus sarba* (Gwo et al., 2004) and 3 mitochondria with diameter of 0.64 μm in *Acanthopagrus latus* (Gwo, 1995).

1.3 Behaviour of fish spermatozoa: mechanism of activation

Fish spermatozoa are immotile in the testis and in the seminal plasma (Billard, 1978; Stoss, 1983). Motility is induced when sperm cells are discharged into the aqueous environment. Changes in factors such as ions, pH and osmolality are believed to depolarize the cells thereby stimulating motility (Cosson et al., 1999). In fish, the activation of a sperm cell usually occurs when the sperm cell in the seminal fluid is diluted into the surrounding water, with changes in ion concentration, or osmotic pressure, as the most common triggering factors. In fresh water spawning fishes, sperm

cell experience hypoosmotic change, when they shift from the seminal fluid into fresh water (Billard, 1978; Morisawa et al., 1983). In contrast, sperm cells of marine fishes, encounter the opposite change, hyperosmotic change, meaning a change to a medium with higher osmolality (Billard, 1978; Morisawa and Suzuki, 1980; Morisawa, 1985). In spawning salmonids e.g. rainbow trout, *Oncorhynchus mykiss*, the sperm motility is regulated by ions such as potassium, hydrogen and calcium of their spawning environment (Morisawa et al., 1983; Morisawa and Morisawa, 1986; Morisawa and Ishida, 1987). In internally fertilising fishes, their sperm cell motility are triggered mostly by the changes in ionic concentrations or oxygen of the female tract (Morisawa and Suzuki, 1980; Morisawa, 1985). Despite triggering sperm cell motility, osmotic shock is also severely damaging to the sperm cell. As the cells are only surrounded by a thin layer of membrane, changes in the osmotic and/or ionic concentration will further alter their movement pattern and duration of motility (Morisawa, 1994). Most fresh water spawning fishes show a short motility duration. For example carp, *Cyprinus carpio* exhibit motility for only 30-40 sec (Perchee et al., 1993). In sperm cells of marine fishes motility is longer in duration. The Mediterranean horse mackerel, *Trachurus mediterraneus* and the plain red mullet, *Mullus barbatus*, sperm are motile for 60 and 125 sec, respectively (Lahnsteiner and Patzner, 1998).

Temperature rise, however, leads to an increase in spermatozoa velocity but with a shorter duration of motility which is due to ATP exhaustion cause by hydrolysis of dynein ATPases (Billard and Cosson, 1990; Perchee et al., 1995). On the other hand, lowering the temperature of the swimming medium results in a prolonged duration of motility with reduced cell velocity (Stoss, 1983).

A negative relationship between the time of sperm activation and the proportion of eggs fertilized has been demonstrated (Hoysak and Liley, 2001), which suggests that the duration of motility is related to fertilization success. Several studies have further demonstrated a positive correlation between sperm cell motility and fertilization success in some fish species (Moccia and Munkittrick, 1987; Ciereszko and Dabrowski, 1995; Lahnsteiner et al., 1998).

In comparison with fish, most mammalian spermatozoa display two types of physiological motility between mating and fertilization. The first phase of motility, the so-called activated motility occurs at ejaculation from the cauda epididymus and the second phase, hyperactivation, occurs in the female reproductive tract (Yanagimachi, 1970; Suarez and Osman, 1987), and enables the sperm to generate adequate force to liberate themselves from the oviductal isthmus, and thus inducing movement towards cumulus matrix or zona pelucida of the oocyte (Suarez, 1996). At the first phase of motility, the activated spermatozoa in mammals exhibit a symmetrical beat with lower amplitude waveform that drives the sperm cells in a relatively straight line while the hyperactivated spermatozoa has asymmetrical beat with higher amplitude waves which results in circular trajectories (Ishijima et al., 2002).

1.3.1 Sperm motility

Motility of sperm cells is used as an indication of sperm fitness and fertility. The motility is basically determined by grading the percentage motile cells (Rana and McAndrew, 1989; Aas et al., 1991; Suquet et al., 1992; Fauvel et al., 1999) or by determining the duration of movement (Ciereszko et al., 2001), or by a combination of these criteria (Turner and Montgomerie, 2002), often simply through observation by

using microscope. These simple visualization methods have limitations mostly due to the large variation in defining the motility itself, as well as varying technician skills (Verstegen et al., 2002). Furthermore, variation in the chemical composition of the extender or activation medium may also produce variation in the duration of spermatozoan motility. Temperature also alters motility duration with low temperatures prolonging the duration of movement therefore reducing sperm cells velocity (Hines and Yashouv, 1971). In addition, variation related to samples with different cell densities, different sample volumes, and surface tension effects further complicate the usual approach of sperm motility assessment (Rana and McAndrew, 1989).

Thus, difficulties in sperm cell motility assessment as per discussed earlier has focussed efforts to develop a new method to determine sperm cell motility with the aim of greater objectivity and quantifiability. A few such methods have been developed e.g. stroboscopy illumination (Cosson et al., 1985) and computer-aided sperm analysis, CASA (Dott and Foster, 1979; Cosson et al., 1997). CASA is more popular among researchers as it can measure several different sperm cell motility characteristics or parameters. CASA, was first introduced by Dott and Foster (1979) and was initially used for andrology purposes in human (Boone et al., 2000; Donnelly et al., 2001; Wroblewski et al., 2003), as well as in several domestic animal species (Farrell et al., 1998; Rasul et al., 2000; Maes et al., 2003; Pena et al., 2003; Suzuki et al., 2003; Quintero-Moreno et al., 2004; Joshi et al., 2005). The essence of this approach is to convey the images of motile sperm cells from a microscope to a video camera, which enables digitization of the image in order to provide a description of the movement of tracked sperm by image analysis software. Apart from being objective and rapid, CASA also has the ability to facilitate the analysis of the kinematic properties of sperm cell

motility by measuring the character of motility itself and thus, may provide greater insight into sperm cell fitness assessments. In fact, correlations between several CASA motility descriptors and *in vivo* fertility in mammals (Wilhelm et al., 1996; Holt et al., 1997; Farrel et al., 1998) as well as in free-spawning invertebrates (Chia and Barker, 1996; Au et al., 2001) and fish (Chechun et al., 1994) have been demonstrated. In addition, CASA has also been used as an important tool to assess teleost sperm cell fitness that have been subjected to pollutants (Van Look et al., 2000; Van Look and Kime, 2003), cryopreservation studies (McNiven et al., 1993; Rurangwa et al., 2001) or prior to fertilization (Duplinsky, 1982; Morisawa et al., 1983; McMaster et al., 1992; Toth et al., 1995). One example of CASA system is the Hobson sperm tracker, which simultaneously generates 15 different spermatozoa motility descriptors (Table 1.1). On a whole, VAP, VSL, STR and LIN are indicators of sperm progression, whereas ALH and BCF, are indicators of sperm vigour. A diagram showing the motility descriptors is shown in Figure 1.3. The sperm tracker offers advantages over other tracker systems such as Cell tracks or Hamilton sperm tracker as it can track up to 200 individual spermatozoa at one time compared to a single sperm frame by frame (Kime et al., 1996). As the tracker analyzes individual components of sperm motility, any changes in the movement related to inhibition or activation is detected directly.

1.3.2 Seminal fluid and osmolality

The effect of osmolality on sperm cells motility in teleosts was first emphasized by Morisawa and Suzuki (1980). Osmolality is considered as the main triggering agent for sperm motility in many fish species (Morisawa, 1985), with the exception of salmonids and viviparous species such as guppy. In goldfish, *Carassius auratus*, duration of sperm

Table 1.1: Definitions of sperm motility descriptors generated by Hobson sperm tracker

Parameter (unit)	Definition
Curvilinear velocity, VCL ($\mu\text{m s}^{-1}$)	the sum of the incremental distances moved in each frame along the sampled path divided by the total time of the track. This parameter considers only the motile spermatozoa
Straight line velocity, VSL ($\mu\text{m s}^{-1}$)	straight line distance between the start and end points of the track divided by the time of the track
Average path velocity, VAP ($\mu\text{m s}^{-1}$)	a derived smoothed path calculated by measuring the angles of the turning points on the track in relation to the start point and dividing by the time of the track
Pause or variation of VSL, PFT ($\mu\text{m s}^{-1}$)	is a measure of how much a spermatozoon changes its movement along its track
Mean angular displacement, MAD ($^{\circ}$)	the average change in direction of the spermatozoa head from frame to frame
Beat cross frequency, BCF (Hz)	is calculated using the turning points on the track (when the track changes from yellow to green and vice versa)
Amplitude of lateral head displacement, ALH (μm),	the average deviation from the smoothed path based on the difference in linearity between the smoothed and sampled paths
Dance mean, DMN (μm)	the ALH of a track divided by its linearity (LIN)
Linearity, LIN (%)	the straight line distance between the start and end points of the track divided by the sum of the incremental distances along the actual path ($VSL/VCL*100$)
Straightness, STR (%)	the straight line distance between the start and end points of the smoothed track divided by the distance along the smoothed path ($VSL/VAP*100$)
Linear morphometry, LIN MOR (μm)	the width and height of every sperm head in every frame
Area morphometry, AREA (μm^2)	the average value of the average width x height derived from the LIN MOR
Per cent motile, %MOT (%)	the number of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the field
Motile concentration, MOTCN (millions ml^{-1})	the derived concentration of motile sperm within the analysis field
Track time, TKTMS (tenths of a sec)	the duration of accepted tracks

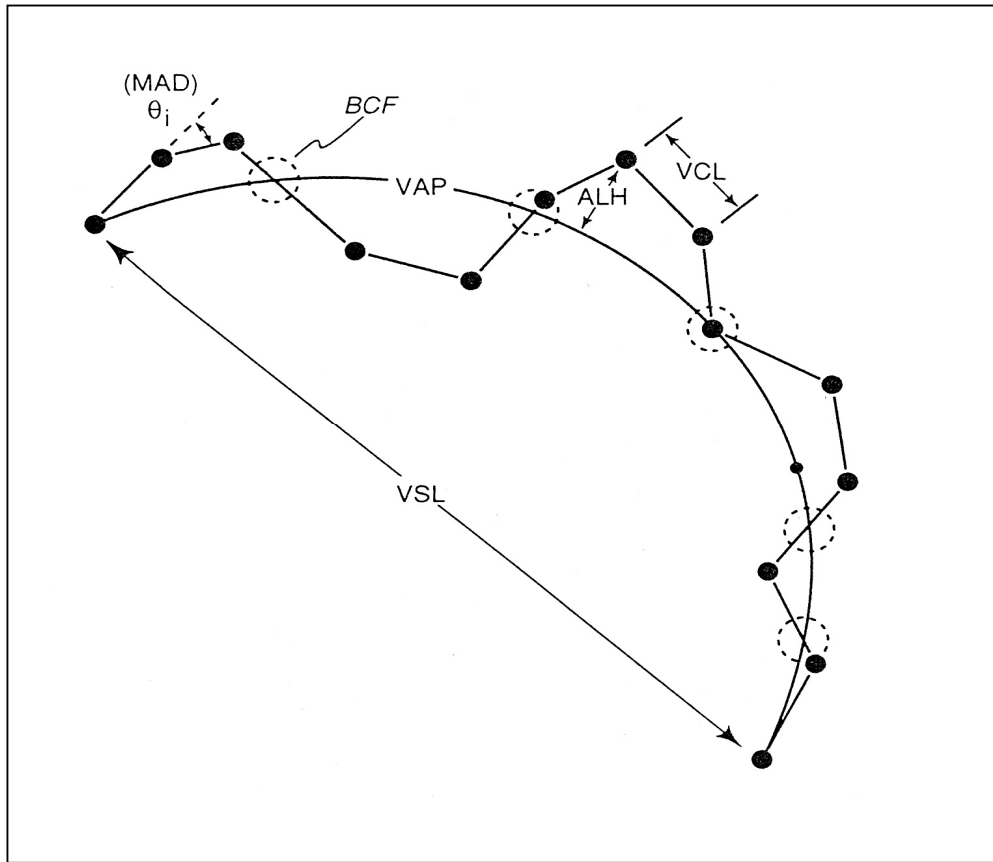


Figure 1.3. A diagram illustrates sperm motility descriptors generated by sperm tracker (Boyers et al., 1989). The black dot represents successive images of the head of a motile sperm and the dashed circle represents the beat cross frequency, BCF. All motility descriptors are described in Table 1.1

motility is longer in medium with an osmolality between 100 and 200 mOsmol/kg, while in marine puffer, *Fugu niphobles*, sperm cells are immotile at 300 mOsmol/kg, quiescent at 1200 mOsmol/kg and active for the longest period at 400 mOsmol/kg. In *O. mossambicus*, duration of the motility of the sperm cell lasted longest in mannitol-based solution with an osmolality between 100 and 200 mOsmol/kg, but declined at 0 and 300 mOsmol/kg (Harvey and Kelley, 1984). A number of spermatozoa were also actively motile at osmolality as high as 450 mOsmol/kg, thus suggesting a high degrees of physiological plasticity in the sperm cells of euryhaline species. Hypotonic media also initiates motility of spermatozoa of freshwater fishes such as *Cyprinus carpio* (Morisawa and Suzuki, 1980; Morisawa et al., 1983; Billard et al., 1995) and *Carassius auratus* (Morisawa and Suzuki, 1980). In general, osmotic changes have consistently been reported to induce sperm cell motility and are regarded as the major triggering factors either in freshwater or marine fish as osmolality decreases below or increases above the threshold level of seminal fluid, respectively.

In sea water-acclimated *O. mossambicus*, sperm showed high motility in calciumchloride plus sodium chloride buffered with 10 mM Hepes in comparison with sodium chloride buffered solution alone. Motility was also decreased as osmolality was increased either with the addition of potassium chloride, or mannitol (Morita et al., 2004). However, in this species potassium is unlikely to regulate the initiation of sperm cell motility as either increased or decreased of potassium did not affect demembranated sperm cell reactivation in either freshwater- or seawater-acclimated *O. mossambicus*. On the other hand, reactivation of demembranated zebrafish sperm cells failed to occur in mannitol solution which implies that suitable ion composition and concentration is critical for the initiation of sperm cell motility in zebrafish (Takai and Morisawa, 1995).

In contrast with oviparous freshwater and marine teleosts, in viviparous species such as topminnow, *Gambusia affinis* and guppy, the breakdown of the spermatozeugma (sperm ball) occurred between an osmolality of 50 and 300 mOsmol/kg in sodium chloride and potassium chloride solution, but not occur in mannitol solution. Thus alteration in ionic composition that induces the release of sperm cells from cell aggregations may participate in the initiation of motility in the sperm of these species (Morisawa and Suzuki, 1980). The factors initiating sperm cell motility in both oviparous and viviparous teleost is likely dependent on adaptations to the nature of the spawning medium.

It is not possible to separate osmolality from ionic or gaseous effects. For instance, sperm cells of marine puffer fish become motile as the osmolality of the surrounding media increase (Oda and Morisawa, 1993) which probably induces an increment of intracellular potassium (Takai and Morisawa, 1995) and intracellular calcium, as well as the decrease of the internal pH, (Oda and Morisawa, 1993). In freshwater fish such as carp, sperm cells that are immotile in the seminal fluid (Redondo-Muller et al., 1991; Morisawa et al., 1993; Perchec et al., 1995) become active and immediately move once exposed to hypoosmotic solution, either with or without electrolytes. Hypoosmotic media may also induce cell swelling and thus, affect membrane permeability (Krasznai et al., 2003).

1.3.3 Seminal fluid and ionic composition

In rainbow trout, *Oncorhynchus mykiss*, a decrease in the concentration of potassium in the seminal fluid, induced a hyperpolarization of the membrane potential (Boitano and Omoto, 1991) thus activating sperm motility. However, excess of potassium

inhibits spermatozoa motility as observed in salmon, *Salmo gairdneri* (Baynes et al., 1981) and sea urchin, *Strongylocentrotus purpuratus* (Labarca et al., 1996; Galindo et al., 2000). This inhibition of motility may be overcome by divalent cations. Thus, Baynes et al. (1981) demonstrated that the addition of 0.3-1.0 mM of calcium, into 1.0 mM potassium-buffered solutions (at pH 9.4 and pH 8.6) that previously inhibited sperm cell motility, induced sperm cell motility in rainbow trout. They also demonstrated that 5.0-12.5 mM magnesium abolished the inhibitory effect of sodium at 130 mM and potassium in 20 mM of buffered solutions at pH 9.0. In fact, it has been reported that the addition of calcium prolonged sperm cell activation by 4-fold, however, calcium concentration of more than 20.0 mM caused the aggregation of the sperm cell thus decreasing their movement. In addition, the removal of external calcium ions by ethylenediaminetetraacetic acid (EDTA) or ethylene glycol tetraacetic acid (EGTA) chelation prevented the initiation of sperm motility in rainbow trout. Furthermore, the initiation of sperm motility in the trout was associated with the increase in intracellular free calcium that was prohibited by the presence of potassium ions in the external medium (Cosson et al., 1989).

The effect of potassium on sperm cells motility in other species of teleosts is less clear, but in some species, potassium is reported neither inhibit nor trigger the motility (Perchee et al., 1993). As such, the complex synergistic effect of ionic composition and concentration on sperm cells motility have led some researchers to suggest that motility is regulated, not directly by osmotic changes, but by changes induced in sperm cell membrane potential (Gatti et al., 1990; Boitano and Omoto, 1991).

In trout sperm cells, experiments using stroboscopic light demonstrated that calcium influx induced a circular movement which becomes smaller with time (Cosson et al., 1989). In spermatozoa of *Salmo gairdnerri* loaded with Quin-2 AM, which is a membrane permeable acetoxymethyl ester calcium chelator, the addition of calcium increased the fluorescent emission intensity up to 6-fold within 40 seconds thus indicating that the motile phase is associated with the increase of calcium. The addition of calcium channel blocker desmethoxyverapamil, however, prevented the initiation of motility and of calcium rise in sperm cells which suggests that there is an influx of extracellular calcium rather than a mobilization of calcium internal stores (Cosson et al., 2005). In addition, the depletion of calcium in sodium chloride saline solution containing 10 mM EGTA, below 10^{-7} M has been reported to inhibit sperm cell movement in trout (Gatti et al., 1990). The requirement for extracellular calcium to initiate a rise in calcium during sperm cell mobilization was also confirmed by Morita et al. (2004). They also reported that there is reactivation of movement in demembrated sperm cells from seawater and freshwater acclimated *O. mossambicus*, which occurs in the presence of more than 10^{-4} mol/l calcium (Morita et al., 2003, 2004). Their studies therefore indicate an involvement of calcium in sperm cell activation in *Oreochromis*. However, whilst modulation of intracellular calcium is regarded as a universal event during sperm cells motility in many teleosts (Cosson et al., 1989; Krasznai et al., 2000; Morita et al., 2004), some studies indicate that these intracellular calcium changes are not associated with the influx of extracellular calcium (Billard et al., 1993; Chauvaud et al., 1995).

Some studies have also indicated the involvement of a calcium -dependent cyclic-adenosine monophosphate (cAMP)-mediated protein phosphorylation axonemal

membrane signalling cascade in sperm motility initiation. For example, in salmonids, motility initiation is induced by phosphorylation of a 15-kDa protein *via* the increase in cAMP (Hayashi et al., 1987) and in *O. mossambicus* protein phosphorylations were also observed (Morita et al., 2004). However, some conflicting evidence has been reported in some fish species. For instance, Krasznai et al. (2000) suggest that cAMP-dependent protein phosphorylation is not required in the regulation of sperm motility in carp. In their studies, the demembrated carp sperm was able to be reactivated with the addition of ATP in the absence of cAMP. Furthermore, the cAMP level was low and no changes of cAMP level were observed during the initiation of sperm motility. As such, the importance of cyclic nucleotides-dependent protein phosphorylation regulatory pathway in sperm motility of fish cannot be ascertained.

1.3.4 Seminal fluid and pH

Other than osmolality and ions, pH also affects sperm cell motility in some teleost species (Hines and Yashov, 1971; Billard et al., 1993; Linhart et al., 1995; Ingermann et al., 2002). Higher pH values (pH range of 8.0-8.5) apparently enhance the motility and fertility of salmonids spermatozoa (Scott and Baynes, 1980; Billard et al., 1981). Some authors however, suggest that ions may influence the sperm cells activation regardless of pH. For example, Baynes et al. (1981) demonstrated that calcium concentration in the range of 3 to 6 mM activate sperm cell of rainbow trout in pH buffered solution of 7.0 or 8.0. Measurements of cell membrane potential by using lipophilic ions such as tetraphosylphonium and thiocyanate showed that the membrane of trout sperm depolarized at either acidic pH or by an elevated concentration of extracellular potassium (Gatti et al., 1990).

In cyprinids, the extracellular and the intracellular pH, influences the initiation and duration of the sperm motility (Marian et al., 1997). However, activation of carp sperm appears to be independent of intracellular pH where their sperm cells are initiated to move in the media of pH range of 6 and 9 (Redondo-Muller et al., 1991). The increment of intracellular pH in sperm cells by 0.15 unit from 7.06 after 6 minutes of activation in 110 mOsmol/kg solution detected by BCECF-AM, a membrane permeant acetoxymethyl ester, that accompanied carp sperm motility initiation however, do not trigger axonemal movement (Krasznai et al., 1995), which is in contrast with trout sperm (Gatti et al., 1990; Boitano and Omoto, 1991).

In salmonids, the seminal fluid concentration of bicarbonate ion and pH elevate as sperm cell translocate from the testis to the sperm duct. Seminal fluid contains about 8 to 10 mM bicarbonate with pH of 7.9 to 8.0. In this fish species, the sperm cells activate within a pH range of 8 to 8.5, but the motility diminishes at more alkaline pH. It was thought that the combination of pH, bicarbonate and cAMP in the seminal fluid help the spermatozoa to preserve their motile ability (Morisawa and Morisawa, 1988). Chavaud et al. (1995) however, found that the percentage of motile spermatozoa depend on the pH of the activating medium, where highest numbers of motile sperm cells were observed at pH close to 8.2. Lahnsteiner et al. (1998) reported a significant correlation between the pH of the seminal fluid and the percentage of motile spermatozoa, linearly motile sperm cell as well as their swimming velocity.

Generally, the extracellular pH probably influences the intracellular ionic concentration of the sperm cell, which in turn modifies the membrane potential and thus sperm motility behaviour (Boitano and Omoto, 1992).

1.4 Pollution in aquatic environment

Fish are major components of aquatic systems and consumed as human food, thus, the bioaccumulation and biomagnification of pollutants in fish tissues could ultimately alter human fitness. Of late, there has been a growing concern over the use of chemicals that may disrupt endocrine functions in fish that control developmental, reproductive, behaviour and immunological-processes.

Numerous studies have reported the incidence of skeletal deformity in organisms exposed to inorganic and organic contaminants (Teh et al., 2002; Sanchez-Bayo and Gouka, 2005). Longer-term, lifecycle laboratory tests exposures have been concurrently used to assess the effects of chronic exposure: growth enhancement, liver enlargement, and decreases in gonad size and fecundity in several invertebrates and fish species (Klumpp et al., 2002; Corsi et al., 2003). The effect on reproductive ability is regarded as the most sensitive endpoint. But determining the thresholds of the effects requires lengthy and expensive tests. Sub-lethal effects occur at lower concentrations than those causing death of an animal. Detection of sub-lethal effects provides an early indication of contaminant bioavailability, thus providing valuable information on the possible consequences of prolonged or continuous exposure to pollutants.

There has been a growing interest to use animals sperm cells as a sentinel for ecotoxicological studies. Compared to eggs, sperm cells are produced in enormous numbers. Furthermore, in teleosts that adopt a reproductive strategy of external fertilization, the sperm cells are released into the environment before fertilization and thus may be subjected to toxicant exposure that may affect their viability. Therefore, abnormalities in sperm cell movement, behaviour and morphology may provide

integrated endpoints that can determine the effects of water quality and habitat conditions. Many toxicological studies, using copper (Cu), methylmercury (meHg), mercuric chloride (HgCl) and acid water, have employed either fertilization rate or assessment of sperm cell motility to determine the effects of pollutants on sperm cells quality (Duplinsky, 1982; Khan and Weis, 1987). However, the assessment of sperm cells motility is often criticised as the standard measurement approach is very subjective and makes for difficulty in performing statistical comparison of the results. Recently, the introduction of computer-aided sperm analysis (CASA) has significantly increased the interest of researchers in studying the effect of various types of pollutants on sperm cell motility in animal species. This technique is also an uncomplicated, relatively low-cost methodology that could be applied in monitoring programs at various temporal and spatial scales.

1.4.1 Cadmium and toxicity

Cadmium (Cd), which has an ecotoxicological importance, was selected as one of the pollutants of the present study. This biologically non-essential heavy metal is usually present in sediments and water at low concentrations that range from 0.2 to 0.9 ppm and less than 0.1ppm, respectively (Cook and Morrow, 1995). Major sources of Cd include chemical sewage particularly those related to plastic and colour pigments industries (Arnason and Fletcher, 2003; Migliarini et al., 2005), as well as in the fabrication of nickel-cadmium batteries and electroplating of electrical components.

The toxic effects of Cd have been well studied in many animals, and significant data from Cd-exposed humans have also been assembled. Occurrence of Cd in the human body is frequently associated with renal dysfunction (Wu et al., 2001; Lu et al., 2005),

osteoporosis (Jin et al., 2004) and metabolic anomalies which is mostly caused by enzyme inhibitions (Casalino et al., 2002). Another known effect due to contamination of Cd in human is itai-itai sickness in Japan (Inaba et al., 2005) which was due to regular consumption of rice that was highly contaminated with Cd derived from a smelter (Ishihara et al., 2001). This disease is characterized by multiple, spontaneous bone fractures due to competition of Cd with calcium, and the symptoms mainly manifest in elderly multiparous women.

Cadmium shows different mechanisms of toxicity under different experimental conditions, and in various species, which makes it difficult to understand and establish its effect and mechanism in general. Many studies have associated Cd with oxidative stress (Almeida et al., 2002) as this metal can also alter the antioxidant defense system (Chandran et al., 2005) and induced peroxidation of polyunsaturated membrane lipids (Bagchi et al., 1997). Cadmium also tends to bind to calcium binding sites on the surface of animal cells (Prozialeck et al., 1996) and plant cells (Faller et al., 2005) and apparently has a high affinity for calcium-ATPase of cell membranes (Verbost et al., 1989; Baldisserotto et al., 2004). In this regard, relatively low Cd exposure concentrations can cause hypocalcemia and mortality in fish at early life stages (Witeska et al., 1995). Furthermore, Cd has been reported to interfere with osmoregulation (Lionetto et al., 2000), and is associated with a low heart rate in fish (Hallare et al., 2005).

Deoxyribinucleic acid (DNA) damage induced by Cd mostly occurs at high cytotoxic concentrations (Takaki et al., 2004; Fotakis et al., 2005) and depend on the concentration and period of incubation (Faverney et al., 2001; Fotakis et al., 2005). In

rat hepatoma cell lines, it is observed that in addition to the loss of lysosomal integrity and functions, DNA damage was detected at 200 μM , 500 μM and 1000 μM of Cd in cells incubated with Cd for 8 hours (Fotakis et al., 2005). Cadmium was previously reported to induce DNA strand breaks but such effect was only observed with cytotoxic concentrations of more than 40 μM (Misra et al., 1998).

At lower Cd concentrations, however, many conflicting data have been obtained. Cd has been demonstrated to induce inhibition of DNA repair systems, particularly by disrupting Zn finger structures of DNA-binding proteins (Predki and Sarkar 1994; Hartwig 1998). In embryo of *Palaemonetes pugio* (grass shrimps) exposed to 5 μM Cd for 12 h, for example, no significant effect on DNA strand breaks or hatching rates were observed (Hook and Lee, 2004). Likewise, in mussels gill cells exposed to 0.88 μM Cd for 4 weeks, no alteration in basal level of DNA damage, or even when the gill cells were exposed to 3.07 μM Cd for 1 week as observed with comet assay. These results suggest a lack of direct effect of low level Cd on DNA (Pruski and Dixon, 2002).

The higher ventilation rate of fish compared to humans may increase their exposure to waterborne contaminants particularly to the respiratory surfaces of the gills. In addition, other features of fish gills (e.g., countercurrent system of blood and water flow, thin epithelial membranes, and a high surface area) may increase the uptake of compounds from the water to their bloodstream. Marine teleosts drink seawater due to their hypotonic nature, which may contribute to their exposure to waterborne substances. In contrast, hypoosmotic freshwater fish move water into their bodies thereby creating a route of exposure to waterborne contaminants. While the obvious sign of highly polluted water (dead fish) is readily apparent, the effect of low-level pollution may have

no apparent impact on the fish itself but may decrease the fecundity of fish populations leading to a long term decline and possibly extinction of these important resources. Low levels of pollution could affect the reproduction either indirectly *via* accumulation in the reproductive organs or directly on gametes which are freely released into the water.

The effects of Cd on reproduction performance has fascinated many researchers in many reproductive toxicology studies. However, much attention had been given to establish the effect of Cd on reproductive organ, gamete production, fertilization and embryogenesis. Nonetheless, the tolerance capabilities of teleost to Cd may also vary between species and developmental stage. In female fish, the chemical dose of pollutants at which early developmental stages of animals are exposed are derived largely *via* maternal transfer associated with deposition of nutrients from the lipid reserves to the maturing oocytes. Therefore, although the egg affords some degree of protection from contaminants in the external environment, it also can subject embryos to elevated, maternally derived, chemical exposures during potentially sensitive early developmental stages. However, the closed environment of the egg (i.e. little excretion) and the minimal biotransformation abilities of young embryos create unpredictable exposure variations as the animals develop. For instance, wild European flounder from heavily industrialized estuaries (the Mersey and Tyne) and a bay (Seine) were reported to have altered spermatogenesis (Lye et al., 1998) and up to 18% of the population showed ovotestis (Matthiessen et al., 1998; Allen et al., 1999; Minier et al., 2000). Lake whitefish collected from Jackfish Bay exhibited reproductive abnormalities and delayed sexual maturation (Munkittrick et al., 1992).

In carp, incubation of fertilized eggs in Cd levels from 0.001 ppm to 0.05 ppm decreased eggs swelling, delayed hatching and lower the survival rate of the embryo. These studies also showed that the susceptibility of the carp larvae to Cd, decreases with larvae age (Witeska et al., 1995). In adult medaka, exposure of 0.001ppm, 0.005ppm and 0.01 ppm of Cd for 7 weeks, did not alter their reproductive capacity, fecundity, egg production, egg fertilization and egg size or even the phenotypic sex ratio. However, a significant effect was demonstrated on hatching although it was not concentration dependent (Tilton et al., 2003), which may be due to the inhibition of astacin, an endopeptidase which is involved in rupture of then egg membrane (S. George, personal communication). These various responses of teleosts in various stages of development to Cd toxicity have resulted in difficulties with intercomparability. Less attention has been given to study the direct effect imposed by Cd on gamete quality, especially on sperm fitness. Only a few studies to date have been performed on the effect of Cd on teleost sperm cells quality or on the other well-being of commercially important freshwater species (Lahnsteiner et al., 2004).

1.4.2 Pesticides and toxicity

Whilst the use of insecticides and other pesticides is often regarded as essential for efficient crop and animal production, and the economic benefits of pesticides in producing high crops yield and in control of disease borne pests are undeniable, their usage has to be carefully controlled to minimize unintended adverse consequences to other plants and animals, and ultimately to man. The global market of pesticides is as shown in Table 1.2. In year 2000, 36.0% of the pesticides market was conferred by herbicides which accounted for 1944 millions lbs worldwide. However, the total market of pesticides has declined in 2001 where herbicides market accounted for only 1870

Table 1.2: Summary of world pesticide markets in 2000 and 2001

Year	Type of pesticides	Millions lbs	Percentage
2000	Herbicides	1944	36.0
	Insecticides	1355	25.0
	Fungicides	516	10.0
	Other	1536	29.0
2001	Herbicides	1870	37.0
	Insecticides	1232	24.0
	Fungicides	475	9.0
	Other	1469	29.0

Source: USEPA 2004; 'other' includes nematicides, fumigants, rodenticides, molluscicides, aquatic and fish/bird pesticides, miscellaneous pesticides

millions lbs. But, even so, the impact of pesticide practice in the previous years should not be overlooked.

Pesticides are used in every realm of the environment to control undesired pests, such as insects, weeds, fungus and rodents. Therefore, it is very likely that many non-target organisms are exposed to multiple pesticides throughout their lifetimes, either sequentially or concurrently. Pesticides can affect fresh water and marine fish either by causing immediate acute toxicity on exposure or if these agents are persistent in the environment, they can cause chronic toxicity. Pesticides can also affect the organisms that fish consume, and so have an indirect impact on fish populations. It must be understood that these compounds are all applied at different rates, often degrade in differing ways under specific conditions, and some may partition and otherwise disperse in the environment entirely differently from each other. Thus their environmental concentrations can be very difficult to predict. Three major categories of pesticides have been intensively studied with regard to detrimental effects on wildlife and the environment: organochlorines (OCs), organophosphates (OPs) and carbamates and synthetic pyrethroids. Other than agricultural applications, pesticides applied around homes, in gardens, and on roadsides also find their way into surface waters.

In order to assess the effect of pesticide exposure to aquatic organisms, it is necessary to monitor water, sediments, and tissue samples. However, there are inherent limitations to these kinds of monitoring studies: they are expensive and time-consuming, and only a few government agencies are equipped and/or funded to carry out comprehensive monitoring. Nevertheless, there is a significant amount of information available on pesticides concentration in surface water and groundwater, sediments, and animal

tissues. The use of many pesticides e.g. OPs to destroy insect pests is based upon their 1000-fold differential toxicity to insects compared with vertebrates, which in the case of some OPs is attributable to the presence of a detoxification system in vertebrates that is absent in invertebrates. Other pesticides, eg pyrethroids may have much greater effects on arthropod target proteins than the vertebrate counterparts, explaining their differential toxicity. Even one particular organism can display a marked difference in sensitivity not only to different pesticides but also, to the same pesticide at different life stages (Dutta et al., 1995).

Recent data by Hayes et al. (2002), suggest that environmentally relevant concentrations of frequently-used pesticides can cause devastating effects to the reproductive potential of amphibians which highlight the potential harmful effects of constant low-dose pesticide exposure.

Sublethal exposure to low levels of pesticides may have a more significant effect on fish populations than acute poisonings. Subtle changes in behaviour and physiology (Fleming et al., 2004; Sindhu et al., 2005) may impair both survival and reproduction. Survival is threatened further by pesticides that suppress the immune systems of fish, resulting in a high mortality rate due to opportunistic disease. Toxic effects of pesticides also influence tolerant species of aquatic communities, in that their qualitative presentation and types of competitors, predators, and prey-organisms may expand or contract as niche space is vacated or eliminated (Kegley et al., 1999). However, minimal work has been conducted on the effect of pesticides compounds on teleost sperm quality to date.

1.4.2.1 Malathion

Malathion is an insecticide in the organophosphate pesticide chemical family. This pesticide is highly lipid soluble and stored in lipophilic tissues (Garcia-Repetto et al., 1995). It is one of the oldest insecticides in the family, and has been used since 1950 for the control of insects such as fruit flies, mosquitoes and aphids. The relatively low toxicity compared to other organophosphates, and a rather short persistence in the environment have led to the extensive use of malathion in many insect eradication programs particularly in the control of adult mosquito, especially in the cases of public health concerns over mosquito vectored diseases (Stahl, 2002). Generally, pesticide breakdown products are less toxic than the pesticide itself, but this is not true in the case of malathion, which along with other related phosphorodithioate insecticides must be oxidized before they have inhibitory potency and toxicity. Malathion is of relatively low acute toxicity and the effects observed are mainly due to its active metabolite, malaoxon. Oxidation occurs *via* cytochrome P450 and results in the conversion of the 'P=S' group in malathion to 'P=O' forming its oxon, malaoxon (Blasiak et al., 1999).

Malaoxon inhibits the enzyme acetylcholinesterase (AChE) activity (Dutta et al., 1995; Panda and Sahu, 2004). Malaoxon covalently (and hence irreversibly) binds to AChE by mimicking the structure of enzyme's natural substrate, acetylcholine, ACh (Cox, 2004). Inhibition of AChE leads to accumulation and continuous action of ACh and therefore causes signals in the nervous system to persist longer than normal.

Several studies have reported developmental and reproductive effects in mammals due to high doses of malathion administration in test animals (Gallo and Lawryk, 1991). For example, pregnant rats fed high doses of 240 mg/kg/day of malathion showed an

increase rate of newborn mortality. In contrast, malathion fed to rats at low dosages caused no reproductive effects (US Public Health Service, 1995). Malathion has also been reported to impair the period of sperm motility as well as to induce cytoplasmic droplets in male rats (Akbarsha et al., 2000). Choudhary et al. (2008) suggest the decrease in sperm motility of wistar rats administered malathion orally with 50 mg/kg/day and more is due to the androgen deprivation effect of this pesticides. In addition, testosterone impairment due to the exposure to pesticide has been demonstrated (Zhang et al., 2007).

While malathion is only slightly toxic to mammalian species at relatively high concentrations, its toxicity is generally much higher in aquatic species. Malathion has a wide range of toxicities in fish, extending from very highly toxic in walleye (PAN Pesticide Database, 2005) to slightly toxic in goldfish (Ecotoxnet, 1996). Summary table showing the relative toxicities of fish species exposed to malathion are shown in Table 1.3. The lowest observable effect concentration (LOEC) values were 3 mg/L and 1.25 mg/L in *Danio rerio* and *Clarias gariepinus* (Nguyen and Janssen, 2001), respectively, which appear to lie within the range of other fish species (Kumar and Ansari, 1984). Neurotoxicity of these both insecticide and the active product can be detoxified by the carboxylesterase (CE) enzyme which were highly found in mammals in compared to insect thus explained why it is highly selective to insect (Pandey et al., 2005). However, as far as we are concerned, to date, there are not many studies performed on the effect of malathion on reproductive performance. Studies by Giri et al., (2002) showed malathion increased the frequency of chromosome aberrations and sperm head morphology which linearly increased with malathion dose (2.5, 5.0 and 10

Table 1.3.: Relative toxicities to various fish species exposed to malathion

Species	Duration	Result	Reference
<i>Stizostedion vitreum</i> (Walleye)	96 hours	96 h LC50=0.06 mg/l	PAN Pesticide Database, 2005
<i>Carrasius auratus</i> (Goldfish)	96 hours	96 h LC50=10.7 mg/l	Ecotoxnet, 1996
<i>Pimephales notatus</i> (Fathead minnow)	96 hours	96 h LC50=8.6 mg/l	Ecotoxnet, 1996
<i>Oryzias latipes</i> (Killifish)	96 hours	96 h LC50=1.8mg/l	Tsuda et al., 1997
<i>Oreochromis niloticus</i> (Tilapia)	96 hours	96 h LC50=2.00mg/l	Pathiratne and George, 1998
<i>Channa punctatus</i> (Green snakeheads)	96 hours	96 h LC50=6.65mg/l	Pandey et al., 2005
<i>Labeo rohita</i> (Indian major carp)	96 hours	96 h LC50=9.0 µl/l	Patil and David, 2008
<i>Clarias batrachus</i> (Catfish)	96 hours	48 h LC50=0.31 mg/l 96 h LC50=0.25 mg/l	Wasu et al., 2009

mg/kg). Their results showed that malathion may disrupt reproductive performance in animals.

In fish, malathion has been associated with muscular damage during embryolarval development (Hong Lien et al., 1997; Bonfanti et al., 2004) and non-muscular effect (Key et al., 1998; Lockbridge et al., 2005). But the effect of malathion on sperm cells motility and behaviour in teleosts particularly in *O. niloticus* has not been investigated thoroughly.

1.4.2.2 Rotenone

Rotenone is a natural product derived from the roots of tropical and subtropical plants of *Lonchocarpus* spp., *Derris* spp. (Tada-oikawa et al., 2003) and *Tephrosia* spp. found in vast areas of South America, East Africa and South East Asia (Finlayson et al., 2000). It is both insecticidal and piscicidal and thus is widely used in fish eradication programs worldwide (Margraf and Knight, 2002) as well as to treat *Gyrodactylus* parasites, commonly found on salmon (Johnsen and Jensen, 1991). Recently it has been applied to control various mites in honey bees (Jiminez et al., 2000). In other countries, it is used usually for crops and vegetation production as well as for stunning fish (Ibrahim et al., 2000). Mechanistically, its mode of action is by inhibition of NADH-ubiquinone reductase that catalyzes the reduction of NADH to ubiquinone (Zhang et al., 2001) which is a component of electron transport complex I of mitochondria (Sherer et al., 2003). Its effects are thus similar to the effects of antimycin, cyanide and dinitrophenol (Ling, 2003). Other reported effects are related to lipid peroxidation and oxidative stress (Sherer et al., 2003) with induction of DNA oxidation leading to DNA ladder formation which precedes to cell apoptosis (Tada-oikawa et al., 2003). In humans,

rotenone has been associated with cases of Non-Hodgkins lymphoma and Parkinson's disease (Kitamura et al., 2002; Sherer et al., 2003).

Female rats fed with 10 mg/kg/day of rotenone on days 6 through 15 of gestation period experienced decreased fecundity, increased fetal resorption and lower birthweight. However, 2.5 mg/kg/day dose of rotenone produced no observable maternal toxicity or adverse effect on fetal development (Ecotoxnet, 2005). In contrast to mammals, rotenone is highly toxic in all fish species as the chemical affects all gill breathing organisms. At 40 µg/l, the rotenone is adequate to kill most species of fish. The 96 hour LC50 lie in the range of 0.02 to 0.2 mg/L (PAN Pesticide Database, 2005). For instance, the reported LC50 of 96-hour were 2.6 µg/l in channel catfish, 23.0 µg/L in bluegill, for 44% formulation (Ecotoxnet, 2005) and 5.8 µg/l in rainbow trout (Cheng and Farrel, 2007).

Although the literature on rotenone is vast, the effects of this chemical towards fish reproduction, in particular to gamete quality and viability, so far have been neglected.

1.5 Nile tilapia

Nile tilapia is the generic name for one group of fish belongs to the class of Actinopterygii under the order Perciformes of Cichlidae family. These fishes are native to Africa but have become one of the most widely farmed fish (Popma and Masser, 1999) with an increasing production output worldwide (Lem and Shehadeh, 1997).

Nile tilapia inhabits fresh and brackish water of Africa, Middle East, Coastal India, Central and South America. It has become one of the most important food fish with an

estimated 2 millions metric tons production worldwide in 2002 (FAO, 1999). Given their importance in the world aquaculture markets, they are being extensively studied among the researchers. Thus, a wealth information has accumulated on the biology and culture of tilapia. Most of the studies carried out on tilapia are basically concerned with their reproductive performance (Campos-Mendoza et al., 2004; Biswas et al., 2005) or nutrition evaluation and growth (Richter et al., 2003; Liebert and Portz, 2005).

These fishes have become increasingly popular for farming as they are able to reproduce rapidly, easily bred in captivity, tolerate to a wide range of environmental conditions, highly resistant to diseases, and most important of all, have good flavour. Though the fish originated in Africa, Asian countries have become the leading producers of these fishes (Rana, 1997; World Seafood Market News Digest, 2005). In fact, Nile tilapia has become one of the most commonly farmed freshwater fish species throughout the world, in particular *Oreochromis niloticus* (Rana, 1997; Popma and Masser, 1999).

1.6 Problem statements

Sperm cells motility of teleosts may be used as indicator for sperm fitness and probably fertility, but, the knowledge of the physiology and biology of spermatozoa in many teleosts is still lacking, particularly in commercialized freshwater fish species such as in Nile tilapia, *Oreochromis niloticus*. Furthermore, although many previous studies has been undertaken to study the effect and environmental cues following sperm cell activation, subjective approaches and measurements as well as diverse definitions of motility are major problems. Osmolality and ions are identified as the main signal for sperm cells motility in many teleosts, but how these factors effects motility

characteristics has not been thoroughly studied. In addition, little attention has also been given to the effect on spermatozoa morphology during motility.

1.7 Aims of study

Sperm of fish show great promise as a useful indicator of low level toxic effects. They can be produced in large amounts, are easy to expose, have few ethical constraints to their use, and potentially effects are easy to score or measure. In order to be used as a tool for ecotoxicology test, the physiology of their activation need to be determined. However, efforts devoted towards understanding of the mechanism of the sperm activation are still scarce. Considering these limitations of studies in *O. niloticus*, the following aims were set:

- i. to identify seminal fluid characteristics and extracellular factors that trigger motility in *O. niloticus* sperm and their morphological changes, and
- ii. to determine the physiological effects on sperm motility parameters associated with *in vitro* sublethal exposure of selected environmentally relevant pollutants i.e. cadmium, malathion and rotenone, using a computer-aided sperm analysis (CASA) system.

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 male fishes

Male Nile tilapia (200-250g), *Oreochromis niloticus* were procured from the tropical hatchery facility at the Tropical Aquarium of Institute of Aquaculture, University of Stirling. The fish were pit-tagged and maintained in 1m x 1m x 0.5 m tank linked with a water recirculatory system that was thermostatically regulated at $27 \pm 1^\circ\text{C}$. Water quality was recorded at regular intervals (water hardness 238-253 mg CaCO_3/l ; pH 7.9-8.2; oxygen 9.0-9.2 mg/l). The fish were reared under a daily photoperiod of 12L:12D controlled by 24-h electronically preset lights and fed *ad libitum* twice daily (1-5% body weight per day) with commercial pelleted feed (Omega, no. 4 protein content 47%, Ewos Bakers, Bathgate, Scotland).

2.1.1 Collection of seminal fluid

Fish were anaesthetized with tricaine methane sulphonate, (MS222, 200 mg/l), and their identities were identified with the pit-tag reader (Trovan, Germany) and recorded. Fish were netted and their abdomen was dried with a soft cloth to avoid contamination of sperm with water or urine. Seminal fluid was collected into capillary tube by pressing the fish abdomen gently using the thumb and forefinger from the direction of the head to tail, then aliquoted to precooled clean 1.5 ml microcentrifuge tubes, and immediately held on crushed ice.

2.1.2 Assessment of seminal fluid quality

Each seminal fluid sample collected was checked for quality and viability by measuring their motility scores, sperm density, osmolality and pH. Only uncontaminated samples i.e. more than 95.0% of immotile cells at collection time and a population of more than 80.0% of motile cells after activation, and osmolality of 300 mOsm/kg of seminal plasma were used for further analyses. Observations were carried out with an inverted microscope (CK Olympus, Tokyo) at 200x magnification without a cover slip. Contaminated samples were discarded.

2.1.3 Estimation of sperm density

Seminal fluid selected were diluted 1,000 fold in an immobilising solution, a modified fish Ringer's solution that consisted of 110.0 mM sodium chloride, 40.0 mM potassium chloride, 2.4 mM calcium chloride and 1.4 mM sodium bicarbonate, at osmolality of 290 mOsmol/kg and pH 7.0 (Khan, 1987; Rana and McAndrew, 1989). Sperm count was made by using a hemocytometer. Sperm in undiluted seminal fluid are difficult to count due to their high concentration. Sperm count was estimated as described by Tvedt et al. (2001). Briefly, 10 µl of diluted samples were pipetted to the underneath of cover slip of hemocytometer chamber and left to stand for a few minutes to settle down. The number of spermatozoa (cell/ml) was counted with a tally counter and determined for each sample by using the following formula:

$$\text{Sperm density} = \frac{\text{average cells count} \times \text{dilution} \times 5 \text{ (number of counted squares)} \times 10}{\text{(volume of diluted samples used)} \times 1000 \text{ (conversion factor from } 1 \text{ mm}^3 \text{ to ml)}}$$

2.1.4 Measurement of sperm motility

Five μl of diluted sample was pipetted onto a glass slide and observed at 200x magnification either without a cover slip (motility score) or with a cover slip (computer-aided sperm analysis, CASA). The time elapsed between activation and observation for motility score and CASA was 10 sec and 5 sec, respectively.

2.1.4.1 Motility score

Sperm were scored according to Fauvel et al. (1999) and Suquet et al. (1992) based on percentage of motile cells (Table 2.1) using inverted microscope (CK Olympus, Tokyo).

Table 2.1: Motility score for sperm motility measurement

Score	Percentage of motile cells, %
0	0
1	>0-20
2	>20-40
3	>40-60
4	>60-80
5	>80-100

2.1.4.2 Computer-aided sperm analysis (CASA)

Sperm motility was assessed at room temperature (18-20°C) using light microscopy at 20X negative-high phase contrast objective via a U-PMTVC adaptor (Olympus) and recorded with video cassette recorder (SONY SLV D950G/I, SONY Corporation). This type of optic gives an even contrast to the head of fish spermatozoa during progressive motility. The recordings were analysed for sperm trajectories using a Hobson Sperm Tracker 7V3B (Hobson Vision Ltd., Baslow, UK) operating at 50Hz within IBM-compatible computer in Institute of Zoology. The distance in the field of view was

calibrated using a calibrated slide (100 x 0.01=1 mm, Pysers-Sgi, Ltd, Tonbridge, Kent, UK) and videotaped prior to each experiment. A diagram showing the equipments used and the workflow during sperm tracking using computer-aided sperm analysis (CASA) system is shown in Figure 2.1. Computer-aided sperm analysis has been shown to be an objective tool for studying sperm motility in fish (Kime et al., 1996, 2001).

The Hobson Sperm Tracker gate parameters were adjusted for tilapia sperm. The search radius used was 5.94 μm with minimum and maximum cell size of 5.9 μm and 11.9 μm , respectively. The final settings were then saved as a disk file, which was then retrieved when each experiment was undertaken. Summary table showing gate parameters used for Hobson Sperm Tracker are shown in Tables 2.2. Sperm trajectories derived from Hobson sperm tracker were exported and remotely sorted and analyzed using the appropriate parametric and non-parametric statistical test.

Twenty individual spermatozoa were analyzed per male for their trajectories quantitatively for each of the treatment. The descriptors of sperm trajectories are summarized in Table 1.1 (Chapter 1). Sperm trajectories were quantified for the first 15 sec following the period of 5 sec of activation, as the most sensitive period of movement for sperm was at 5 to 20 sec after activation (Rurangwa et al., 2001). All experiments were performed in triplicates.

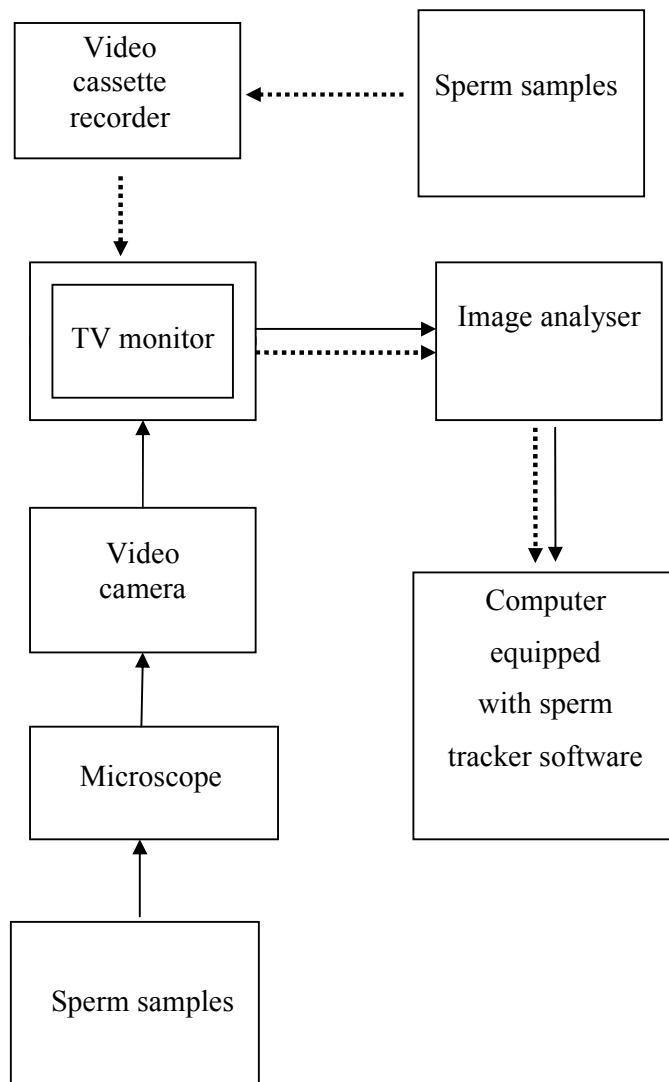


Figure 2.1 A schematic diagram of equipments used for computer-aided sperm analysis, CASA. *Single lines* denote the output flow from none tape-recorded samples directly observed under microscope; *Dashed lines* denote the output flow from tape-recorded samples

Table 2.2: Gate parameters of Hobson sperm tracker

Parameter	setting			
Search radius	5.94 μm			
Thresholds	+30/-100			
Reference time	1 sec			
Process window	0.8 sec			
Immotile process shape				
	Maximum size	11.9 μm		
	Minimum size	5.9 μm		
Video enhancement				
	Ratio	1.0		
	Brightness	121		
	Contrast	52		
Filter weightings	1:2	2:3	3:1	4:1
On time	0 min 15 sec			
Minimum t, time	1.4			
Maximum t, time	100			
Trails	Normal			
Off time	0 min 0 sec			
Trail draw	4 sec			

2.1.5 Measurement of seminal plasma osmolality and pH

Osmolality of samples was measured with an osmometer (3MO PLUS Advance Instrument, USA) and expressed as mOsmol kg⁻¹. Calibration of the osmometer was carried out routinely using standard solution of 50 mOsmol/kg, 800 mOsmol/kg and clinitrol reference solution, 290 mOsmol/kg (Advanced Inst. Inc., MA, USA) as well as deionised distilled water (0 mOsmol/kg). Twenty µl samples were injected with injection sampler into the sample chamber and the reading was noted. As only a minute amount of sample obtained, pH indicator strip papers (Type CS, pH 6.0-8.1, Whatman Int. Ltd., England) were used to determine the pH of seminal fluid collected.

2.1.6 Determination of ionic composition in seminal plasma

Twenty µl of samples or blank were digested at 80°C with 100 µl HNO₃ (Aristar 69%) in clean tubes until the solutions became clear. The samples were then stored at 4°C until further analysis. The properties of the determined element/ion for flame spectrophotometer were shown as below (Table 2.3).

Table 2.3: Features of each element for PERKIN ELMER 2280 for flame spectrophotometry

Element	Wavelength	Absorbance	Slit width
Na	589.2	0.23	0.7
K	766	0.22	2.0
Ca	423	0.20	0.7
Mg	285	0.19	0.7

2.1.7 Sperm morphology

Sperm morphology was evaluated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, all abnormalities on any spermatozoon observed were counted and then were divided into 2 groups that is normal spermatozoa and abnormal spermatozoa (abnormalities of sperm head and acrosome, coiled sperm tail, etc.). Morphological abnormalities were expressed as a

percentage of the total number of all counted spermatozoa. Ultrastructural sperm morphology was evaluated by TEM and was photographed with a TECNAI G2 Spirit BioTWIN (FEI Company, USA).

2.2 Preparation of experimental solutions

Analytical grade reagents were used in all experiments conducted. Deionised distilled water (Milli-Q water purification system, Millipore) was used for preparation of stocks solutions and experimental solutions. The concentration of solutions for ion channel studies and pollutants studies were chosen accordingly to those commonly used in the literature. Stock solutions for ion channel studies and pollutants were prepared fresh, immediately prior to each experiment.

2.2.1 Ion channel studies

2.2.1.1 Lanthanum

Lanthanum chloride heptahydrate (Lot No. 0230K1088, FW: 371.38, Sigma, St Louis, USA) was used to prepare the stock solution. Approximately 3.7 mg of lanthanum was added to 10.0 ml of deionised dilution water to produce 1.0 mM stock solution. An appropriate amount of this stock solution was mixed with deionised distilled water to prepare working solutions of 5.0 μ M, 10.0 μ M, 25.0 μ M and 50.0 μ M.

2.2.1.2 Flunarizine

Flunarizine dihydrochloride (Lot No. 117H4642, FW: 477.4, Sigma, St Louis, USA) was used to prepare the stock solution. Approximately 4.8 mg of flunarizine was added to 10.0 ml of deionised distilled water to produce 1.0 mM stock solution. An

appropriate amount of this stock solution was mixed with deionised distilled water to make up working solutions of 5.0 μM , 10.0 μM , 25.0 μM and 50.0 μM .

2.2.1.3 Amiloride

Amiloride hydrochloride hydrate (Lot No. 064K0743, FW: 266.1, Sigma, St Louis, USA) was used to prepare the stock solution. Approximately 2.7 mg of amiloride was added to 10.0 ml of deionised distilled water to produce 1.0 mM stock solution. An appropriate amount of this stock solution was mixed with deionised distilled water to make up working solutions of 10.0 μM , 50.0 μM , 100.0 μM and 200.0 μM .

2.2.1.4 Quinine

Quinine (Lot No. 13224E0194, FW: 324.44, Aldrich, Germany) was used to prepare the stock solution. Approximately 32.444 mg of quinine was added to 1.0% ethanol in 1.0 ml deionised distilled water to produce 10.0 mM stock solution. An appropriate amount of this stock solution was mixed with deionised distilled water to make up working solutions of 10.0 μM , 100.0 μM , 500.0 μM and 1000.0 μM .

2.2.1.5 Ouabain

Ouabain (Lot No. 074K1426, FW: 584.7, Sigma, St Louis, USA) was used to prepare the stock solution. Approximately 5.847 mg of ouabain was added to 1.0 ml of deionised distilled water to produce 10.0 mM stock solution. An appropriate amount of this stock solution was mixed with deionised distilled water to make up working solutions of 1.0 μM , 10.0 μM , 50.0 μM and 100.0 μM .

2.2.2 Pollutant studies

2.2.2.1 Cadmium

Cadmium chloride (Lot No. 10064, FW: 228.35, AnalaR BDH, England) was used to prepare the stock solution. Approximately 0.10 mg of anhydrous cadmium chloride was added to 10.0 ml of deionised distilled water to produce 10.0 mg/l (or 0.044 mM as Cd^{2+}) stock solution. An appropriate amount of this stock solution was mixed with mannitol solution, in order to maintain the osmolality at 300 mOsmol/kg or mixed with deionised distilled water to make up the working solutions of 10.0 $\mu\text{g/l}$ (0.04 μM), 50.0 $\mu\text{g/l}$ (0.22 μM), 100.0 $\mu\text{g/l}$ (0.44 μM) and 500.0 $\mu\text{g/l}$ (2.20 μM).

2.2.2.2 Malathion

Malathion (Lot No. 3126X, FW: 330.36, Riedel de Haen) was used to prepare the stock solution. Approximately 0.10 mg of anhydrous malathion was added to 10.0 ml of deionised distilled water to produce 10.0 mg/l (0.303 mM) stock solution. The appropriate amount of this stock solution was mixed with mannitol solution, in order to maintain the osmolality at 300 mOsmol/kg or mixed with deionised distilled water to make up the working solutions of 0.1 $\mu\text{g/l}$ (0.003 μM), 1.0 $\mu\text{g/l}$ (0.03 μM), 10.0 $\mu\text{g/l}$ (0.3 μM) and 50.0 $\mu\text{g/l}$ (1.5 μM).

2.2.2.3 Rotenone

Rotenone (Lot No. 45656, FW: 394.42, Riedel de Haen) was used to prepare the stock solution. Approximately 0.10 mg of rotenone was added to 10.0 ml of deionised distilled water to produce 10.0 mg/l (0.025 mM) stock solution. An appropriate amount of this stock solution was mixed with mannitol solution, in order to maintain the osmolality at 300 mOsmol/kg or mixed with deionised distilled water to make up the

working solutions of 5.0 µg/l (0.013 µM), 10.0 µg/l (0.025 µM) , 25.0 µg/l (0.063 µM) and 50.0 µg/l (0.13 µM).

2.3 General statistical analyses

Motility score was assessed in two ways: 1) The number of motile spermatozoa was expressed as a percentage of the total number of spermatozoa following 10 sec period of activation; 2) The number of motile spermatozoa was expressed as a percentage of the total number of spermatozoa following 10 sec period of activation on a given time point. The scoring in these two categories was based on a subjective scale between 0 and five, zero being no motility and five maximum (80-100%) following Suquet et al. (1992) and Fauvel et al. (1999). Test of motility scores of spermatozoa was carried out in triplicates. Significant differences among groups were determined using the Kruskal–Wallis test. Post-hoc comparisons were conducted with Mann–Whitney U tests. For sperm morphology analysis, mean values of the measured parameters and standard deviation (s.d.) were calculated per sample. Group difference on data from sperm morphology were analysed by Kruskal-Wallis. Effects of dilution rate, osmolality, pH and ion channel inhibitors on a given sperm trajectories assessed by CASA technique as described in detail in Section 3.2.6. Similarly, the effects of pollutants on a given sperm trajectories were assessed by CASA technique as described in details in Section 4.2.

CHAPTER 3 SPERM QUALITY OF NILE TILAPIA

3.1 Introduction

Gamete quality is strongly influenced by broodstock wellbeing and nourishment. Consequently, poor gamete quality can result in embryo mortality. This is one of the main problems encountered by fish culturists worldwide. Since greater emphasis is placed on the study of female broodstock, with an emphasis on fertilization and embryogenesis, these problems have been largely explained by low quality of female gametes. In these respects, research efforts on male gamete quality for maximising sperm fitness and reproductive success should be given equal effort.

Although assessment methods of sperm quality based on fertilisation rate of eggs are widely used, these methods may not always be practical due to constraints on the availability of eggs, which are produced in lower amounts than sperm and are required for culture purposes. Furthermore, in fertilization experiments for sperm quality assessment, it is not always easy to control the sperm-egg ratio, a prerequisite for rigorous evaluation (Kime et al., 2001).. Thus, it is important to develop methods of sperm quality evaluation that can be applied to a batch of sperm without the need for eggs. This would enable the sperm to be analyzed prior to fertilization and circumvents the difficulties of using eggs in fertilization tests (Rurangwa et al., 2004). As there is a strong positive correlation between fertilization rate and sperm motility (Rurangwa et al., 2001), sperm motility can be used independently to assess sperm quality.

3.1.1 Sperm quality in fish

As discussed, the ultimate measure of sperm quality is defined as the ability of sperm to fertilise an egg (Rurangwa et al., 2004). However, given the constraints on egg availability, sperm quality can alternatively be determined through assessment of seminal plasma osmolality, sperm cell density/concentration, seminal plasma pH, seminal plasma ion composition, sperm motility and sperm morphology (Table 3.1). Knowledge on chemical and physical characteristics of the seminal fluid is necessary to understand the physiology of spermatozoa. In addition, the information is also important for insemination and sperm storage purpose.

Seminal plasma has a specific composition regarding the presence of substances which support the viability of spermatozoa and substances which ensure reproductive function (Piironen and Hyvarinen, 1983; Lahnsteiner et al., 1993). The correlation between the composition of the seminal plasma and the motility of spermatozoa has been investigated only in few species: mostly Salmonidae (Hwang and Idler, 1969), Cyprinidae (Kruger et al., 1984; Lahnsteiner et al., 1996) and Acipenseridae (Toth et al., 1997).

Table 3.1: Sperm quality biomarkers in fish species

Species	Sperm quality biomarker	References
Rainbow trout	Motility, DNA fragmentation, fertilising ability	Dietrich et al., 2005
Striped Bass	Duration of motility and percentage of motile cells	Castranova et al., 2005
Stickleback	CASA and fertilization	Le Comber et al., 2004
Turbot	Sperm concentration, motility score and duration	Piferrer et al., 2004
Catfish	Spermotocrit, sperm concentration, percentage of motile cells, fertilising ability	Viveiros et al., 2000
Turbot	Osmolality, pH, ions, glucose, urea and protein content	Dreanno et al., 1998
Carp	Percentage of motile cells, swimming speed, flagellar beat frequency and sperm ATP content	Perchee et al., 1998
White bass	Volume, sperm concentration, percentage of motile cells and fertilising ability	Mylonas et al., 1997

3.1.2 Sperm motility score

Albeit subjective, the estimation of the percentage of motile sperm in a semen sample is the most common laboratory assay utilised on a routine basis. Sperm motility is usually estimated in on an arbitrary scale or index ranging from 0 to 5 (Billard, 1978; Suquet et al., 1998; Liley et al., 2002; Fitzpatrick et al., 2005). Sperm that are motile can be observed under 100x or 200x magnification of dark field microscopy with standard microscope slides or may be counting using a hemocytometer and tally counter (Stoss, 1983).

Spermatozoa of fish species with external fertilization are motile the instant they enter the spawning or aquatic environment. In salmon, motility ceases after 15 sec (Billard and Breton, 1976) and up to 2-3 min in other species (Billard, 1978; Chowdhury and Joy, 2001; Burness et al., 2005). Sperm longevity can be evaluated by periodically sampling semen in activation solution and measuring the duration of sperm motility. Osmolality (Billard and Cosson, 1990) and ionic composition (Cosson et al., 1991; Morita et al., 2003) are reported to influence sperm motility duration in freshwater fish species.

3.1.3 Computer-aided sperm analysis (CASA)

Computer-aided sperm analysis, widely known as CASA, has increasingly become one of the important means to determine sperm quality in domestic mammals (Farrell et al., 1998; Hirano et al., 2001) and in fish (Cosson et al., 1985; Kime et al., 1996, 2001). It enables rapid and subjective quantification of sperm movement characteristics. In addition, CASA has been used as important tool for ecotoxicological studies in fish sperm (Van Look et al., 2000; Rurangwa et al., 2004) as well as for cryopreservation studies (Rurangwa et al., 2001).

Compared to the more subjective method of visual motility score determination, CASA provides the classification several different motion parameters of spermatozoa. In fact, CASA parameters have been modelled and refined mathematically to describe the spermatozoa motion as it moves through a microscopic field (Boyers et al., 1989). Many reproductive studies conducted on African catfish, carp, goldfish, roach, Eurasian perch, trout, lake sturgeon, using sperm tracking systems have reported that the most useful parameters of sperm motility are the curvilinear velocity (VCL, the actual velocity along the trajectory) and the straight line velocity (VSL, the straight line distance between the start and end points of the track divided by the time of the track) (Ciereszko et al., 1996; Kime et al., 2001; Rurangwa et al., 2001, 2002). The average path velocity (VAP, the velocity along a derived smoothed path) is reported to be of little use in most fish sperm studies in contrast with mammalian spermatozoa, as the generated tracks are smooth curves in fish sperm, making both VAP and VCL identical (Rurangwa et al., 2001). Furthermore, as teleost spermatozoa exposed to pollutants showed increasingly curved sperm trajectories, the linearity (LIN, the ratio of net distance moved to total path distance (VSL/VCL)) can be a very useful indicator of curvature of the trajectory (Kime et al., 2001; Rurangwa et al., 2001). Importantly, several studies have reported a positive correlation between sperm motility variables of CASA and fertilization success in fish (Lahnsteiner et al., 1998; Linhart et al., 2000).

3.1.4 Mechanism of fish sperm activation

In mammals, sperm cells released into the lumen of the seminiferous tubules in the testis are immotile and incompetent to interact with the oocyte and its zona pellucida (Toshimori, 1998). The mammalian sperm cells require two physiological maturation phases in order to acquire optimal fertilization properties: i) epididymal maturation, a

process in which several surface protein and lipid modifications take place (Toshimori, 1998), which results in the generation of sperm cell motility (Amann et al., 1982); ii) further surface modifications and activation in the female genital tract, particularly in the lumen of the oviduct (Yanagimachi, 1994). *In vitro* activation of sperm cells triggers diverse signalling pathways such as cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) and induced protein tyrosine phosphorylation (Visconti et al., 1995) and leads ultimately to the generation of sperm cells with high binding affinity for the zona pellucida.

Fish spermatozoa, in contrast to mammalian spermatozoa, are immotile in the seminal plasma. The spermatozoa only become motile when they are in contact with the external medium, either freshwater or saltwater. However, some exceptions have been demonstrated in a few marine species such as herring, *Clupea harengus membras* of the Baltic Sea. Herring spermatozoa are motionless in seawater and only becoming motile near the micropyle region of the egg upon physical contact (Griffin et al., 1998). On the other hand, in salmonid fishes such as rainbow trout, *Oncorhynchus mykiss*, sperm activation is triggered by alteration of ionic composition where a decrease in extracellular potassium in the spawning medium result in a potassium efflux and an increment of intracellular calcium (Tanimoto et al., 1994). Although some aspects of teleosts sperm activation has been comprehensively studied, little effort has been focussed on the mechanism of *O. niloticus* spermatozoa activation and their morphological changes associated with sperm activation.

Environmental cues activate sperm motility through transduction events involving ion channels (Quill et al., 2006). Various systems of ion channels in sperm have been well

described in invertebrates (Labarca et al., 1996; Nishigaki, et al., 2004; Schulz, et al., 2004) and in some vertebrates (Beltran et al., 1994; Espinosa et al., 1998).

In sea urchin sperm, an increase in internal sodium, activates a sodium-hydrogen pump that increases the intracellular pH, which further stimulates sperm cell motility through cAMP-dependent phosphorylation processes (Labarca et al., 1996; Sanchez et al., 2001).

Only a few studies to date have been carried out to study the role of ion channels during sperm activation in teleosts. For example, calcium influx has been suggested as an important activation process following experiments which reversibly inhibited calcium channels with specific blockers (eg verapamil and conotoxin) during sperm activation in carp (Krasznai et al., 2000). Stretch-activated membrane channels and sodium-calcium exchanger have also been suggested to influence sperm activation of carp (Krasznai et al., 2003). Similarly, a membrane bound sodium-calcium exchanger has been identified on the surface of the midpiece region of herring spermatozoa (Vines et al., 2002). Thus, in both marine and freshwater fish, it seems that calcium channels and sodium calcium exchangers probably regulate sperm motility.

3.1.5 Morphology of fish spermatozoa

The morphology of fish spermatozoa has become a subject of interest from a taxonomic point of view in the past few years (Jamieson, 1991; Marco-Jiminez et al., 2006). It is suggested that the divergence in fish sperm ultrastructure are influenced mainly by phylogenetic factors (Cruz-Landim et al., 2003) although some reported that the

morphology of the spermatozoa is dependent on the reproduction mode and the fertilization environment (Eckelberger and Young, 2002; Vladic et al., 2002).

Ultrastructural studies of the spermatozoa in many fish species shown that in general the organization of the spermatid organelles are conserved in members of the same fish family or subfamily (Jamieson, 1991; Abascal et al., 2002; Quagio-Grassiotto et al., 2003; Gwo et al., 2005). In taxonomically unresolved groups, such as the Perciformes, spermatozoa can display a great variety of spermatozoon types, structures and spermiogenesis processes (Jamieson, 1991).

The spermatozoa of many fish species, particularly teleosts, have been extensively examined by electron microscopy (Jamieson, 1991; Gwo et al., 2004, 2005; Marco-Jimenez et al., 2006). Spermatozoa in most teleosts are generally of basic form, consisting of a short, spherical head without an acrosome, a short midpiece containing a few numbers of mitochondria and a tail with a simple flagellum (Stoss, 1983; Jamieson, 1991). Apart from studies of Chao et al. (1987) and Don and Avtalion (1993), no information on tilapia sperm morphology particularly of *Oreochromis niloticus* is available.

3.1.6 Aims of study

Manipulation of sperm *in vitro* either for storage or artificial insemination requires the determination of factors (internal or external) that regulates the initiation of their motility. Therefore, the present study aims to examine the external factors that may contribute to sperm activation in *O. niloticus* using CASA and to identify the type of ion

channels involved during sperm cell activation through manipulation of ion channel blockers. Thus, the specific objectives of this chapter were set:

- i. to determine the physiochemical characteristic of *O. niloticus* seminal fluid, and the effect of stripping frequency on physiochemical characteristic of seminal fluid in relation to osmolality and sperm density;
- ii. to determine factor (s) that trigger sperm activation by studying the effect of dilution rate, osmolality of non electrolytes and electrolytes solution, and pH by using motility score and CASA;
- iii. to identify ion channel(s) that may play important role in sperm activation by looking at the effects imposed on sperm motility characteristics upon using selected ion channel inhibitors, and
- iv. to determine the morphological alteration during sperm activation by observation using electron microscopy.

3.2 Materials and methods

3.2.1 Physiochemical characteristics of seminal fluid

3.2.1.1 Seminal plasma osmolality

To obtain seminal plasma, seminal fluid samples were centrifuged at 7000 rpm (5625 g) at 4° C (Eppendorf Centrifuge 5417R, Eppendorf AG, Germany) for 20 mins to pellet the sperm. Seminal plasma collected was re-centrifuged to prevent any presence of sperm which this was further verified by observation under the microscope. The osmolality was measured with a microosmometer (3MO Advance Inst. USA) of depressing freezing point and expressed as mOsmol/kg.

3.2.1.2 Density of spermatozoa

One µl of seminal fluid samples was diluted (1:500, v/v) with modified fish ringer's and a volume of 10 µl was placed on the hemocytometer chamber at x200 magnification, covered with cover slip and number of cells observed were counted with a tally counter.

3.2.1.3 Ionic composition

Seminal plasma, contaminated seminal plasma (samples with motile cells score of 5), washed sperm cells and a batch of tank water were sampled and subjected to ion analyses (Na, Ca, K, Mg) using flame spectrophotometer. All glasses equipment for ionic measurement purpose were previously soaked in 10% HNO₃ Aristar (69%) for 24 hrs, and rinsed twice with deionised water to minimise the possibility of ionic contamination (Gaspic et al., 2002).

3.2.2 Effect of seminal fluid stripping frequency on osmolality and sperm density

The tag fishes were grouped into 4 with 4 individual fish each. The first group composed of fishes that hand-stripped twice for 8-10 days, the second group composed of fishes that hand-stripped twice for 18-20 days, the third group composed of fishes that hand-stripped twice for 28-30 days and the fourth group composed of fishes that hand-stripped twice for 48-50 days. Fish weights were also determined. Seminal fluids collected were examined for osmolality and sperm density (Section 3.2.1.1 and 3.2.1.2).

3.2.3 Sperm motility assay

For motility score, seminal fluid samples were diluted directly in various test solutions (i.e. immobilising medium or extender and activation medium). Five μl of diluted aliquot samples were immediately placed on a clean glass slide, observed without cover slip at x200 magnification by using inverted microscope. The proportion of motile sperm was scored from 0-5 with 0 representing all immotile, 1: 0-20%, 2: 20-40%, 3: 40-60%, 4: 60-80% and 5: 80-100% motile (Suquet et al., 1992; Fauvel et al., 1999). Duration of sperm motility i.e. the time taken from activation until all the movement stopped, was determined using motility score, measured at various time point (0, 30, 60, 180, 360 and 540 sec). Osmolality of immobilising solutions was adjusted to approximately 300 mOsmol/kg using mannitol and modified fish Ringer's solution was used as a control unless otherwise stated. Deionised distilled water was used as activation medium. All observation of each samples were done in triplicates.

For CASA analysis using Hobson Sperm tracker, 1 μl of seminal fluid was diluted 500-fold with test solutions in an eppendorf tube and a volume of 5 μl of the diluted seminal fluid was immediately pipetted into one well of a 12-well multitest glass slide (ICN

Biomedicals Inc., Basingtoke, UK), quickly covered with a coverslip and immediately videotaped for up to 60 sec. For CASA analysis, the following sperm motility variables were examined: straight line velocity, VSL ($\mu\text{m}/\text{sec}$); beat cross frequency, BCF (Hz) and percentage of motile sperm, MOT (%) except in ion channel inhibitors experiment where VSL($\mu\text{m}/\text{sec}$), VCL ($\mu\text{m}/\text{sec}$) and LIN (%) were determined. CASA experiments on sperm motility was assessed at room temperature (18-20°C) using light microscopy at 20X negative-high phase contrast objective via a U-PMTVC adaptor (Olympus) and recorded with video cassette recorder (SONY-SLVD950G/I, SONY Corporation). This type of optic gives an even contrast to the head of fish spermatozoa during progressive motility. The Hobson Sperm Tracker gate parameters were adjusted for tilapia sperm. The search radius used was 5.94 μm with minimum and maximum cell size of 5.9 μm and 11.9 μm , respectively. The final settings were then saved as a disk file, which was then retrieved when each experiment was undertaken. The recordings were analyzed for sperm trajectories using a Hobson Sperm Tracker 7V3B (Hobson Vision Ltd., Baslow, UK) operating at 50Hz within IBM-compatible computer. The distance in the field of view was calibrated using a calibrated slide (100 x 0.01=1 mm, Pyser-Sgi, Ltd, Tonbridge, Kent, UK) and videotaped prior to each experiment.

3.2.3.1 Effect of dilution rate on sperm motility

A volume of 1 μl seminal fluid samples was mixed with deionised distilled water at various dilution ratio (250; 500; 750; 1000, v/v) and sperm motility was immediately determined as mentioned in subchapter 3.2.3. Comparisons of sperm motility were also made between deionised distilled water and tank water.

3.2.3.2 Effect of osmolality of non-electrolytes and electrolytes solution on sperm motility

To test the effect of osmotic pressure on sperm activation, a volume of 1 μ l seminal fluid samples was mixed with mannitol (1:500, v/v) and modified fish Ringer's solution (1:500, v/v) at osmolality ranging from 0 to 300 mOsmol/kg. Sperm motility was determined as mentioned in Section 3.2.3.

3.2.3.3 Effect of pH on sperm motility

Hepes-buffer solutions (10 mM with 16 mosmol/kg) were prepared using HCl or NaOH at various pH (6, 8, 10). A volume of 1 μ l seminal fluid samples was diluted 500-fold in the buffered solutions and sperm motility was immediately determined as mentioned in Section 3.2.3.

3.2.4 Effect of membrane ion channel inhibitors on sperm motility

Lanthanum, flunarizine, amiloride, ouabain and quinine were chosen to study the effect of ion channel blockers on sperm activation. In these trials, spermatozoa was directly exposed to the required concentration of ion channel inhibitors solutions as selected (Chapter 2.2.1). A volume of 1 μ l seminal fluid samples was diluted 500-fold in deionised distilled water containing the required concentration of ion channel inhibitors and videotaped for CASA as described above (Section 3.2.3).

3.2.5 Effect of activation on sperm morphology

A volume of 1 μ l seminal fluid was diluted and activated at 500-fold in deionised distilled water and sub sampled at various time point (0, 30, 60, 120, 480 sec), immediately fixed and observed for morphological changes. Sperm morphology was

evaluated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For these trials, three individual fish samples were sampled.

For SEM, samples were immediately collected on 12 µm thick polyester hydrophilic cyclopore membrane (Whatman Int. Ltd, England) and processed as followed (Nation, 1983). Briefly, sperm were fixed for 1 hour in 1.0% glutaraldehyde buffered with sodium cacodylate 0.1 M at 4° C and then with 3.0% gluteraldehyde for 2-3 days, rinsed and post fixed with 1.0% osmium tetroxide for 2 hours. After dehydration through an ascending ethanol series and hexamethyldisilazine (HMDS), samples were dried and gold coated. Samples were examined with JSM 6460 LV JEOL (JEOL Company). The percentage of normal and abnormal sperm (abnormalities of sperm head and acrosome, coiled sperm tail, etc.) were calculated from the total of 100 sperm observed, starting from the centre of the SEM grid.

Ultrastructural sperm morphology was evaluated by transmission electron microscopy (TEM). The activated samples were immediately fixed with 2.5% glutaraldehyde, centrifuged at 7000 rpm (5625 g) to pellet the cells and processed as followed (Hayat, 1986). Briefly, sperm cells were fixed for 2 hours in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 4°C and rinsed in buffer overnight. The samples were then postfixed for 1 hour in 1.0% osmium tetroxide, enbloc stained with 2% uranyl acetate in 30% acetone, washed and subjected to dehydration in acetone series. Samples were then embedded in Spurr resin and 90 nm (gold sections, Reichert Ultracut E, Leica) were mounted on 200 mesh formvar coated copper grids and stained with 4% uranyl acetate in 50% ethanol and Reynold's lead citrate. Diameter of nucleus and mitochondria were

measured from longitudinal sagittal section of spermatozoa at 26500x using TEM TECNAI G2 Spirit BioTWIN (FEI Company, USA).

3.2.6 Statistical analyses

All data for sperm motility score and CASA analysis except for sperm morphology, were obtained from 5 different individual samples each of which was run in triplicates. Prior to analysis, all the data were tested for normality and homogeneity in variances. For sperm motility assay using motility score and CASA analysis, the value was determined after 10 sec and 5 sec of activation, respectively. Significant differences among groups were determined using one way ANOVA or Kruskal–Wallis test. Post-hoc comparisons were conducted with Tukey test or Mann–Whitney U tests. Significance level was set at $P < 0.05$.

For sperm morphology analysis, the data were first tested for normality using a Kolmogorov-Smirnov test. Mean values of the measured parameters and standard deviation (s.d.) were calculated per sample. Group difference on data from sperm morphology were analysed by a one-way ANOVA. A probability of $P < 0.05$ was accepted as significant.

Multivariate analyses of individual sperm trajectories (VSL, VCL and LIN) for ion channel inhibitors experiment were carried out using the computer program PATN (CSIRO, Canberra, Australia) following Abaigar et al. (1999). Data sets for analysis were prepared by merging raw data files from every measured sperm sample. In the present study, the groups were distinguished on the basis of multivariate combinations of motion parameters. Once the sub-populations had been identified, the effect of treatment/time combinations on the frequency of the spermatozoa belonging to the fast-

linear subpopulation (only) was analyzed by one-way ANOVA using general linear models (Statistica version 9.0) after log transformation of percentage data. Post-hoc multiple comparison tests for differences of population means were performed using Tukey test. Data were expressed as the mean \pm standard deviation (s.d.). A probability of $P < 0.05$ was accepted as significant.

3.3 Results

3.3.1 Physiochemical characteristics of seminal fluid

Seminal fluid of *Oreochromis niloticus* is creamy fluid of white to grey, with viscous consistency. Creamy white seminal fluid usually shows higher sperm density and osmolality value. The average pH of seminal fluid is around 7.4 (\pm 2.0). Average density of spermatozoa on the other hands was 3.59×10^9 cells/ml with highest and lowest density obtained at 7.75×10^9 cells/ml and 6.83×10^8 cells/ml respectively. Mean osmolality of seminal plasma was $305 (\pm 9)$ mOsmol/kg for good quality samples of seminal fluid (in terms of motility) and the highest osmolality during the study period recorded was $326.0 (\pm 2.0)$ mOsmol/kg. Contaminated seminal plasma (motility score at 5, 80-100% motile cells with clear transparent fluid with less viscosity) on the other hand has lower osmolality with mean of 60.0 ± 8.0 mOsmol/kg and mean pH of $6.6 (\pm 1.49)$. Summary table showing the characteristic of the seminal fluid is shown in Table 3.2. In the seminal plasma, sodium shows the highest concentration (167.2 ± 3.7 mM) followed by potassium (64.3 ± 0.2 mM), calcium (2.4 ± 1.0 mM) and magnesium (2.0 ± 0.2 mM). A lower concentration of ions was observed in contaminated seminal plasma when compared to seminal plasma of good quality, with exception of Mg where the value remain constant in each type of fluid (Table 3.3).

Table 3.2: Seminal fluid characteristic of *Oreochromis niloticus* (mean \pm s.d.)

Osmotic pressure, mOsmol/kg	305 \pm 9
pH	7.4 \pm 1.2
spermatozoa density, (x 10 ⁹ /ml)	3.59 \pm 0.72

*n=5 fish, 60 spermatozoa each

Table 3.3: Mean (\pm s.d.) comparison of major ion composition (mM) in various types of seminal fluid compartment

Compartment	Sodium (Na ⁺)	Potassium (K ⁺)	Calcium (Ca ²⁺)	Magnesium (Mg ²⁺)
Seminal plasma	167.2 \pm 3.7	64.3 \pm 0.2	2.4 \pm 1.0	2.0 \pm 0.2
Contaminated seminal plasma	37.6 \pm 9.3	5.4 \pm 0.6	1.0 \pm 0.5	2.2 \pm 0.5
Tank water	4.6 \pm 1.0	0.4 \pm 1.0	1.6 \pm 0.5	1.8 \pm 0.6
**Sperm cell	18.7 \pm 5.0	10.1 \pm 0.4	3.4 \pm 0.4	2.0 \pm 0.5

*n=5 fish, 60 spermatozoa each; contaminated seminal plasma refers to seminal plasma with osmotic pressure of 60 mOsmol/kg with motility score of 5; **average sperm density at 3.27 x 10⁹/ml, volume for measurement 20 μ l

3.3.2 Effect of stripping frequency on seminal plasma osmolality and sperm density

The values of seminal plasma osmolality and spermatozoa density were continuously stable over the two stripping times. The spermatozoa density were slightly higher for 28-30 days and 48-50 days group but the values had no significant changes ($P>0.05$; Table 3.4).

Seminal plasma osmolality and spermatozoa density were slightly increased as the fish size increased during sequential stripping of 8-10 days, but no significant changes was observed. The spermatozoa density also remained stable over the stripping times ($P>0.05$; Table 3.5).

Table 3.4: Mean (\pm s.d.) values of seminal plasma osmolality and sperm density (per ml) during sequential stripping from tilapia

Stripping frequency (x10 ⁸)	n	mOsmol/kg		Spermatozoa density	
		1 st collection	2 nd collection	1 st collection	2 nd collection
8-10 days	4	303 \pm 5 ^a	307 \pm 3 ^a	22.6 \pm 4.7 ^a	25.5 \pm 8.5 ^a
18-20 days	4	304 \pm 10 ^a	308 \pm 11 ^a	19.5 \pm 3.5 ^a	29.1 \pm 7.7 ^a
28-30 days	4	300 \pm 8 ^a	303 \pm 8 ^a	32.0 \pm 7.4 ^a	41.7 \pm 10.7 ^a
48-50 days	4	314 \pm 10 ^a	308 \pm 15 ^a	29.0 \pm 6.3 ^a	45.8 \pm 11.9 ^a

*n=number of fish; the values with the same letter are not significantly different

Table 3.5: Mean (\pm s.d.) values of seminal plasma osmolality, sperm density (per ml) and fish size during sequential stripping of 8-10 days from tilapia sampled from January 2005 to June 2005

Month	n	mOsmol/kg	spermatozoa density (x10 ⁸)	Fish size (g)
January	14	300 \pm 11 ^a	34.8 \pm 5.3 ^a	186 \pm 5
March	17	302 \pm 6 ^a	33.1 \pm 7.4 ^a	201 \pm 8
June	10	307 \pm 9 ^a	30.3 \pm 4.9 ^a	224 \pm 9

*n=number of fish; the values with the same letter are not significantly different

3.3.3 Effect of dilution rate on sperm motility

Mean of sperm motility score after 10 sec of activation, determined at various dilution rate are shown in Figure 3.1. Approximately 40–60% of sperm became motile upon activation with deionised distilled water when seminal fluid was diluted at 1:250. At dilutions of 1:500 and 1:750, however, almost 100% of the tilapia sperm were activated, and only 60-80% became motile at 1:1000. Sperm motility score at 250-fold was significantly lower in comparison to 500-fold, 750-fold and 1000-fold (Figure 3.1; $P < 0.05$).

Mean of motility score after 10 sec of activation, at 500-fold of dilution, for deionised distilled water and tank water as activation medium were determined at various motility time point are shown in Figure 3.2. Almost 100% of sperm were activated from 0 up to 180 sec, however, only 40% and less than 20% were motile at 360 and 540 sec, respectively. In both activation medium of deionised distilled water and tank water, sperm motility score at 0, 30, 60 and 180 sec were significantly higher in comparison to 360 and 540 sec ($P < 0.05$; Figure 3.2).

The effects of dilution on mean sperm motility variables determined by CASA are shown in Figure 3.3. Mean (\pm s.d) VSL were 21.16 (\pm 4.89) $\mu\text{m}/\text{sec}$, 21.96 (\pm 5.26) $\mu\text{m}/\text{sec}$, 20.66 (\pm 6.313) $\mu\text{m}/\text{sec}$ and 20.45 (\pm 3.15) $\mu\text{m}/\text{sec}$ after 5 sec of activation in

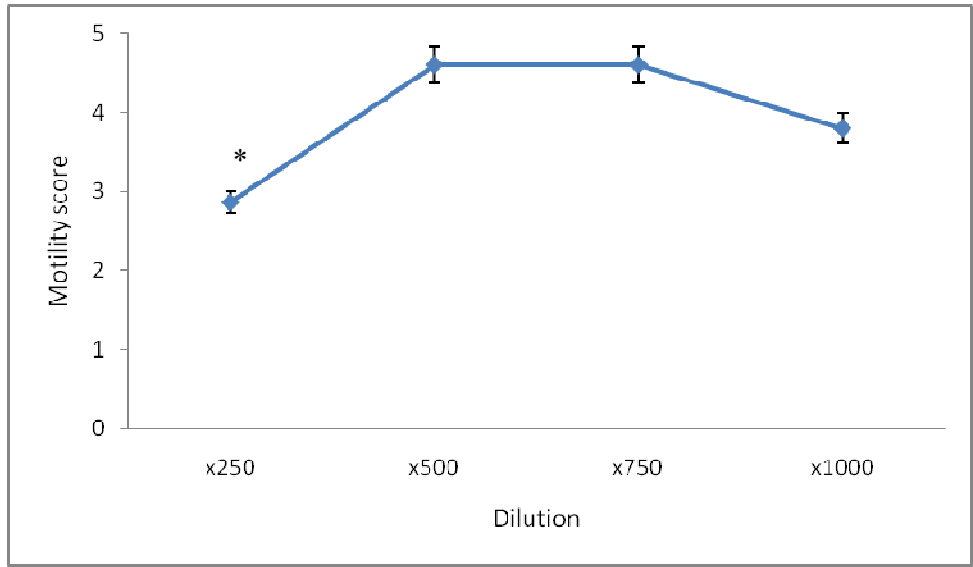


Figure 3.1. Effect of dilution rate on sperm motility score, determined at 10 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; *means are significantly different at $P < 0.05$

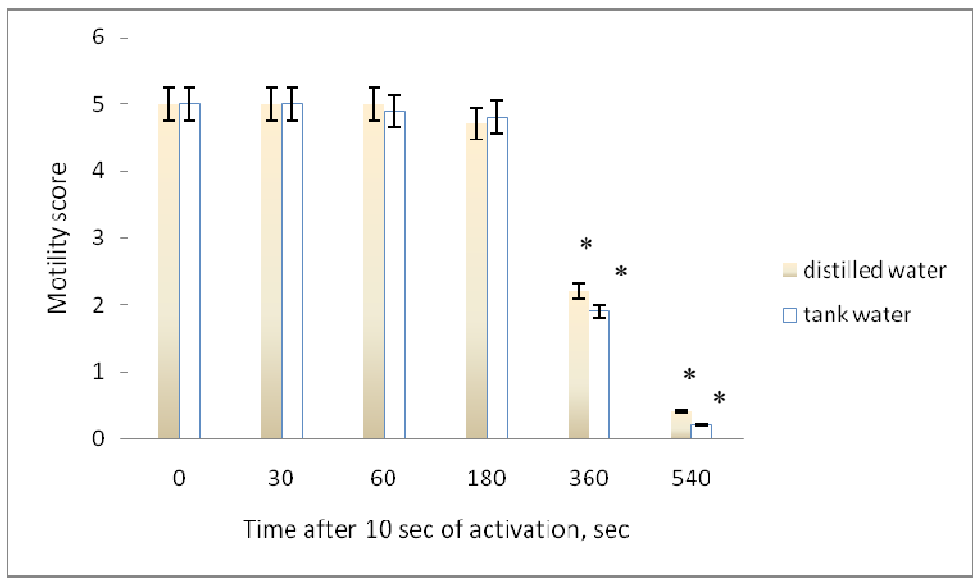


Figure 3.2: Effect of tank water and deionised distilled water on sperm motility score and motility duration determined until 540 sec. Values are expressed as mean (\pm s.d) of 5 fish; *means are significantly different at $P < 0.05$

distilled water for 250, 500, 750 and 1000-fold dilution, respectively, where the mean were not statistically significant ($P>0.05$). In contrast to VSL, mean BCF (\pm s.d.) was slightly decreased at 1000-fold dilution (14.94 ± 3.32 Hz), in comparison to 250-fold (16.68 ± 4.05 Hz), 500-fold (17.50 ± 3.11 Hz) and 750-fold dilution (17.32 ± 3.53 Hz) but no significant different was found ($P>0.05$). The percentage of motile sperm (MOT) was 91.98% (± 6.48), 95.42% (± 5.15), 93.23% (± 6.49) and 89.76% (± 7.31) at dilution rates of 250, 500, 750 and 1000-fold, respectively. The mean differences, were however not statistically significant ($P>0.05$; Figure 3.3).

Mean of motility variables after 5 sec of activation, at 500-fold of dilution, for deionised distilled water and tank water as activation medium are shown in Figure 3.4. In general, mean differences of VSL, BCF and MOT were not significantly different ($P>0.05$) when compared between distilled water and tank water.

These results imply that dilution rate in the range of 250-fold to 1000-fold did not influence the sperm motility variables of *O. niloticus* after 5 sec of activation in contrast to the result of sperm motility score. However, as the dilution rate of 500-fold showed the highest values in both sperm motility score and motility variables, this dilution rate was selected for further experiments. Similarly, as both deionised distilled water and tank water showed comparable results, deionised distilled water was selected for further experiments.

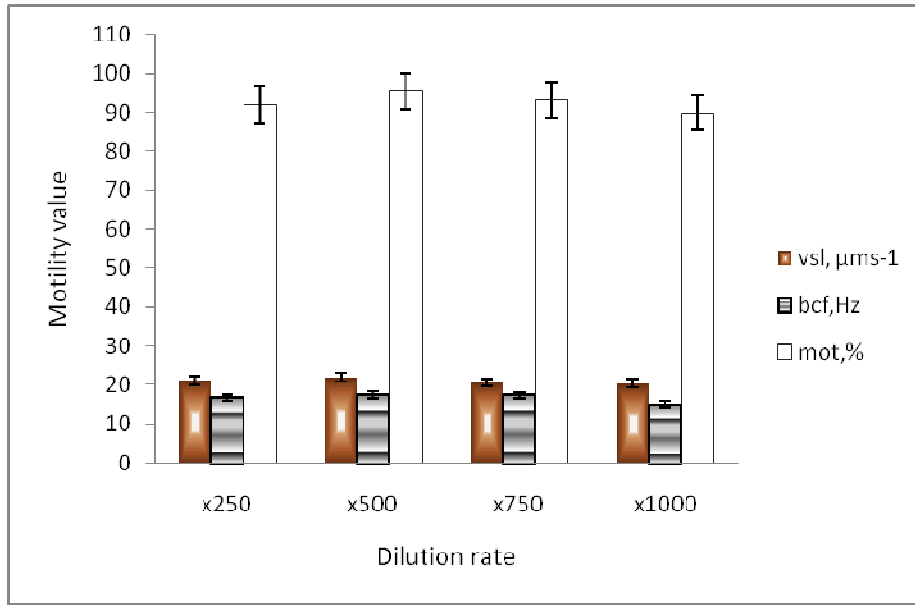


Figure 3.3: Effect of dilution rate on sperm motility variables determined at 5 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; *means are significantly different at $P < 0.05$

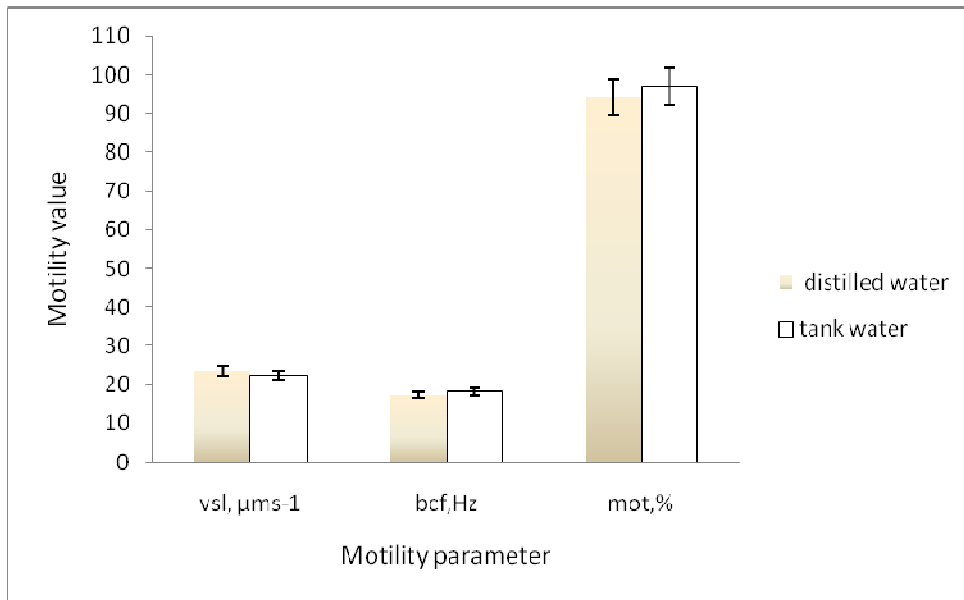


Figure 3.4: Effect of tank water and deionised distilled water on sperm motility variables determined at 5 sec after activation.. Values are expressed as mean (\pm s.d) of 5 fish; * means are significantly different at $P < 0.05$

3.3.4 Effect of osmolality of non-electrolytes and electrolytes solution on sperm motility

Mean of sperm motility score after 10 sec of activation, determined in mannitol at various osmolality range are shown in Figure 3.5. Approximately 80-100% of sperm became motile upon diluted 500-fold in mannitol at 0, 50 and 100 mOsmol/kg. At 150 mOsmol/kg and above, gradually less number of the tilapia sperm were motile. At 300 mOsmol/kg, less than 20% of the sperm were motile. Mean differences were highly significant at 250 and 300 mOsmol/kg (Figure 3.5; $P < 0.05$).

Mean of sperm motility score after 10 sec of activation, determined in modified fish ringer's at various osmolality range are shown in Figure 3.6. Similarly to mannitol, 80-100% of sperm became motile upon diluted 500-fold in modified fish ringer's at 0, 50 and 100 mOsmol/kg. At 150 mOsmol/kg and above, gradually less number of the tilapia sperm were motile. At 300 mOsmol/kg, less than 20% of the sperm were motile. Mean differences were found to be highly significant at 250 and 300 mOsmol/kg (Figure 3.6; $P < 0.05$).

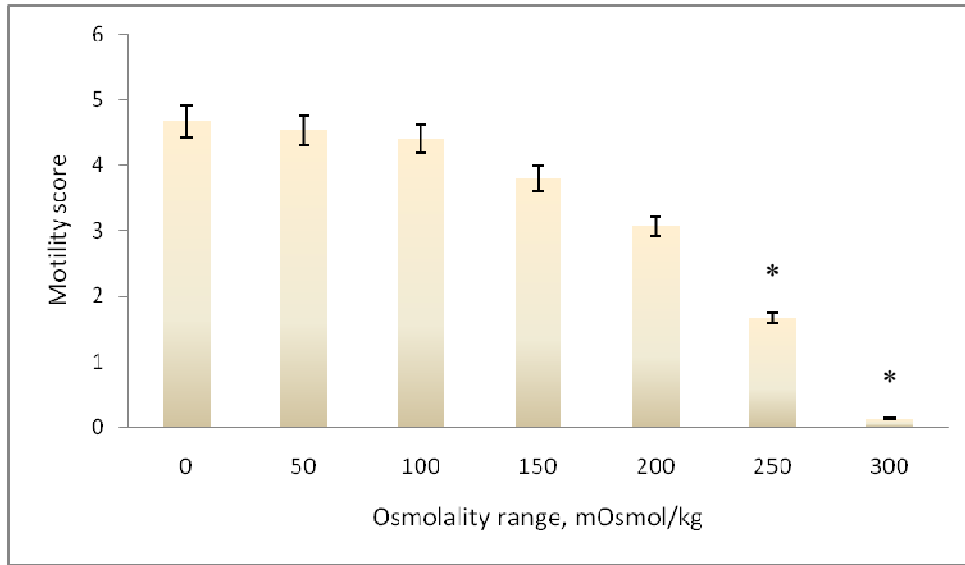


Figure 3.5. Effect of osmolality of mannitol on sperm motility score, determined at 10 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; *means are significantly different at $P < 0.05$

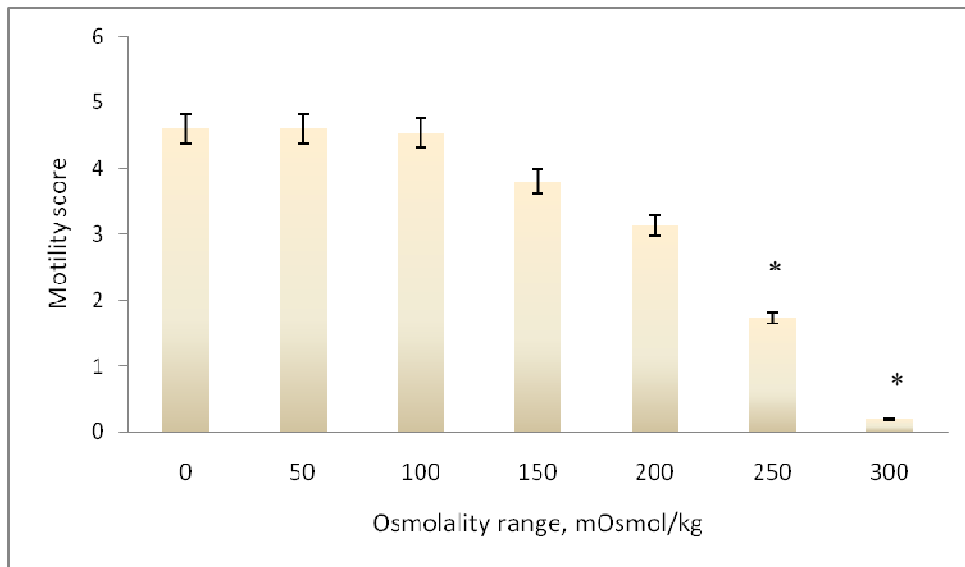


Figure 3.6. Effect of osmolality of modified fish ringer's on sperm motility score, determined at 10 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; *means are significantly different at $P < 0.05$

The effects of osmolality of mannitol on mean sperm motility variables determined by CASA are shown in Figure 3.7. Mean (\pm s.d) VSL were 21.10 (\pm 3.14) $\mu\text{m}/\text{sec}$, 21.57 (\pm 4.31) $\mu\text{m}/\text{sec}$, 21.04 (\pm 6.17) $\mu\text{m}/\text{sec}$, 22.93 (\pm 2.14) $\mu\text{m}/\text{sec}$, 22.92 (\pm 4.14) $\mu\text{m}/\text{sec}$, 18.78 (\pm 4.72) $\mu\text{m}/\text{sec}$ and 4.52 (\pm 2.25) $\mu\text{m}/\text{sec}$ after 5 sec of activation for 0, 50, 100, 150, 200, 250 and 300 mOsmol/kg, respectively, where the means differences were statistically significant at 300 mOsmol/kg ($P < 0.05$; Figure 3.7). Mean BCF (\pm s.d.) was significantly decreased at 300 mOsmol/kg (7.96 ± 1.29 Hz), in comparison to other osmolality ($P < 0.05$; Figure 3.7). Meanwhile means MOT (\pm s.d.), were significantly different at 250 and 300 mOsmol/kg with 65.80% (\pm 10.22) and 6.83% (\pm 0.72), respectively ($P < 0.05$; Figure 3.7).

The effects of osmolality of modified fish ringer's on mean sperm motility variables determined by CASA are shown in Figure 3.8. Mean (\pm s.d) VSL were 21.31 (\pm 1.27) $\mu\text{m}/\text{sec}$, 22.45 (\pm 2.05) $\mu\text{m}/\text{sec}$, 22.71 (\pm 2.88) $\mu\text{m}/\text{sec}$, 23.08 (\pm 1.98) $\mu\text{m}/\text{sec}$, 23.26 (\pm 2.32) $\mu\text{m}/\text{sec}$, 17.10 (\pm 2.59) $\mu\text{m}/\text{sec}$ and 8.56 (\pm 3.24) $\mu\text{m}/\text{sec}$ after 5 sec of activation for 0, 50, 100, 150, 200, 250 and 300 mOsmol/kg, respectively, where the means differences were statistically significant at 300 mOsmol/kg ($P < 0.05$; Figure 3.8). Mean BCF (\pm s.d.) was significantly decreased at 300 mOsmol/kg (8.12 ± 0.99 Hz), in comparison to other osmolality ($P < 0.05$; Figure 3.8). Meanwhile means MOT (\pm s.d.), were significantly different at 250 and 300 mOsmol/kg with 60.03% (\pm 6.58) and 7.20% (\pm 2.10), respectively ($P < 0.05$; Figure 3.8).

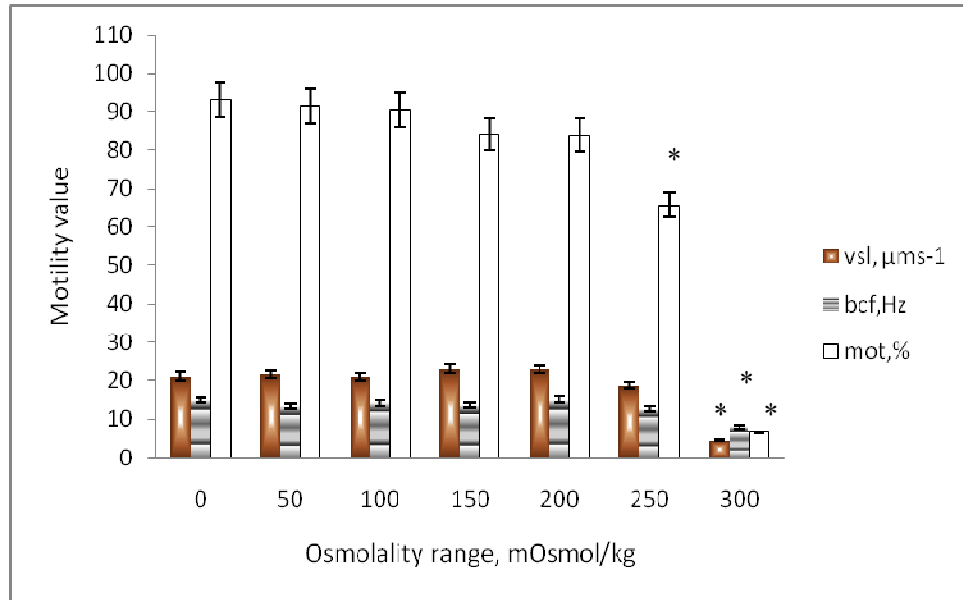


Figure 3.7. Effect of osmolality of mannitol on sperm motility variables, determined at 5 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; * means are significantly different at $P < 0.05$

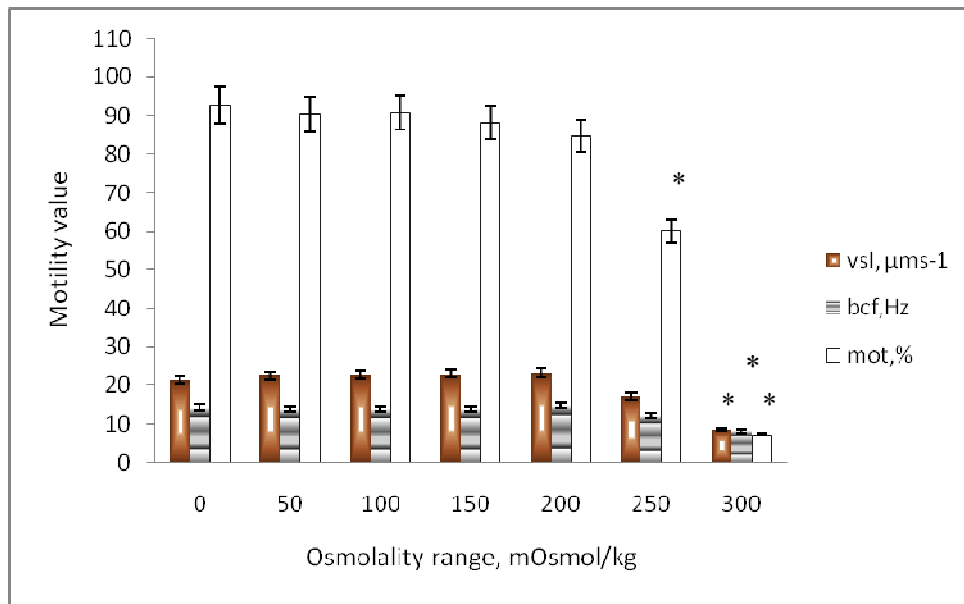


Figure 3.8. Effect of osmolality of modified fish ringers' on sperm motility variables, determined at 5 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; * means are significantly different at $P < 0.05$

These results imply that osmolality affect both sperm motility score and variables of *O. niloticus* with both solutions i.e. mannitol and modified fish ringer showed comparable results.

3.3.5 Effect of pH on sperm motility

Mean of sperm motility score after 10 sec of activation, determined in buffer hepes solution at pH range of 6 to 10 are shown in Figure 3.9. Approximately 80-100% of sperm became motile upon diluted 500-fold in mannitol and hepes solution at pH 6 and pH 8. However, at pH 10, only 20-40% of the sperm were motile. Mean differences were highly significant at pH 10 (Figure 3.9; $P < 0.05$).

The effects of pH on sperm motility variables determined by CASA are shown in Figure 3.10. Mean (\pm s.d) VSL were 18.72 (\pm 8.40) $\mu\text{m}/\text{sec}$, 18.95 (\pm 6.84) $\mu\text{m}/\text{sec}$, 19.62 (\pm 6.61) $\mu\text{m}/\text{sec}$ and 20.10 (\pm 5.53) $\mu\text{m}/\text{sec}$ after 5 sec of activation for control (mannitol), pH 6, pH 8 and pH 10, respectively, where the means differences were not statistically significant ($P > 0.05$; Figure 3.10). Similarly, mean BCF (\pm s.d.) and MOT were not statistically different compared to control ($P > 0.05$; Figure 3.10). These results imply that pH affect both sperm motility score and motility variables of *O. niloticus* with pH range of 6 to 8 showed comparable results to control although significantly different means was obtained for pH 10 with motility score.

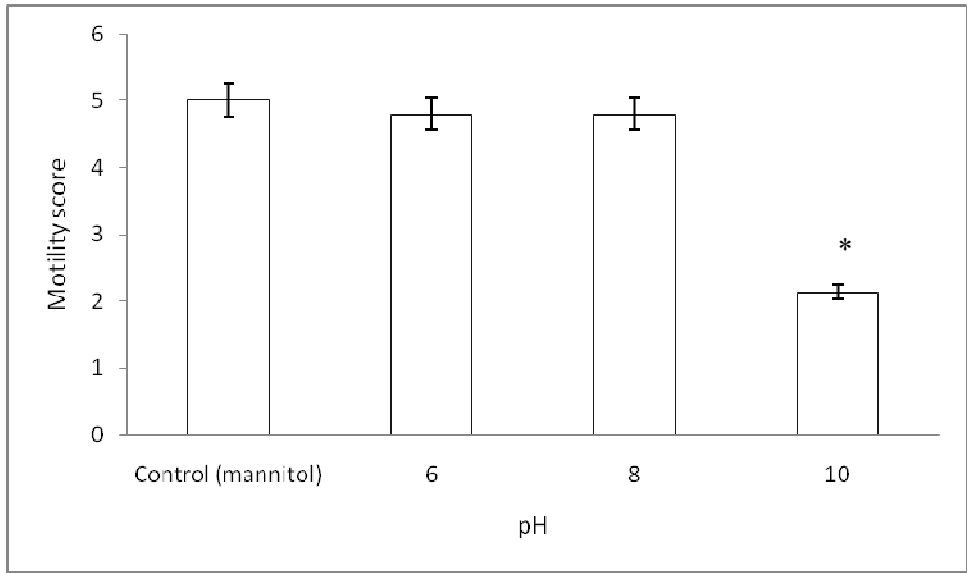


Figure 3.9. Effect of pH on sperm motility score, determined at 5 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; * means are significantly different at $P < 0.05$

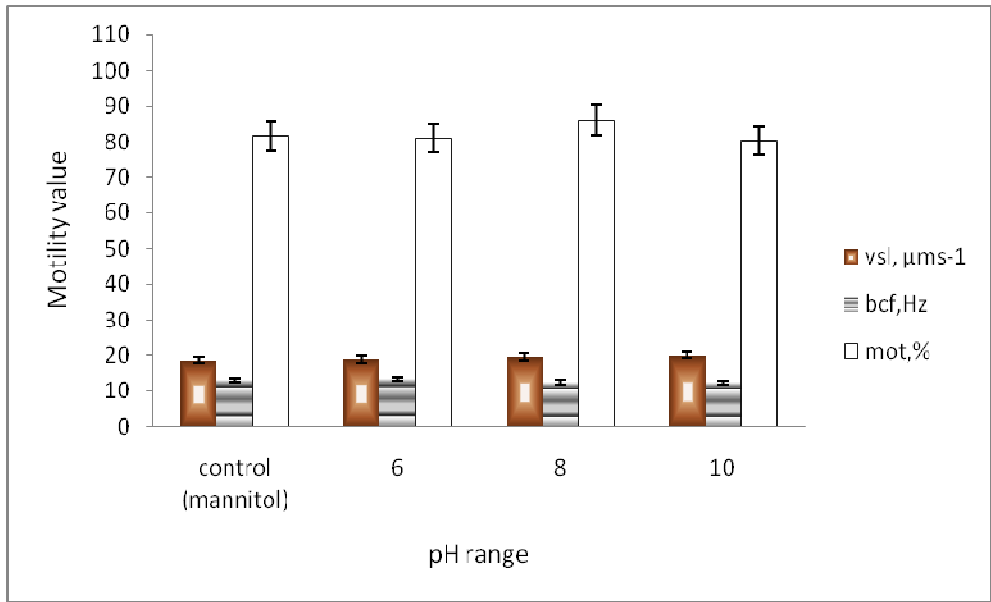


Figure 3.10. Effect of pH on sperm motility variables, determined at 5 sec after activation. Values are expressed as mean (\pm s.d) of 5 fish; * means are significantly different at $P < 0.05$

3.3.6 Effect of membrane ion channel inhibitors on sperm motility

3.3.6.1 Effect of lanthanum on sperm motility

Data were obtained from 1500 individual motile spermatozoa and subjected to PATN analysis using the motility data obtained from CASA analysis: VSL, straight line velocity (μms^{-1}); VCL, curvilinear velocity (μms^{-1}) and; LIN, linearity (%). Summary table showing PATN group motility characteristics derived from lanthanum experiment is shown in Table 3.6. Each subpopulation was characterized by a mean value of the motion parameters distinguished by their velocity and linearity rendered by CASA. The first subpopulation (group 1) contained the largest number of spermatozoa (1090) that included spermatozoa covering long distances (high VSL), vigorous (high VCL) and progressive (high LIN), and hence they were labeled as fast- linear spermatozoa. The second subpopulation (group 2) contained 410 spermatozoa covering short distances (low VSL), less vigorous (low VCL) and less progressive (low LIN): slow-nonlinear spermatozoa.

Frequency variations of fast linear spermatozoa along the dose response experiment showed that when lanthanum was absent, there was a high proportion (76.67%) of group 1, fast linear spermatozoa (Figure 3.11). However, exposure to lanthanum induced a considerable change in the profile. In the presence of 25 μM lanthanum, only 50.0% spermatozoa was classified as group 1. As lanthanum concentration was increased to 50 μM , only 35.0% of the spermatozoa was classified as group 1. At 25 and 50 μM , the means differences were highly significant ($P < 0.05$; Figure 3.11). Testing the dose response (0, 5, 10, 25 and 50 μM), there is a clear evident that lanthanum dosage

Table 3.6 Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of lanthanum

Source of variation	No. of sperm in subset	VSL μms^{-1}	VCL μms^{-1}	LIN (%)
Group 1 (fast-linear sperm)	1090	31.61 \pm 11.04	64.87 \pm 13.18	48.99 \pm 13.92
Group 2 (slow-non linear sperm)	410	9.03 \pm 3.74	57.12 \pm 15.54	17.30 \pm 8.34

*n=5 fish, 60 spermatozoa each

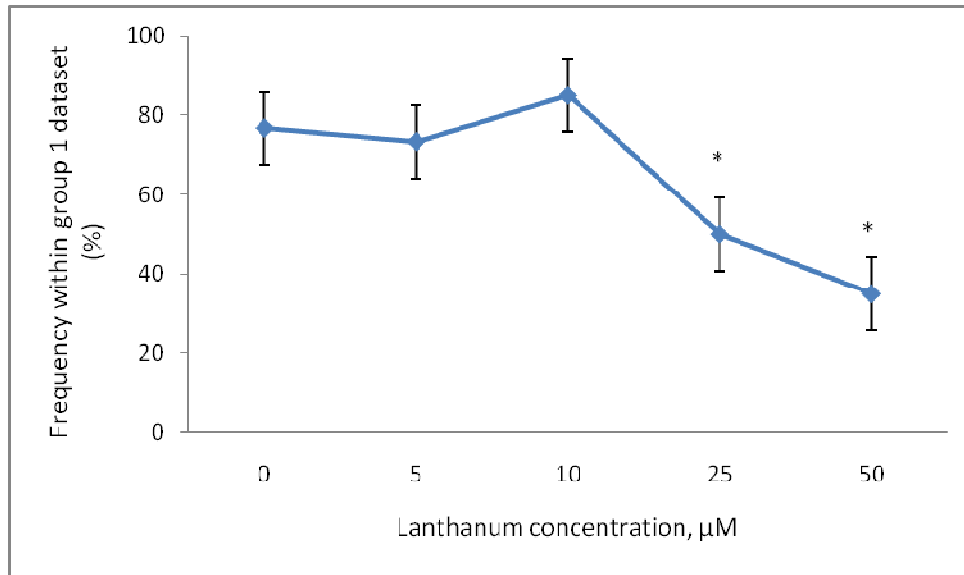


Figure 3.11. Frequency (percentage) of fish spermatozoa in data set classified as PATN group 1 in relation to lanthanum treatment; vertical bars denote 95% C.I.; *mean s are significantly different at $P < 0.05$

significantly affected the frequency of sperm subpopulation of group 1 ($F_{4,20}=17.990$, $P=0.000$).

3.3.6.2 Effect of flunarizine on sperm motility

Data were obtained from 1500 individual motile spermatozoa and subjected to PATN analysis using the motility data obtained from CASA analysis: VSL, straight line velocity (μms^{-1}); VCL, curvilinear velocity (μms^{-1}) and; LIN, linearity (%). Summary table showing PATN group motility characteristics derived from flunarizine experiment is shown in Table 3.7. Each subpopulation was characterized by a mean value of the motion parameters distinguished by their velocity and linearity rendered by CASA. The first subpopulation (group 1) contained the largest number of spermatozoa (939) that included spermatozoa covering long distances (high VSL), vigorous (high VCL) and progressive (high LIN), and hence they were labeled as fast- linear spermatozoa. The second subpopulation (group 2) contained 561 spermatozoa covering short distances (low VSL), less vigorous (low VCL) and less progressive (low LIN): slow-nonlinear spermatozoa.

Frequency variations of fast linear spermatozoa along the dose response experiment showed that when lanthanum was absent, there was a high proportion (75.0%) of group 1, fast linear spermatozoa (Figure 3.12). The numbers of spermatozoa dropped to 51.67% as flunarizine were added at 5 μM . The value remain stable up to 25 μM . However, in the presence of 50 μM flunarizine , only 30.0% spermatozoa were classified as group 1. At 5 and 50 μM , the means differences were highly significant ($P<0.05$; Figure 3.12).

Table 3.7. Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of flunarizine

Source of variation	No. of sperm in subset	VSL μms^{-1}	VCL μms^{-1}	LIN (%)
Group 1 (fast- linear sperm)	939	29.59 \pm 11.71	73.84 \pm 15.68	42.26 \pm 16.57
Group 2 (slow-non linear sperm)	561	8.71 \pm 4.10	71.12 \pm 18.70	13.41 \pm 7.43

*n=5 fish, 60 spermatozoa each

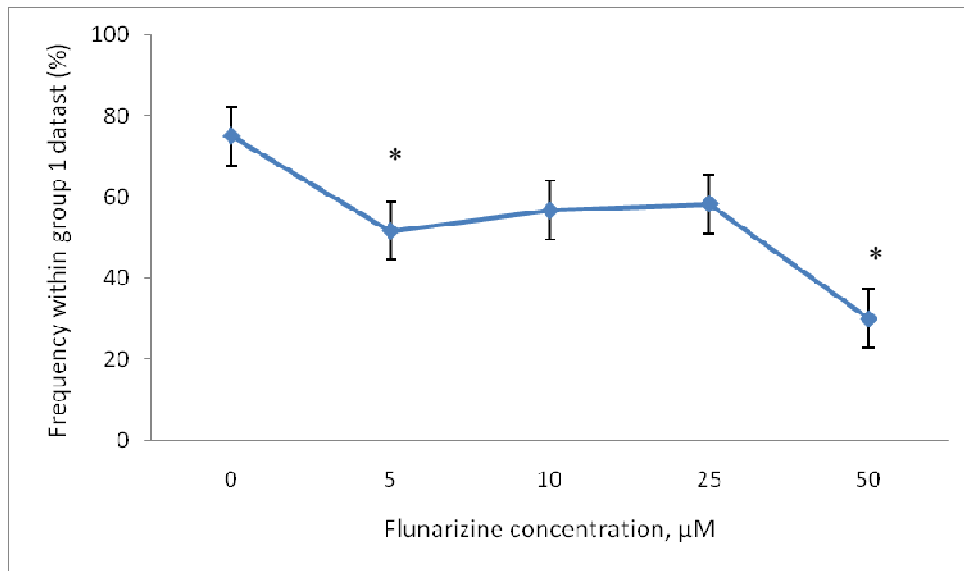


Figure 3.12 Frequency (percentage) of fish spermatozoa in data set classified as PATN group 1 in relation to flunarizine treatment; vertical bars denote 95% C.I; *means are significantly different at $P < 0.05$

Testing the dose response (0, 5, 10, 25 and 50 μM), there is a clear evident that flunarizine dosage significantly affected the frequency of sperm subpopulation of group 1 ($F_{4,20}=17.621$, $P=0.000$).

3.3.6.3 Effect of amiloride on sperm motility

No significant difference was observed in terms of sperm motility

3.3.6.4 Effect of ouabain on sperm motility

No significant differences was observed in terms of sperm motility

3.3.6.5 Effect of quinine on sperm motility

No significant difference was observed in terms of sperm motility

3.3.7 Effect of activation on sperm morphology

The morphological characterization of *Oreochromis niloticus* is shown in Figure 3.13 to Figure 3.16. Spermatozoa had no acrosome and the head region approximately oval (2.18 ± 0.54 μm in length and 1.89 ± 0.31 μm in width) (Figure 3.13). The head is covered by cytoplasmic membrane and has a nucleus in the form of an inverted U in longitudinal section which is located perpendicular to the flagellar axis (Figure 3.14). The midpiece was approximately cylindrical in shape (1.16 ± 0.18 μm in length and 0.88 ± 0.34 μm in width) and it contained numerous tightly packed spherical mitochondria (average diameter of 0.29 ± 0.01 μm) arranged in 2-3 layers. The midpiece was pervaded by cytoplasmic channel, an invagination of plasma membrane that separate flagellum from the midpiece (Figure 3.15). The spermatozoa are uniflagellate and the flagellum was very long (16.92 ± 2.64 μm in length) with two lateral side fins

and was composed of two central and nine peripheral pairs of microtubule (9+2 axoneme construction) (Figure 3.16).

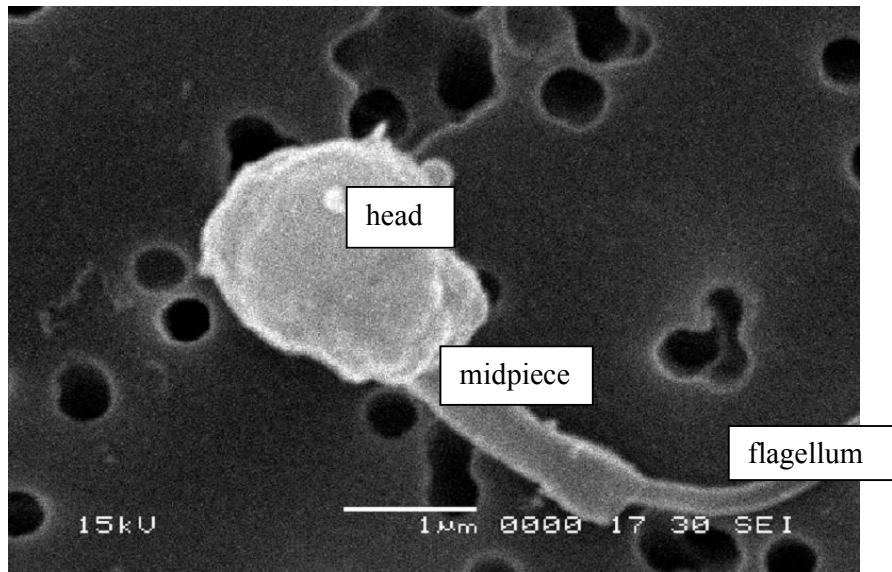


Figure 3.13. SEM imagery of *Oreochromis niloticus* spermatozoa; scale bar= 1µm

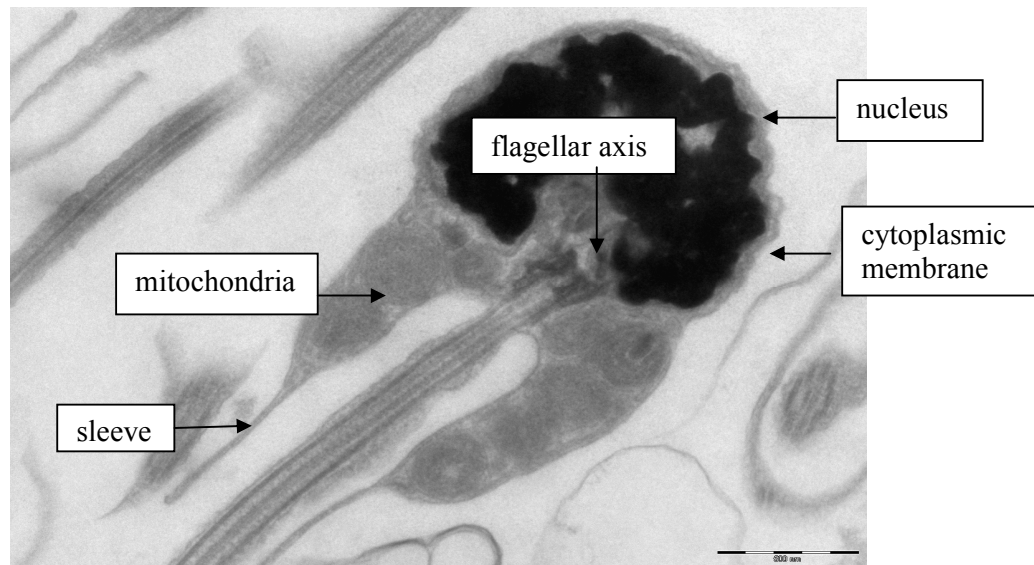


Figure 3.14. TEM imagery of *Oreochromis niloticus* spermatozoa. Longitudinal sagittal section of the spermatozoa showing sleeve, mitochondria, flagellar axis, nucleus and cytoplasmic membrane; scale bar= 500 nm

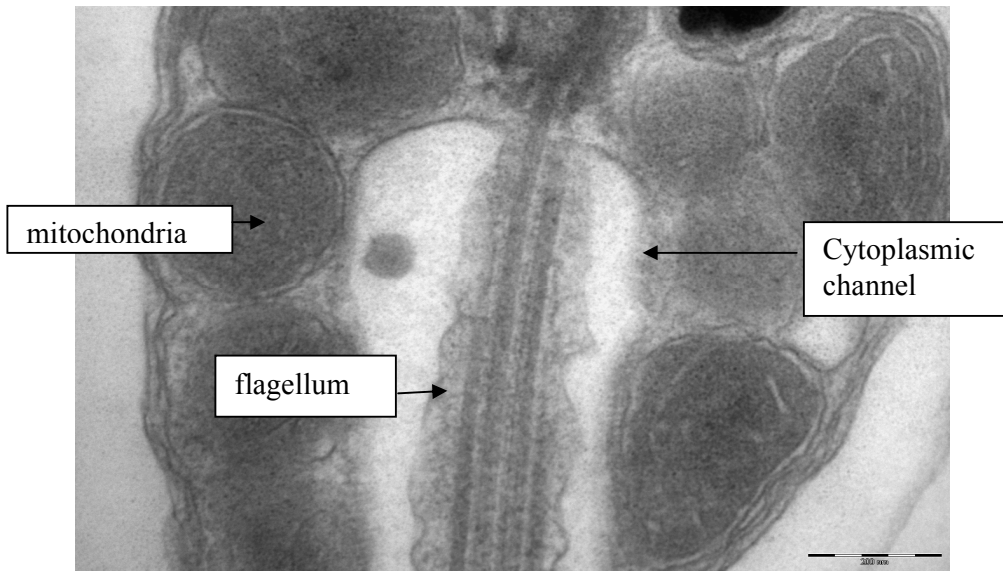


Figure 3.15. TEM imagery of *Oreochromis niloticus* spermatozoa. Longitudinal sagittal section of the spermatozoa showing mitochondria, flagellum and cytoplasmic channel scale bar= 200 nm

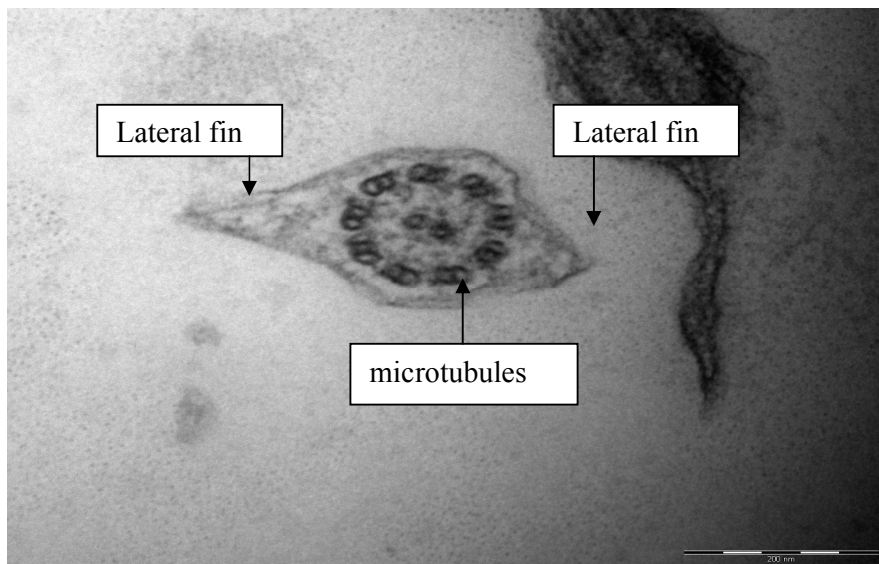


Figure 3.16. TEM imagery of *Oreochromis niloticus* spermatozoa. Cross section of the flagellum showing lateral fins, peripheral and central microtubules ; scale bar =200nm

Samples were collected and fixed at various time points after activation i.e. 0, 30, 60, 120 and 480 sec. The effect of activation on sperm morphology alteration are shown in Figure 3.17 to 3.24. As early as 30 sec of activation, 17.0% of the spermatozoa showing the swollen of the midpiece (Figure 3.17). Observation by TEM revealed that the sleeve or cytoplasmic sheath was contracted probably due to absorption of water by the midpiece area (Figure 3.18). At 60 sec, 34.0% of the spermatozoa population showing the contraction of the cytoplasmic membrane, where the upper part of the flagellum started to bend and coiled (Figure 3.19). TEM observation revealed that the structure of sleeve or cytoplasmic sheath and cytoplasmic channel were undistinguished (Figure 3.20). At 120 sec of activation, 75.0% of the population showed coiling of the flagellum over the head as a result of the massive swelling of the midpiece (Figure 3.21) and the plasma membrane started to detach from the midpiece (Figure 3.22). Finally, at 480 sec, 98.0% of the abnormal spermatozoa population were represented by fully coiled flagellum (Figure 3.23). At this stage, the cytoplasmic membrane was fully detached from the spermatozoa (Figure 3.24).

However, no significant differences were observed on the means of diameter of the nucleus and mitochondria along the activation period ($P>0.05$; Table 3.8). These results imply that sperm structure was affected during activation period without the effect on diameter of nucleus and mitochondria.

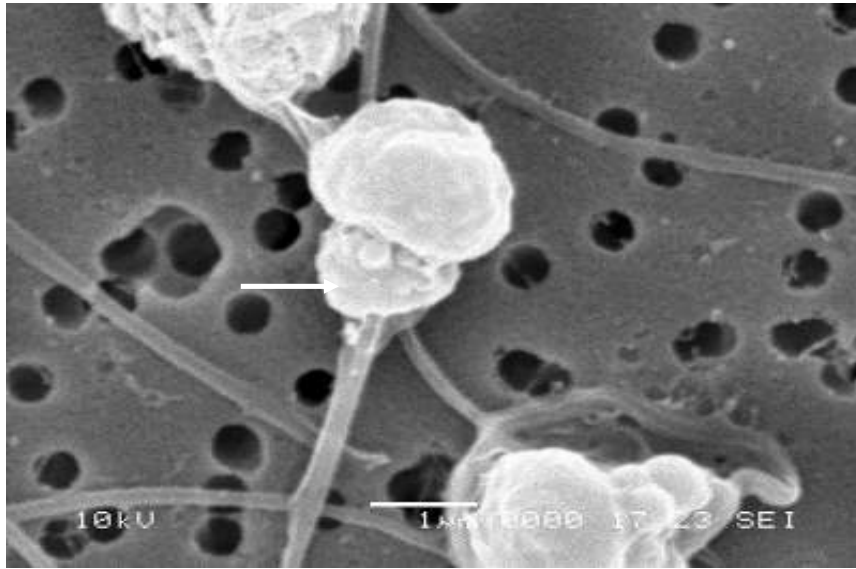


Figure 3.17. SEM imagery of *Oreochromis niloticus* spermatozoa at 30 sec of activation. An arrow showing the swelling midpiece; scale bar=1 μ m

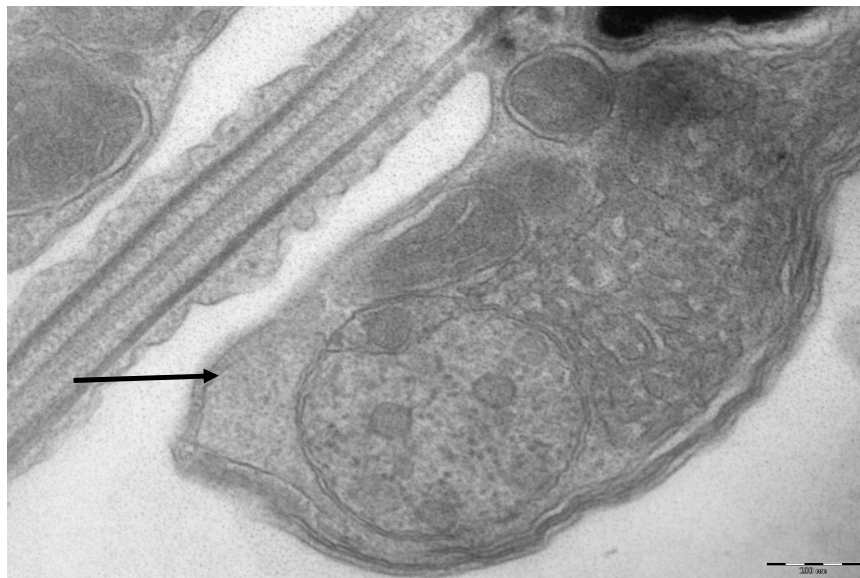


Figure 3.18. TEM imagery of longitudinal sagittal section of *Oreochromis niloticus* spermatozoa at 30 sec of activation. An arrow showing the contracting sleeve or cytoplasmic sheath; scale bar=200 nm

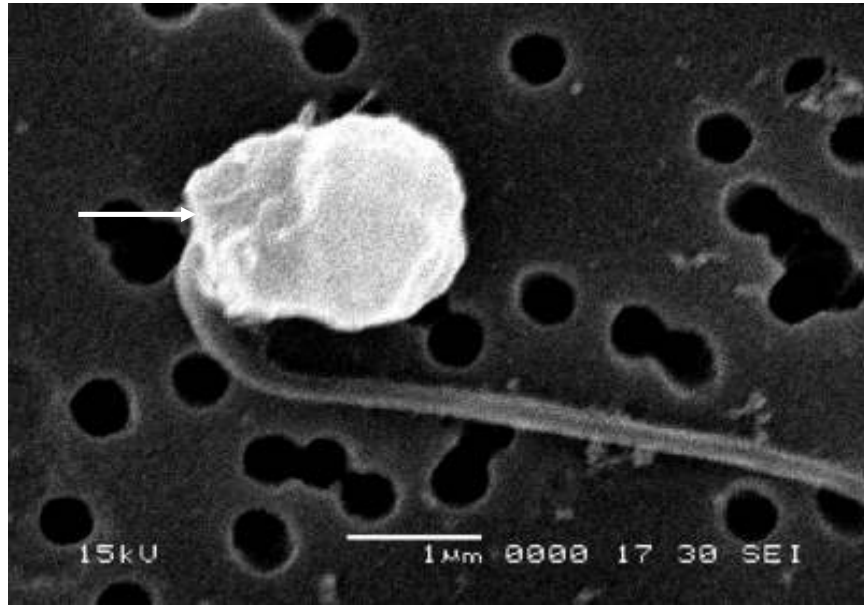


Figure 3.19. SEM imagery of *Oreochromis niloticus* spermatozoa at 60 sec of activation. An arrow showing the swelling midpiece; scale bar=1 µm



Figure 3.20. TEM imagery of longitudinal sagittal section of *Oreochromis niloticus* spermatozoa at 60 sec of activation. An arrow showing the loss of cytoplasmic sheath (sleeve) structure; scale bar=500 nm

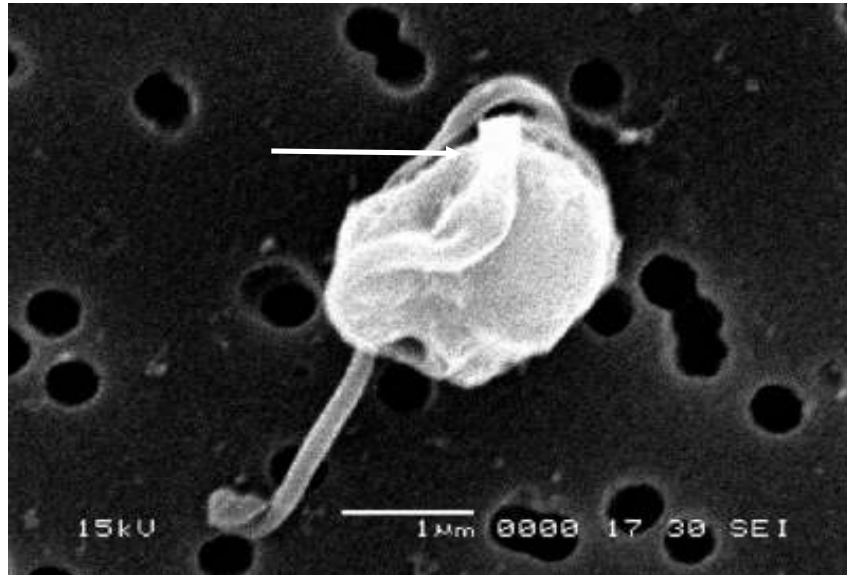


Figure 3.21. SEM imagery of *Oreochromis niloticus* spermatozoa at 120 sec of activation. An arrow showing the coiling of the flagellum around the head ; scale bar=1 µm



Figure 3.22. TEM imagery of *Oreochromis niloticus* spermatozoa at 120 sec of activation. An arrow showing the detaching cytoplasmic membrane; scale bar=500 nm

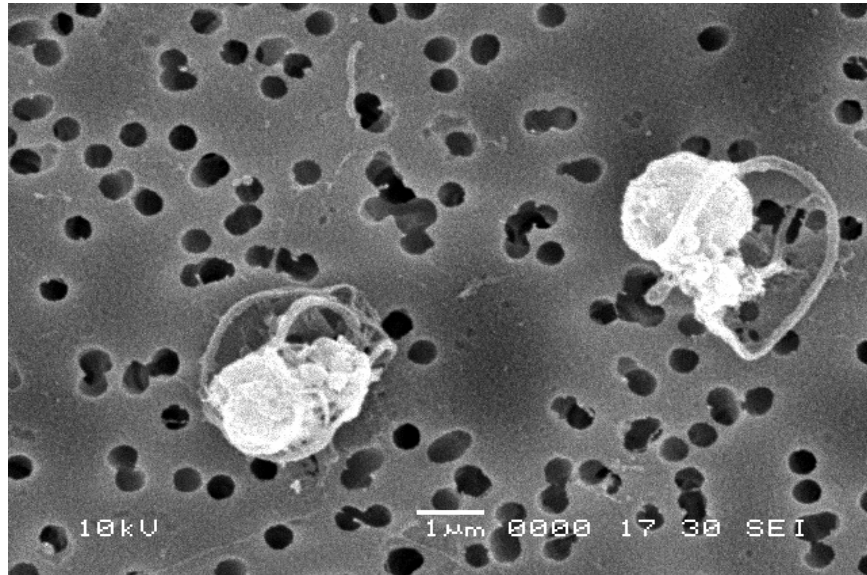


Figure 3.23. SEM imagery of *Oreochromis niloticus* spermatozoa at 480 sec of activation showing the flagellum-coiled spermatozoa without cytoplasmic membrane ; scale bar=1 μ m

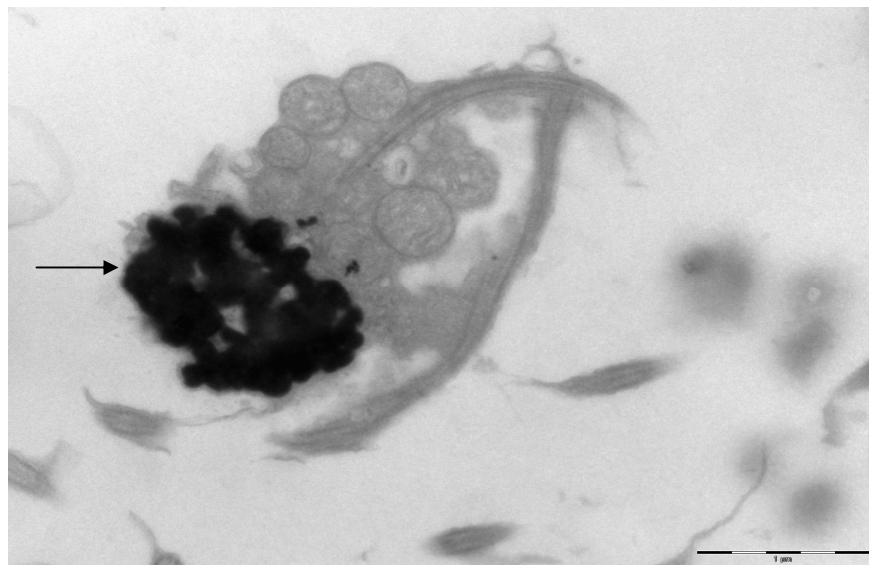


Figure 3.24. TEM imagery of longitudinal sagittal section of *Oreochromis niloticus* spermatozoa at 480 sec of activation. An arrow showing the head of a spermatozoa without cytoplasmic membrane; scale bar=1 μ m

Table 3.8 : Mean (\pm s.d.) diameter of spermatozoa mitochondria and nucleus of *Oreochromis niloticus* as observed by transmission electron microscopy (TEM)

Parameter, μm	time after activation, sec					
	before	0	30	60	120	480
Nucleus, diameter	0.94 \pm 0.16 ^a	0.92 \pm 0.20 ^a	0.95 \pm 0.19 ^a	0.95 \pm 0.25 ^a	0.98 \pm 0.12 ^a	0.99 \pm 0.13 ^a
Mitochondria, diameter	0.29 \pm 0.01 ^a	0.30 \pm 0.04 ^a	0.30 \pm 0.02 ^a	0.33 \pm 0.05 ^a	0.37 \pm 0.04 ^a	0.38 \pm 0.03 ^a

*n=3 fish; 100 spermatozoa each; *means with the same superscript are not significantly different at P<0.05

3.4 Discussion

The first aim of the present study was to determine the composition of the seminal fluids of Nile tilapia, *Oreochromis niloticus* with respect to the osmolality, concentrations of the major inorganic ions (sodium, potassium, calcium and magnesium), and pH. This information is necessary for better understanding of the physiology of the spermatozoa and development of efficient artificial insemination techniques. Furthermore, despite their function for spermatozoa protection, information is only available for some cultivated freshwater species (Piironen and Hyvarinen, 1983; Morisawa, 1985; Billard et al., 1986). Additionally, the ionic components of the seminal fluid can reflect the efficiency of fertilization ability (Ciereszko et al., 2000).

Sperm of tilapia are quiescent in undiluted seminal fluid. Sperm activation occurs when seminal fluid is subjected to lower osmolality as in other fresh water species. Our results show that osmolality of tilapia seminal plasma (305 mOsmol/kg) are within the reported range. Suquet et al. (1994) reported that in other fresh water species the osmolality observed was 297 ± 15 in rainbow trout; 302 ± 5 in carp; and 317 ± 11 in goldfish. In *Acipenseridae*, the osmolality of the seminal plasma is reported in the range of 30-80 mOsmol/kg (Gallis et al., 1991; Alavi et al., 2004) which probably due to lower ion concentrations in seminal plasma (Alavi et al., 2004). The pH value of the seminal fluid as observed in the present study are similar to turbot (7.3; Suquet et al., 1993), but is in contrast to burbot (8.5; Lahnsteiner et al., 1997) and silver catfish (8.0; Borges et al., 2005). These differences are mainly related to the ionic and biochemical composition of the seminal plasma which besides being species-specific (Ciereszko et al., 2000), also vary seasonally as well as due to aging of spermatozoa (Alavi and

Cosson, 2006). pH most likely influences the spermatozoan protonic composition which affects the membrane potential, and thus motility behavior (Boitano and Omoto, 1992).

The observed sperm densities for tilapia (3.59×10^9 /ml) are within the magnitude (10^9) reported for other teleost species (Aas et al., 1991; Fauvel et al., 1999). In carps species, sperm density varied between 2.6×10^{10} /ml to 3.5×10^{10} /ml (Verma et al., 2009). In contrast, sperm density in halibut was an order of magnitude higher within the range from 2 to 6×10^{11} spermatozoa per ml (Tvedt et al., 2001). The differences in sperm densities probably reflect differences in the ecology and spawning behavior of the species (Piironen and Hyvarinen, 1983).

Like in other teleosts, sodium and potassium ions predominate in tilapia seminal plasma compared to calcium and magnesium. In our study, sodium and potassium concentration of tilapia seminal plasma were very high, with 167 and 64 mM, respectively. These ions are most likely responsible for the inhibition of sperm motility due to their osmotic effect (Morisawa, 1985). Moreover, our results show that seminal plasma of tilapia has a higher sodium content compared to common carp (75 mM; Morisawa et al., 1983) and perch (124 mM; Lansteiner et al., 1995) but comparable to catfish (164 mM; Tan-Fermin et al., 1999). On the other hand, potassium content was higher than those reported for catfish (18 mM; Tan-Fermin et al, 1999) where the differences most likely represent species-specific characteristics (Ciereszko et al., 2001). On the contrary, low contents of sodium and potassium in seminal plasma are associated with low quality seminal fluid as observed in contaminated samples.

According to our results, the values of osmolality and sperm density remained constant by increasing of stripping frequency as previously reported for over weekly stripping from landlocked salmon (Piironen, 1985) and bi-weekly stripping from rainbow trout (Sanchez-Rodriguez et al., 1978) but unlike that seen in Atlantic salmon (Aas et al., 1991). Repeated sampling of individual males revealed no obvious pattern of change in osmolality and sperm density throughout the study period. Similarly, no changes were recorded in terms of the seminal plasma osmolality and sperm density of various fish size at repeated stripping frequency of 8-10 days. The differences between our results and those published in literature could be related to several factors, for example contamination of semen by urine during stripping (Suquet et al., 1994) as well as degeneration stage of spermatogenesis (Piironen and Hyvarinen, 1983; Lahnsteiner et al., 1993).

Our second aim is to determine the factors that affect sperm motility in Nile tilapia. Sperm dilution is a key factor in the induction of sperm motility (Billard and Cosson, 1992) and the continuation of fertilizing capability in freshwater (Billard, 1983) and marine fishes (Suquet et al., 2000). A relatively high dilution is necessary to initiate synchronous motility of all spermatozoa (Billard et al., 1995). In *Acipenseridae* the sperm becomes motile at low dilution rates (Linhart et al., 1995).

The motility study concentrated on sperm movements in the first minutes post-activation with water because this is likely to be the most vital period to attain fertilization (Bekkevold et al., 2002). Therefore, the 5 and 10 sec delay in the beginning of sperm observations for sperm motility variables analysis (CASA) and motility score, were unlikely to influence our comparative estimations of sperm motility. In mammals,

the straight line velocity (VSL) is the most reliable indicator of fertility (Moore and Akhondi, 1996). In fish, higher spermatozoa velocities are often associated with high fertilization success (Gage et al., 2004). Furthermore, Iwamatsu et al. (1997) found that linear swimming velocity is correlated with the ability of spermatozoa to enter the micropyle.

Our results of motility score show that dilution rate of 1:500 and above, produce best sperm activity. This is, however, in contradict to CASA results where no changes of motility velocity and percentage of motile sperm was observed at all dilution rates tested. The differences between the results of motility score and CASA probably due to the different approach of each method present where motility score is a rather subjective method compared to CASA. Apart being rapid, CASA methodology has high repeatability and provides a more discriminating estimate of the sperm motility than microscopic subjective methods (Rurangwa et al., 2004).

In freshwater aquatic species with external fertilization, the spermatozoa are released into a hypoosmotic environment where they usually activated for a short time of 1 to 2 min in case of freshwater fish (Holt and Van Look, 2004). Inspection of the motility score shows that the motility of spermatozoa upon activation was more than 80% in which the motility remains for 180 sec. After this period, most of the spermatozoa become motionless. Both distilled water and tank water showed comparable results where the rate of motile spermatozoa decreased to less than 20% in 540 sec.

The seminal plasma osmolality plays an important role in spermatozoa activation. When seminal fluid was diluted in non-electrolyte (mannitol) or electrolyte solutions

(modified fish ringer`s) with osmolalities higher than 200 mOsmol/ kg, sperm motility score and velocities as well as percentage of motile cells decreased, and at osmolalities of 300 mOsmol/kg motility was suppressed. In carp, the motility is initiated in media below 150 mOsmol/kg (Plouidy and Billard,, 1982). The seminal plasma osmolality in the European eel has been reported to be in the range of 325-330 mOsm/kg and extenders with this range of values helped in reversibly suppressing the motility of spermatozoa (Asturiano et al., 2004). Previous reports of African catfish, *Clarias gariepinus* (Mansour et al., 2002), common carp, *Cyprinus carpio* (Krasznai et al., 2000) and sea lamprey, *Petromyzon marinus* (Ciereszko et al., 2002), demonstrating that electrolytes are necessary to initiate sperm motility, our results indicate that electrolytes is not necessary for sperm motility which is in agreement with those of others (Hu et al., 2009). Thus, our results suggest that osmolality, irrespective of ion content, is the principal factor regulating sperm motility in Nile tilapia.

Our results show that sperm motility score was not affected by pH 6.0 to 8.0, but at pH 10 only 40% of the sperm were motile. Furthermore, sperm velocity and percentages of motile sperm were not affected by pH range of 6 to 10. Optimum sperm motility has been reported at pH 7.0 and 8.0 in *Cyprinus carpio* (Cosson et al., 1991) and at pH 8.0 in *Acipenser persicus* (Alavi et al., 2004). On the other hand, mannitol solution showed comparable results to buffered hepes solution. Therefore, the pH of dilution water did not have any influence on the sperm motility and velocity as well as percentages of motile sperm, between the ranges of 6 and 8. Similar results were observed by Kime and Tveiten (2002) with spermatozoa of *Anarhichas minor* submitted to variation of pH between 6.0 and 9.0. Thus, pH appears to have a slight effect on motility activation.

The Hobson Sperm tracker automatically analyzes 14 different parameters of motility, but only VSL, VCL and LIN are referred to in this chapter. These sperm trajectories variables were selected as they are correlated with fertility (Moore and Akhondi, 1996; Rurangwa et al., 2001) in the first 5- 20 sec, and provide the most useful data (Kime et al., 2001). Motility variables of VSL, VCL and LIN data obtained from sperm tracker analysis were subjected to multivariate analysis using PATN software. In this study, we applied a comparable multivariate statistical analysis to the one used by Abaigar et al. (1999). PATN analyses clearly confirmed the existence of a heterogeneous sperm population within sperm samples of tilapia. In most earlier analyses of sperm motility in fish species, the occurrence of the sperm subpopulations has been neglected, hampering the thorough assessment of sperm quality.

Our results show that in samples exposed to lanthanum and flunarizine, two sperm subpopulation or group were identified. Majority of the spermatozoa are classified as group1 which represented the fast linear sperm. On the other hand, group 2 represented the slow non-linear sperm. In the presence of both ion channel inhibitors, a lower proportion of fast linear spermatozoa were seen when compared to control. For lanthanum and flunarizine, the effect can be clearly seen at 25 μ M and 5 μ M, respectively. This result could be due to the spermatozoa in group 1 altering their motility behaviors when exposed to both ion channel inhibitors. While other channel inhibitors (amiloride, ouabain and quinine) did not affect motility, calcium channel inhibitor (lanthanum) and sodium-calcium channel exchanger inhibitor (flunarizine) decreased the sperm swimming velocity of some proportion of fast linear group of motile sperm. The effects on motility depended on the dose indicating the possible involvement of calcium channel and sodium calcium exchanger in the activation

mechanism of sperm. The lack of effect of amiloride, ouabain and quinine indicate that epithelial sodium channels, sodium potassium ATPase and voltage gated potassium channels respectively are unlikely to have major roles in sperm activation or motility.

Lanthanide ions usually block the sites for calcium entry in the cell membrane. However, in the present study, the sperm was activated with deionised distilled water therefore, another possibility is that these ions entered the spermatozoa and exerted an inhibitory effect on the metabolic pathway which is essential for motility activation. Alternatively, these ions might also inhibit the generation of cyclic adenosine 3',5' monophosphate which has been proposed as the intracellular mediator of motility in teleost sperm (He et al., 2004; Zilli et al., 2008). On the other hand, sodium-calcium exchanger is a bidirectional transporter that normally catalyzes the movement of 3 sodium into a cell in exchange for the extrusion of 1 calcium. Alternatively, other than the effect on sodium –calcium exchanger, flunarizine also block L-type calcium channels as well as T-type calcium channels (Tytgat et al., 1988). This might be the reason why the effect of flunarizine occurs at lower concentration compared to lanthanum.

The role of membrane- bound ion channels on sperm activation has been investigated in salmonids and carps. In salmonids, sperm motility is suppressed by potassium-membrane transport blockers and the calcium-membrane channel blockers i.e. verapamil and desmethoxyverapamil (Cosson et al., 1986; Morisawa and Morisawa, 1990) in which a simultaneous efflux of potassium and an influx of calcium occurs through membrane-bound ion channels has been demonstrated (Tanimoto et al., 1994). Krasznai et al. (1995) suggest that a similar ion flux may also occur in cyprinids

because both potassium and calcium channel blockers (4-aminopyridine and verapamil) decrease sperm motility in carp. However, our results show that potassium channel blocker, quinine was ineffective in inhibiting motility of Nile tilapia sperm with amiloride and ouabain posed a similar results. Amiloride was also ineffective in inhibiting motility of carp sperm (Krasznai et al., 1995). Similarly, the sodium potassium ATPase ion transport blocker, ouabain, were ineffective at inhibiting motility of croaker sperm (Detweiler and Thomas, 1998). In marine fishes, herring, a sperm-surface sodium calcium exchanger has been identified in the midpiece region of spermatozoa that regulates motility initiation (Vines et al., 2002). Krasznai et al. (2000) demonstrated that the activation of voltage-gated calcium channels is required in carp sperm for motility initiation where the activation depends upon membrane hyperpolarization through opening of potassium channels and a potassium efflux. Linhart et al. (1999) reported that motility of spermatozoa of *Oreochromis mossambicus* is controlled by osmotic pressure and by calcium ions combined with sodium ions. This is in support with our results and suggests that calcium ion channel and sodium calcium exchanger regulates sperm motility in Nile tilapia.

The spermatozoa of *Oreochromis niloticus* were uniflagellate with clearly differentiated oval-shaped head, midpiece and flagellum. Similarly, the spermatozoa of *O. nilotica* has been reported to consists of a round or spherical head of 2.2 to 2.5 μm (Chao et al., 1987), a middle piece and a tail (Don and Avtalion, 1993).

The morphology of *O. niloticus* spermatozoa conforms to that of a primitive type I, typically found in fish species using external fertilization (Jamieson, 1991) in which the sperm were characterized by an ovoid head, a small midpiece and the absence of an

acrosome. A mid-piece length of 3.5 μm with a numerous mitochondrial count (of 70) were reported for the chondrichthyan (Jamieson, 1991), whilst a single large mitochondrion with a diameter of 0.92 μm was found in Clupeiformes (Gwo et al., 2006). In Senegalese sole, more than eight electron-dense mitochondria have been reported (Medina et al., 2000). Turbot spermatozoa on the other hand, have eight to ten spherical mitochondria located in the midpiece (Suquet et al., 1993) which emphasizes the mitochondrial variability among species. A single flagellum was encased by a plasma membrane, which extended as one, two or three lateral fins (Jamieson, 1991). Our observation shows that the cytoplasmic expansion (lateral fins) is present on both sides of flagellum which is similar to those demonstrated in sturgeons (Psenicka et al., 2008).

Reduced sperm swimming velocity are often linked to abnormal sperm morphology. In both freshwater and marine fishes, exposure of sperm to hypoosmotic environment has led to different types of structural damages after activation. However, there is less information about the effects of activation on sperm morphology. Morphological alteration of carp sperm after motility initiation was studied by Perchee et al (1996). Earlier reports showed that sperm motility in hypoosmotic solution was accompanied by reorganization of membrane structure (Marian et al., 1993). Damages such as swelling of the midpiece and curling of the flagellum as observed in the present study present a great impact towards the swimming velocity of spermatozoa as it shortens the duration of the sperm movement. However, we have not observed any significant changes on mitochondria and nucleus.

Altogether, our data demonstrate that the qualitative inorganic composition of seminal fluid was similar as in other fresh water fish species. In addition, the mechanism of Nile

tilapia sperm motility is regulated by osmolality and membrane ion channels namely, calcium channel and sodium-calcium exchanger. The morphology of Nile tilapia sperm is similar for data describing cyprinid sperm. Finally, to our knowledge, this is among the first study showing the changes in spermatozoa morphology of Nile tilapia during activation using electron microscopy techniques.

CHAPTER 4 POLLUTANTS AND THEIR EFFECTS ON SPERM MOTION AND MORPHOLOGY

4.1 Introduction

Aquatic environments serve as receiving sinks for almost all man-made chemicals, thus growing emphasis has been placed on the toxic evaluation of industrial and municipal effluents. Pollutants can cause lethal toxicity to aquatic organisms at high dose, and even minute amounts may affect cellular or subcellular organization, causing damage to organelles such as mitochondria, ribosomes, endoplasmic reticulum and lysosomes (Li et al., 2003; Xia et al., 2004; Sokolova et al., 2005). Even when environmental concentrations are below toxic thresholds some pollutants, for example metals and lipid soluble pesticides, can accumulate and undergo food chain magnification (Miramand et al., 1998; Muir et al., 2003), reaching toxic levels at higher trophic levels.

All of these toxic processes may affect higher-order physiological pathways and thus compromise organismal fitness. Therefore, the effects of pollutants on organisms are reflected in neurophysiological, behavioural and reproductive abnormalities which are often inter-related.

Fish reproduction is one of the most ecologically relevant indicators of exposure to sublethal concentrations of environmental chemicals (Arcand-Hoy and Benson, 1998). Effects of environmental stressors on reproduction ultimately affect population levels of biota (Donaldson, 1990), but conducting studies at this level is expensive and time consuming. Alternative methods to fish life cycle tests should include basic screening tests which are relatively simple and less costly, and should be able to identify and

elucidate mechanisms of toxicant action. In fish gamete biology, assessments of sperm quality include measures of concentration, aspects of motility and fertilization ability. For instance, reduced sperm quantity or quality in animals can be caused by alterations in testicular development, intrinsic defects in the ability of germ cells to divide and differentiate, or defects or impacts on the hormonal regulatory pathway (Kime and Nash, 1999). In male reproductive toxicity, when damage to sperm cells occurs at later stages of spermiogenesis in the testis or in the epididymal sperm, sperm numbers may be normal, but sperm viability or motion characteristics, and therefore sperm function, may be impaired. In aquatic species where sperm are released directly into the environment they may encounter pollutants, whose effects on sperm function are not well understood. In fish exhibiting external fertilization strategies, the sperm are released in a potentially hostile aquatic environment. In these fishes, sperm motility is induced upon contact with the external environment. As motile sperm would be more likely to produce fertilization success compared to non-motile sperm, the abnormalities in their swimming velocity or morphology could compromise the fertilization rate. Therefore, sperm studies may provide a valuable technique for determining the effect of water pollutants and for assessing ecological risk.

In the present study, heavy metal i.e. cadmium and pesticides, malathion and rotenone were selected as the representatives for pollutant studies.

4.1.1 Heavy metals and pesticides

Metals are present in very low concentrations in natural aquatic ecosystems (Nussey et al., 2000), usually at the nanogram (ng) to microgram (μg) per litre level, but recently the occurrence of heavy metals in excess of natural loads has become an increasing concern for aquatic ecosystem health (Fleeger et al., 2003). The most important heavy metals in water pollution are zinc, copper, lead, cadmium, mercury, nickel and chromium (Toribio and Romana, 2006). The high toxicity of some metals could be due to the inhibition of enzymatic systems in both vertebrate and invertebrate organisms, but may also interact with components of the cell membrane, affecting permeability to organic and inorganic substances (Stanish and Monbouquette, 2000). Metals are also unique environmental pollutants in that they are neither created or destroyed by humans but are only transported and transformed into various products which in turn directly or indirectly affect the growth and longevity of aquatic or terrestrial animals. Metals are also non biodegradable and persist in the environment. Cadmium is also known as a carcinogen in many experimental animals. Furthermore, cadmium modifies the function of calcium channels and is also used as a calcium channel blocker in *in vitro* experiments (Verbost et al., 1989).

The effects of pesticides on aquatic ecosystems are relatively well-known, because considerable attention has been paid to dose–response relationships resulting in both safe and economical levels of pesticide application (Aydin and Koprucu, 2005). However, unwanted side-effects of even strictly controlled uses of pesticides on non-target aquatic organisms are extremely difficult to avoid. Uptake and accumulation of a pesticide by aquatic organisms seem to be more likely a function of habitat, habits, life cycle, and exchange equilibrium than of food uptake; but they are also affected by many

other factors, such as size of the organism, pharmacokinetics, and physical and chemical properties of the pesticide (Timchalk, 2006).

The effects of pesticide to aquatic animals, thus depends on their bioavailability, bioconcentration, biomagnification, and persistence in the environment. Malathion is used widely in aquatic systems because of its high water solubility and it is metabolized quickly with short persistence (half-lives of days to months), and thus, residues are assumed not to pose long-term problems for aquatic animals.

Rotenone on the other hand, which is an ichthyotoxic substance mainly produced by leguminous plants, has been used in fishery practice for experimental fishing, as well as for the elimination of undesirable fish populations in natural waters (McClay, 2000). Fish are particularly susceptible to poisoning because rotenone is absorbed *via* the gill membrane causing immediate death due to suffocation (Ibrahim et al., 2000).

For pesticides that are highly soluble in water, monitoring must be closely linked to periods of pesticide use. The significance for monitoring is that many newer and soluble pesticides can only be detected shortly after application therefore, monitoring programmes that are operated on a monthly or quarterly basis are unlikely to be able to quantify the presence of pesticides in surface waters. Thus, the danger lies in the presumption of non-detectable values which implies that the pesticides are absent.

Sperm might provide an important test tool for toxicity studies as they are easily attainable. However, as far as we are concerned, studies on animal sperm as a bioindicator of pollution, particularly of cultured freshwater fish is still lacking (Kime

et al., 1996; Rurangwa et al., 2002). The few previous studies reported have used either fertilization rate or a subjective assessment of motility to measure the effects of pollutants on sperm quality (Duplinsky, 1982; Khan and Weis, 1987). These methods apart from being time consuming, are rather subjective and thus the results are open to question, particularly with regard to the requirement for accuracy and repeatability in any test for ecological risk assessment. Importantly, the introduction of computer-aided sperm analysis (CASA) system has facilitated the quantification of sperm motility due to its rapidity and objectivity.

Some studies have utilized CASA for toxicity testing with sperm. Kime et al. (1996) reported a significant decrease in motility of catfish sperm exposed to sublethal levels of waterborne Cd and Zn as determined by CASA. Furthermore, recent data by Hayes et al. (2002), suggest that environmentally relevant concentrations of commonly-used pesticides can cause devastating effects to the reproductive potential of amphibians which highlight the potential harmful effects of constant low-dose pesticide exposure.

Several studies have used electron microscopy techniques to investigate the effects of pollutants and environmental stress on the morphology of fish spermatozoa (Abdelmeguid et al., 2007) as well as automated sperm morphology analyses, ASMA (Van Look and Kime, 2003). Gill et al. (2002) which has been used to describe sperm abnormalities (big heads and fuzzy tails) in flounders sampled from the Tyne estuary in northeast England, a polluted site known to contain xenoestrogens.

Although the effect of pollutants on fish sperm motility has been studied by subjective scoring (Abascal et al., 2007; Singh et al., 2008), pollutant effects on a wider range of

objectively measured sperm motility variables have yet to be ascertained. Furthermore, interspecies variation of sperm types in teleost has been also demonstrated (Jamieson, 1991; Munoz et al., 2002) which suggests variation in motility behavior. Moreover, several recent studies demonstrated the presence of different sperm subpopulations within the same fish sample (Cabrita et al., 2008; Martinez-Pastor et. al., 2008). Both studies examined sperm motility in Senegalese sole using computer-aided sperm analyses (CASA). As such the occurrence of sperm subpopulations of fish sperm and their reaction to pollutants could enable a more detailed characterization of sperm motility behavior.

We therefore, tested the hypothesis that spermatozoa differ in their pattern of movement, depending on subpopulation, and exposure to pollutant. To test this hypothesis, spermatozoa considered motile by a computer-assisted motility analyzer (CASA) were allotted to different sperm populations according to their individual kinematic parameters using a series of multivariate pattern cluster analyses (Abaigar et al., 1999).

4.1.2 Aims of study

Aquatic ecosystems are complex environments due to the interaction of many biotic and abiotic factors. As toxicants in sublethal concentration may affect the cellular function of an exposed organism, the present study aimed to examine the effect of selected environmentally relevant pollutants on *Oreochromis niloticus* sperm motility as well as their effects on sperm morphology. Thus, the specific objectives of this chapter were set:

- i. to determine the effect of cadmium, malathion and rotenone at environmentally relevant concentration on subpopulation composition of a sperm sample using the motility data (VSL, VCL and LIN) obtained from CASA analysis, and
- ii. to determine the effect of environmentally relevant pollutants on sperm morphology

4.2 Materials and methods

4.2.1 Effect of cadmium on sperm motility

For exposure of sperm to cadmium, seminal fluid from five animals (ie five biological replicates) was diluted 1:499 (v/v) in distilled water-contained cadmium of selected concentration. The final concentration of CdCl₂ solutions were 0, 0.04, 0.22, 0.44 and 2.20 μM. CdCl₂ was first added to mannitol (300 mOsmol/kg) to obtain 0.04, 0.22, 0.44 and 2.20 μM of cadmium. Seminal fluid was diluted 1:1 (v/v) with CdCl₂-contained mannitol and kept on ice for 5 or 15 mins. CdCl₂ was also added to distilled water to give the final concentrations of 0.04, 0.22, 0.44 and 2.20 μM when in contact with sperm. The distilled water-contained cadmium was later added to cadmium-treated samples at 2:498 (v/v). Immediately, 5 μl of the activated cadmium-treated and control samples were transferred into one well of a 12-well multitest glass slide (ICN Biomedicals Inc., Basingtoke, UK), covered with a coverslip and videotaped for 15 sec. All experiments were performed as three technical replicates.

4.2.2 Effect of malathion on sperm motility

Experiments were performed as described for cadmium, on five biological replicates. The final concentration of malathion solutions were 0.003, 0.03, 0.3 and 1.5 μM. Immediately, 5 μl of the activated malathion-treated samples were transferred into one well of a 12-well multitest glass slide (ICN Biomedicals Inc., Basingtoke, UK), covered with a coverslip and videotaped for 15 sec.

The sperm suspensions were also subsampled for 5 and 15 mins incubation. Malathion was first added to mannitol (300 mOsmol/kg) to obtain 0.003, 0.03, 0.3 and 1.5 μM of malathion. Seminal fluid was diluted 1:1 (v/v) with malathion -contained mannitol and

kept on ice for 5 or 15 mins. Malathion was also added to distilled water to give the final concentrations of 0.003, 0.03, 0.3 and 1.5 μM when in contact with sperm. The distilled water-contained malathion was later added to malathion -treated samples at 2:498 (v/v). Immediately, 5 μl of the activated malathion-treated samples were transferred into one well of a 12-well multitest glass slide (ICN Biomedicals Inc., Basingtoke, UK), covered with a coverslip and immediately videotaped for 15 sec. All experiments were performed in triplicates.

4.2.3 Effect of rotenone on sperm motility

Experiments were performed as described for cadmium, on five biological replicates. The final concentration of rotenone solutions were 0.013, 0.025, 0.063 and 0.13 μM when in contact with sperm. Immediately, 5 μl of the activated rotenone -treated samples were transferred into one well of a 12-well multitest glass slide (ICN Biomedicals Inc., Basingtoke, UK), covered with a coverslip and immediately videotaped for 15 sec.

The sperm suspensions were also subsampled for 5 and 15 mins incubation. Rotenone was first added to mannitol (300 mOsmol/kg) to obtain 0.013, 0.025, 0.063 and 0.13 μM of rotenone. Seminal fluid was diluted 1:1 (v/v) with rotenone -contained mannitol and kept on ice for 5 or 15 mins. Rotenone was also added to distilled water to give the final concentrations of 0.013, 0.025, 0.063 and 0.13 μM when in contact with sperm. The distilled water-contained rotenone was later added to rotenone-treated samples at 2:498 (v/v). Immediately, 5 μl of the activated rotenone-treated samples were transferred into one well of a 12-well multitest glass slide (ICN Biomedicals Inc.,

Basingtoke, UK), covered with a coverslip and immediately videotaped for 15 sec. All experiments were performed in triplicates.

4.2.4 Effect of cadmium, malathion and rotenone on sperm morphology

Milt was diluted and activated 500-fold in pollutants-contained deionised distilled water at a given concentration and incubation period. The sperm were also sub-sampled at various time points (0, 30, 60, 120, 480 sec), immediately fixed and observed for morphological changes. Sperm morphology was evaluated by scanning electron microscopy (SEM). For these trials, three different individual samples were collected for SEM and TEM.

For SEM, samples were immediately collected on 12 µm thick polyester hydrophilic cyclopore membrane (Whatman Int. Ltd, England) and processed as followed (Nation, 1983). Briefly, sperm were fixed for 1 hour in 1.0% glutaraldehyde buffered with sodium cacodylate 0.1 M at 4° C and then with 3.0% gluteraldehyde for 2-3 days, rinsed and post fixed with 1.0% osmium tetroxide for 2 hours. After dehydration through an ascending ethanol series and hexamethyldisilazine (HMDS), samples were dried and gold coated. Samples were examined with JSM 6460 LV JEOL (JEOL Company). The percentage of normal and abnormal sperm (abnormalities of sperm head and acrosome, coiled sperm tail, etc.) were calculated from the total of 100 sperm observed, starting from the centre of the SEM grid.

Ultrastructural sperm morphology was evaluated by transmission electron microscopy (TEM). The activated samples were immediately fixed with 2.5% glutaraldehyde, centrifuged at 7000 rpm (5625 g) to pellet the cells and processed as followed (Hayat,

1986). Briefly, sperm cells were fixed for 2 hours in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 4°C and rinsed in buffer overnight. The samples were then postfixed for 1 hour in 1.0% osmium tetroxide, enbloc stained with 2% uranyl acetate in 30% acetone, washed and subjected to dehydration in acetone series. Samples were then embedded in Spurr resin and 90 nm (gold sections, Reichert Ultracut E, Leica) were mounted on 200 mesh formvar coated copper grids and stained with 4% uranyl acetate in 50% ethanol and Reynold's lead citrate. Diameter of nucleus and mitochondria (μm) were measured from longitudinal sagittal section of spermatozoa at 26500x using TEM TECNAI G2 Spirit BioTWIN (FEI Company, USA).

4.2.5 Hobson Sperm Tracker and sperm motility analysis

All experiments carried out on sperm motility were assessed at room temperature (18-20°C) using light microscopy at 20X negative-high phase contrast objective via a U-PMTVC adaptor (Olympus) and recorded with video cassette recorder (SONY-SLVD950G/I, SONY Corporation). This type of optic gives an even contrast to the head of fish spermatozoa during progressive motility. The Hobson Sperm Tracker gate parameters were adjusted for tilapia sperm. Thus, the search radius used was 5.94 μm with minimum and maximum cell size of 5.9 μm and 11.9 μm , respectively. The final settings were then saved as a disk file, which was then retrieved when each experiment was undertaken. The recordings were analyzed for sperm trajectories using a Hobson Sperm Tracker 7V3B (Hobson Vision Ltd., Baslow, UK) operating at 50Hz within IBM-compatible computer. The distance in the field of view was calibrated using a calibrated slide (100 x 0.01=1 mm, Pyser-Sgi, Ltd, Tonbridge, Kent, UK) and videotaped prior to each experiment.

4.2.6 Statistical analyses

Three hundred individual sperm trajectories were analysed quantitatively for each of the treatment/time combinations, using a Hobson Sperm Tracker (20 sperm in each of five biological replicates X 3 technical replicates). The sperm trajectories used for analysing the motility of tilapia sperm were straight line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) of motile sperm, and the data presented are the 5–20 s tracking interval after activation. Multivariate analyses of individual sperm trajectories were carried out using the computer program PATN (CSIRO, Canberra, Australia). Data sets for analysis were prepared by merging raw data files from every measured sperm sample. In the present study, the groups were distinguished on the basis of multivariate combinations of motion parameters. Once the sub-populations had been identified, the effect of treatment/time combinations on the frequency of the spermatozoa belonging to the fast-linear subpopulation (only) was analyzed by factorial ANOVA using general linear models (Statistica version 9.0) after log transformation of percentage data. Post-hoc multiple comparison tests for differences of population means were performed using Tukey test. Data were expressed as the mean \pm standard deviation (s.d.). A probability < 0.05 was accepted as significant.

For sperm morphology analysis, the data were first tested for normality using a Kolmogorov-Smirnov test. Mean values of the measured parameters and standard deviation (S.D.) were calculated per sample. Data were then statistically analysed by a one-way ANOVA followed by Tukey's multiple comparison post-test or by a Kruskal–Wallis test. A probability < 0.05 was accepted as significant.

4.3 Results

4.3.1 Effect of cadmium on sperm motility

Data were obtained from 4500 individual motile spermatozoa (ie all sperm for all times and treatments) and subjected to PATN analysis using the motility data obtained from CASA analysis: VSL, straight line velocity (μms^{-1}); VCL, curvilinear velocity (μms^{-1}) and; LIN, linearity (%). A summary table showing PATN group motility characteristics derived from cadmium experiment is shown in Table 4.1. Each subpopulation was characterized by a mean value of the motion parameters distinguished by their velocity and linearity rendered by CASA. The first subpopulation (group 1) contained the largest number of spermatozoa (3420) and included spermatozoa covering short distances (low VSL), vigorous (high VCL) and poorly progressive (low LIN), and hence they were labeled as slow-non linear spermatozoa. The second subpopulation (group 2) contained 1080 spermatozoa covering long distances (high VSL), less vigorous (low VCL) but very progressive (high LIN): fast-linear spermatozoa (Figure 4.1).

The occurrence of sperm subpopulation group 2 (fast-linear) were consistent among replicate fish, although there was a small variation of numbers of spermatozoa in a group among individual fish, even though inter-individual variation was not evident ($F_{4,70} = 0.363$, $P = 0.834$).

Overall examination of the cadmium treatment effects upon frequency distribution of individual spermatozoa revealed that, cadmium treatment induced a shift of spermatozoa out of group 2 (fast-linear) into group 1 (slow-non linear). Frequency variations of fast linear spermatozoa along the dose response experiment showed that when cadmium was absent, there was a high proportion (24.80%) of group 2, fast linear

spermatozoa. However, exposure to cadmium induced a considerable change in the profile. In the presence of 0.04 μM cadmium, only 18.89% spermatozoa of group 2 was classified as group 2. As cadmium concentration was increased to 0.22 μM , 19.07% of the spermatozoa was classified as group 2. However, at 0.44 μM , the effect was highly significant ($P=0.001$) where only 18.70% exist as group 2 when compared to control (without cadmium treatment) (Figure 4.2). Testing the dose response (0, 0.04, 0.22, 0.44 and 2.2 μM), there is clear evidence that cadmium dosage significantly affected the frequency of sperm subpopulation of group 2 ($F_{4,60}=4.454, P=0.003$).

However, when the sperm cells were pretreated with cadmium for 5 mins, less fast-linear spermatozoa were observed (21.0%) compared to the control (29.0%). The trend in the reduction of sperm subpopulation of group 2 due to shifting to group 1 remained evident as the length of cadmium exposure was increased to 15 mins. Testing the incubation response (0, 5 and 15 min) confirmed that the length of cadmium incubation directly affected the frequency of sperm in subpopulation group 2, which was evident at 5 mins ($P<0.05$) and 15 mins ($P<0.05$) of incubation period ($F_{2,60}=11.158, P=0.020$; Figure 4.3).

Inspection of the interaction plot of cadmium treatment against incubation suggested that cadmium reduced the proportion of fast linear spermatozoa. This was confirmed by statistical analysis where cadmium treatment and incubation render differences for sperm subpopulation group as evident by factorial ANOVA ($F_{8,60}=14.640, P=0.000$). These results support the hypothesis that pre exposure of cadmium acts upon a particular subset of spermatozoa and alters their trajectories. The results also indicate that a small proportion of fast linear spermatozoa change their motility behavior in the

presence of cadmium. Furthermore, factorial ANOVA demonstrated, with the evidence of dose-duration interaction, that the sperm group 2 subpopulation are significantly affected by cadmium.

Table 4.1: Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of cadmium

Source of variation	No. of sperm in subset	VSL μms^{-1}	VCL μms^{-1}	LIN (%)
Group 1 (slow-non linear sperm)	3420	19.68 \pm 0.14	64.13 \pm 8.15	30.15 \pm 3.38
Group 2 (fast-linear sperm)	1080	29.71 \pm 0.00	60.68 \pm 0.00	49.93 \pm 0.00

*n=5 fish, 60 spermatozoa each

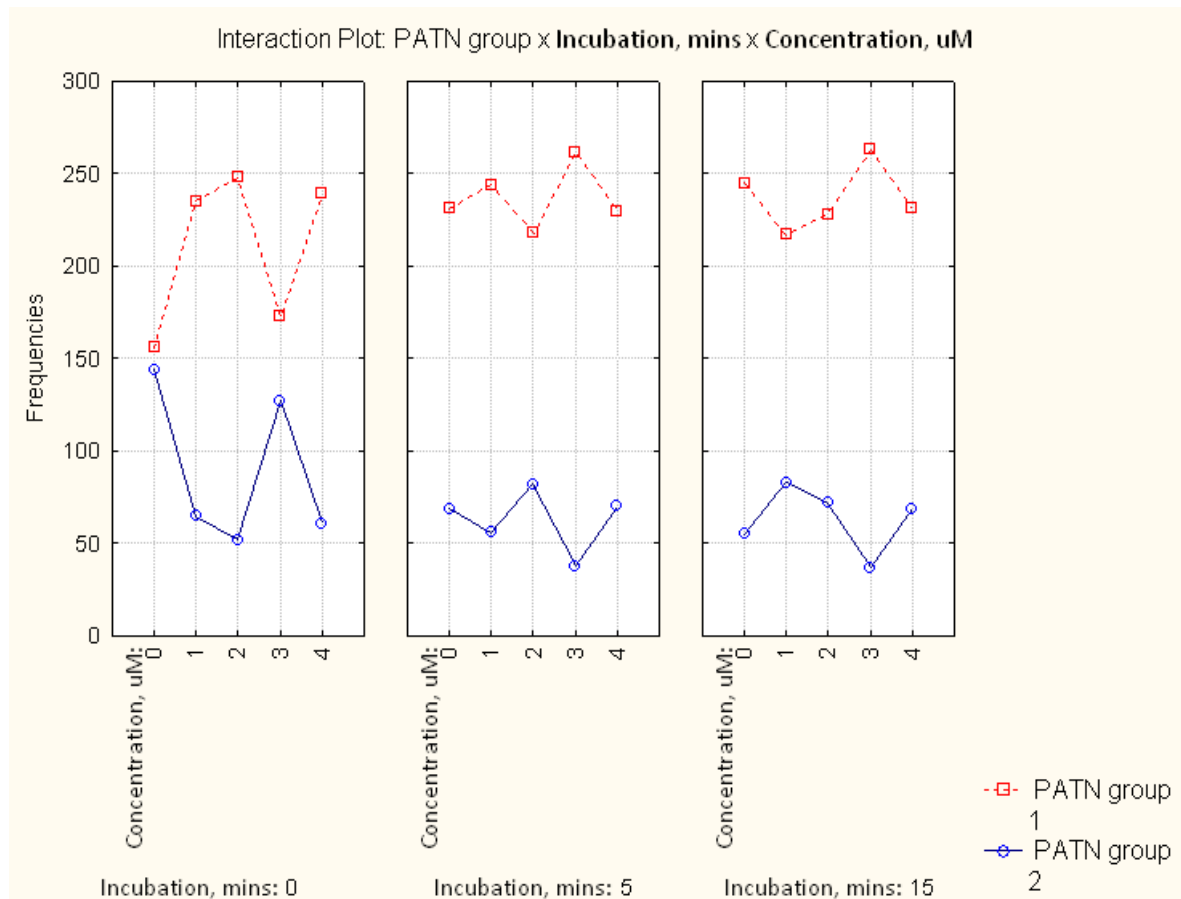


Figure 4.1. Interaction plot of frequency of spermatozoa classified as PATN group 1 and 2 in relation to cadmium treatment and pre exposure at 0, 5 and 15 mins; (0=0 μM ; 1 = 0.04 μM ; 2=0.22 μM ; 3= 0.44 μM ; 4 =2.2 μM)

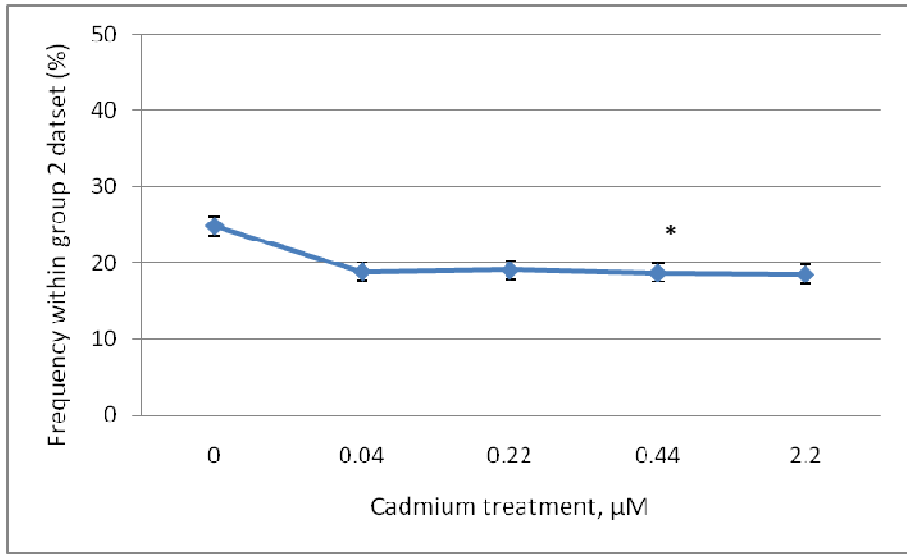


Figure 4.2 Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to cadmium treatment; vertical bars denote 95% C.I; *P<0.05

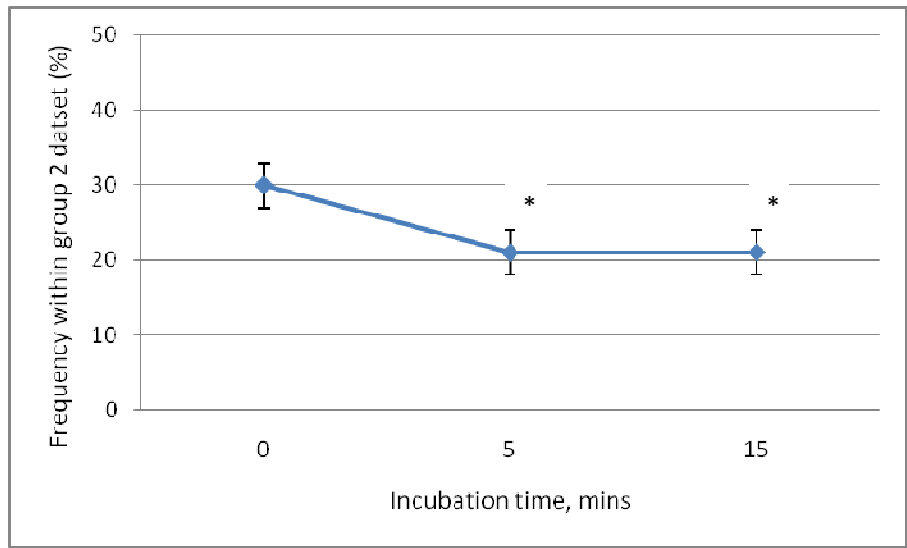


Figure 4.3 Frequency (percentage) of fish spermatozoa in data set classified as PATN group 2 in relation to the period of pre exposure to cadmium; vertical bars denote 95% C.I; *P<0.05

4.3.2 Effect of cadmium on sperm morphology

No significant difference was observed in terms of sperm morphology ($p > 0.05$).

4.3.3 Effect of malathion on sperm motility

Data were obtained from 4500 individual motile spermatozoa and subjected to PATN analysis using the motility data obtained from CASA analysis: VSL, straight line velocity (μms^{-1}); VCL, curvilinear velocity (μms^{-1}) and; LIN, linearity (%). Summary table showing PATN group motility characteristics derived from malathion experiment is shown in [Table 4.2](#). Each subpopulation was characterized by a mean value of the motion parameters distinguished by their velocity and linearity rendered by CASA. The first subpopulation (group 1) contained the largest number of spermatozoa (3346) that included spermatozoa covering long distances (high VSL), less vigorous (low VCL) and progressive (high LIN), and hence they were labeled as fast- linear spermatozoa. The second subpopulation (group 2) contained 1154 spermatozoa covering short distances (low VSL), vigorous (high VCL) but less progressive (low LIN): slow-nonlinear spermatozoa (Figure 4.4).

The occurrence of sperm subpopulation group 1 (fast-linear) were consistent among fish, there was a small variation of spermatozoa group among individual fish where inter individual variation was not evident ($F_{4,70} = 0.006$, $P = 0.999$).

Table 4.2: Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of malathion

Source of variation	No. of sperm in subset	VSL μms^{-1}	VCL μms^{-1}	LIN (%)
Group 1 (fast-linear sperm)	3346	24.76 \pm 9.28	66.43 \pm 10.94	38.29 \pm 13.61
Group 2 (slow-non linear sperm)	1154	7.41 \pm 3.61	70.53 \pm 18.97	11.86 \pm 7.38

*n=5 fish, 60 spermatozoa each

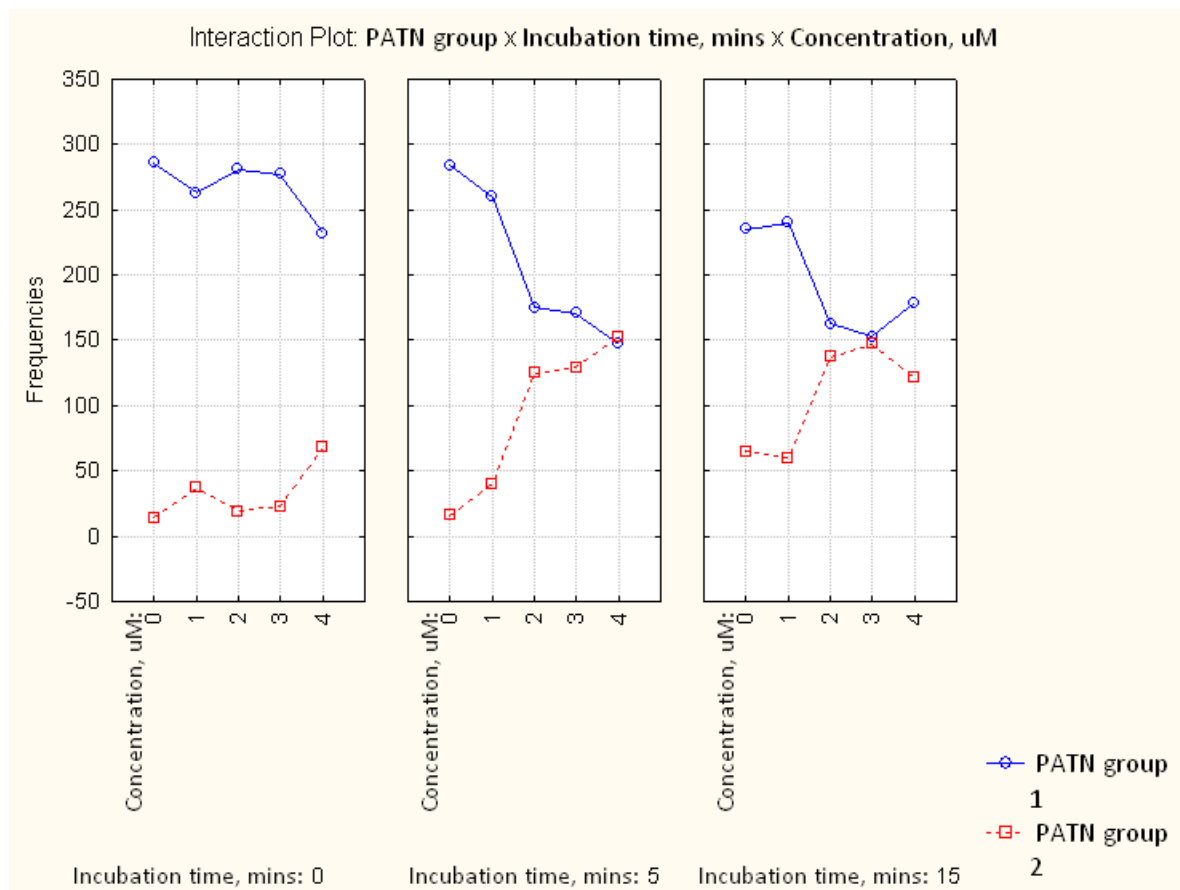


Figure 4.4. Interaction plot of frequency of spermatozoa classified as group 1 and 2 in relation to malathion treatment at 0, 5 and 15 mins; (0=0 μM ; 1 = 0.003 μM ; 2=0.03 μM ; 3= 0.03 μM ; 4 =1.5 μM)

Frequency variations of fast linear spermatozoa along the dose response experiment showed that when malathion was absent, there was a high proportion (24.06%) of group 1, fast linear spermatozoa. However, exposure to malathion induced a considerable change in the profile. In the presence of 0.003 μM malathion, only 22.80% spermatozoa was classified as group 1. As malathion concentration was increased to 1.5 μM , only 16.68% of the spermatozoa was classified as group 1. Testing the dose response (0, 0.003, 0.03, 0.3 and 1.5 μM), there is a clear evident that malathion concentration of $\geq 0.03 \mu\text{M}$ significantly ($P < 0.05$; Figure 4.5) affected the frequency of sperm subpopulation of group 1 ($F_{4,60} = 77.394$, $P = 0.000$).

However, when the sperm cells were pretreated with malathion for 5 mins, less number of fast-linear spermatozoa were observed (69.13%) compared to the control (89.27%). The trend in the reduction of sperm subpopulation of group 1 remained evident as the length of malathion exposure was increased to 15 mins where only 64.67% of group 1 was available. Testing the incubation response (0, 5 and 15 min) confirmed that the length of malathion incubation directly affected the frequency of sperm subpopulation in group 1 which was evident at 5 and 15 min ($P < 0.05$; Figure 4.6) of incubation period ($F_{2,60} = 144.40$, $P = 0.000$).

Inspection of the interaction plot of malathion treatment against incubation suggested that malathion reduced the proportion of fast linear spermatozoa. This was confirmed by statistical analysis where malathion treatment and incubation render differences for sperm subpopulation group as evident by factorial ANOVA ($F_{8,60} = 19.568$, $P = 0.000$).

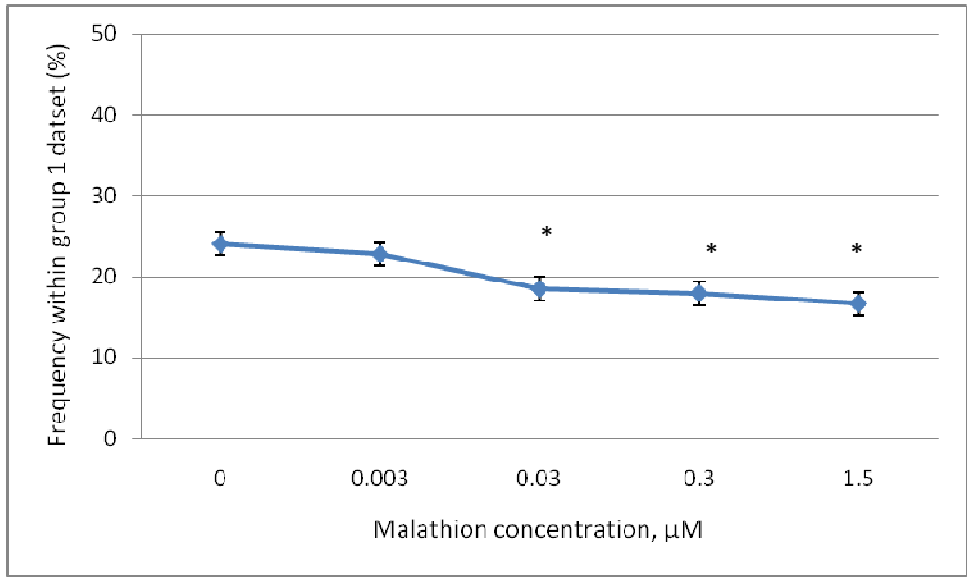


Figure 4.5 Frequency (percentage) of fish spermatozoa in data set classified as group 1 in relation to malathion treatment; vertical bars denote 95% C.I.; * $P < 0.05$

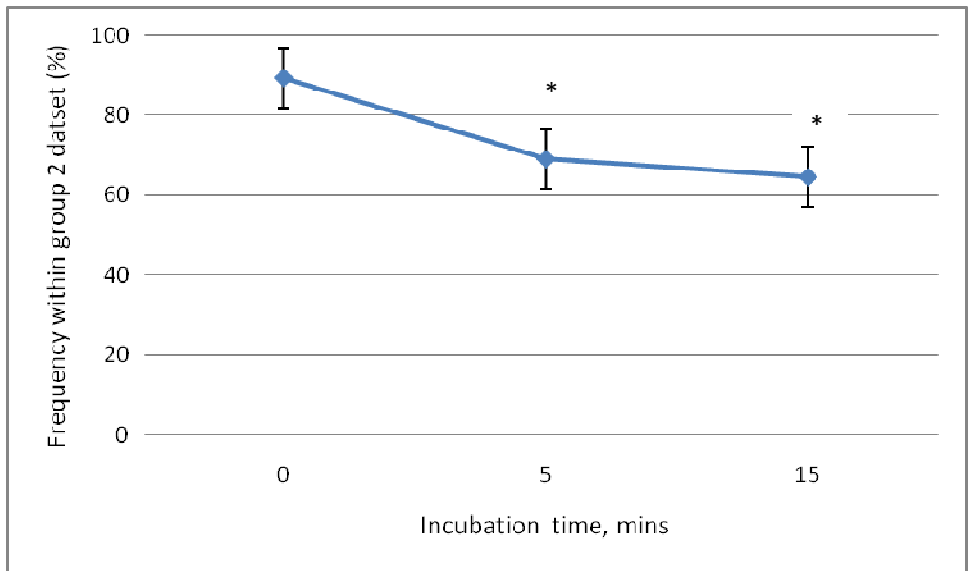


Figure 4.6 Frequency (percentage) of fish spermatozoa in data set classified as group 1 in relation to the period of pre exposure to malathion; vertical bars denote 95% C.I.; * $P < 0.05$

These results support the hypothesis that pre-exposure to malathion acts upon a particular subset of spermatozoa and alters their trajectories. The results also indicate that a small proportion of fast linear spermatozoa change their motility behavior in the presence of malathion. Furthermore, factorial ANOVA demonstrated, that the sperm subpopulation are significantly affected by malathion dosage with variation due to duration of the treatment, with the evidence of dose-duration interaction.

4.3.4 Effect of malathion on sperm morphology

No significant difference was observed in terms of sperm morphology ($p > 0.05$).

4.3.5 Effect of rotenone on sperm motility

Data were obtained from 4500 individual motile spermatozoa and subjected to PATN analysis using the motility data obtained from CASA analysis: VSL, straight line velocity (μms^{-1}); VCL, curvilinear velocity (μms^{-1}) and; LIN, linearity (%). Summary table showing PATN group motility characteristics derived from rotenone experiment is shown in Table 4.3. Each subpopulation was characterized by a mean value of the motion parameters distinguished by their velocity and linearity rendered by CASA. The first subpopulation (group 1) contained the largest number of spermatozoa (3403) that included spermatozoa covering long distances (high VSL), less vigorous (low VCL) and progressive (high LIN), and hence they were labeled as fast- linear spermatozoa. The second subpopulation (group 2) contained 1097 spermatozoa covering short distances (low VSL), vigorous (high VCL) but less progressive (low LIN): slow-nonlinear spermatozoa (Figure 4.7).

The occurrence of sperm subpopulation group 1 (fast-linear) were consistent among fish, there was a small variation of spermatozoa group among individual fish where inter individual variation was not evident ($F_{4,70}= 0.661$, $P=0.620$).

Frequency variations of fast linear spermatozoa along the dose response experiment showed that when rotenone was absent, there was a high proportion (22.10%) of group 1, fast linear spermatozoa (Figure 4.8). However, exposure to rotenone induced a considerable change in the profile. In the presence of 0.013 μM rotenone, only 21.07% spermatozoa was classified as group 1. As rotenone concentration was increased to 0.125 μM , only 18.16% of the spermatozoa was classified as group 1. At 0.063 and 0.125 μM , the effect was highly significant ($P<0.05$; Figure 4.8). Testing the dose response (0, 0.013, 0.025, 0.063 and 0.125 μM), there is a clear evident that rotenone dosage significantly affected the frequency of sperm subpopulation of group 1 ($F_{4,60}=11.331$, $P=0.000$).

However, when the sperm cells were pretreated with rotenone for 5 mins , less number of fast-linear spermatozoa were observed (80.0%) compared to the control (80.30%). The trend in the reduction of sperm subpopulation of group 1 remained evident as the length of malathion exposure was increased to 15 mins where only 66.53% of group 1 was available. Testing the incubation response (0, 5 and 15 min) confirmed that the length of malathion incubation directly affected the frequency of sperm subpopulation in group 1 which was evident at 15 mins ($P<0.05$; Figure 4.9) of incubation period ($F_{2,60}=28.587$, $P=0.000$).

Table 4.3: Summary of group means (\pm s.d.) of spermatozoa motility parameters derived from PATN analysis: effect of rotenone

Source of variation	No. of sperm in subset	VSL μms^{-1}	VCL μms^{-1}	LIN (%)
Group 1 (fast-linear sperm)	3403	25.41 \pm 9.97	62.66 \pm 12.64	40.39 \pm 14.59
Group 2 (slow-non linear sperm)	1097	9.19 \pm 5.11	76.07 \pm 10.17	12.27 \pm 6.47

*n=5 fish, 60 spermatozoa each

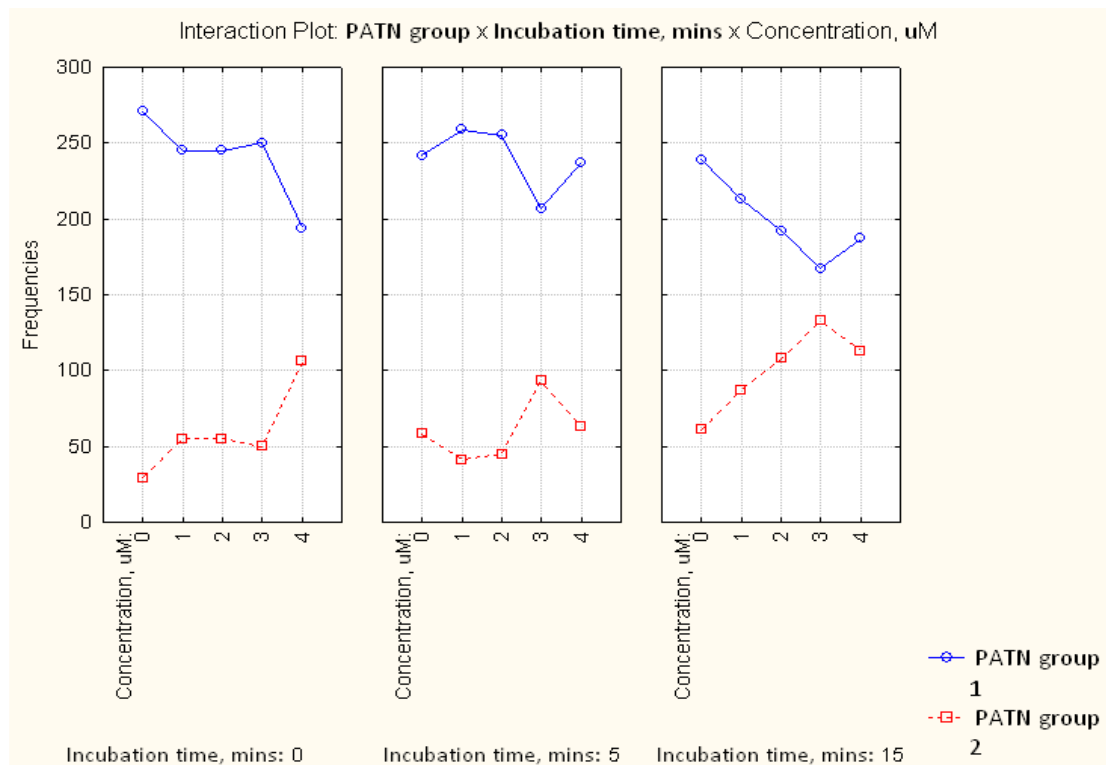


Figure 4.7. Interaction plot of frequency of spermatozoa classified as group 1 and 2 in relation to rotenone treatment at 0, 5 and 15 mins ; (0=0 μM ; 1=0.013 μM ; 2= 0.025 μM ;3= 0.063 μM ; 4= 0.125 μM)

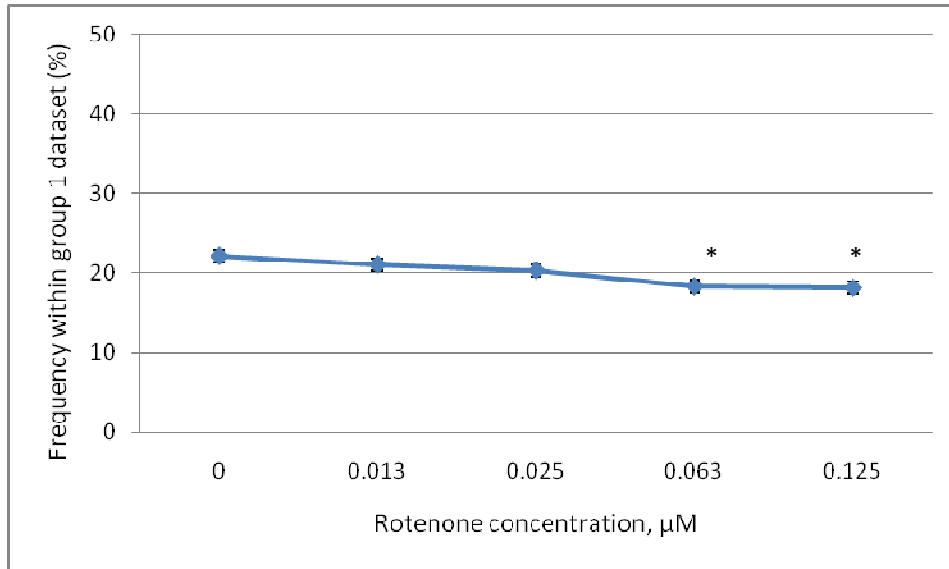


Figure 4.8 Frequency (percentage) of fish spermatozoa in data set classified as group 1 in relation to rotenone treatment; vertical bars denote 95% C.I.;*P<0.05

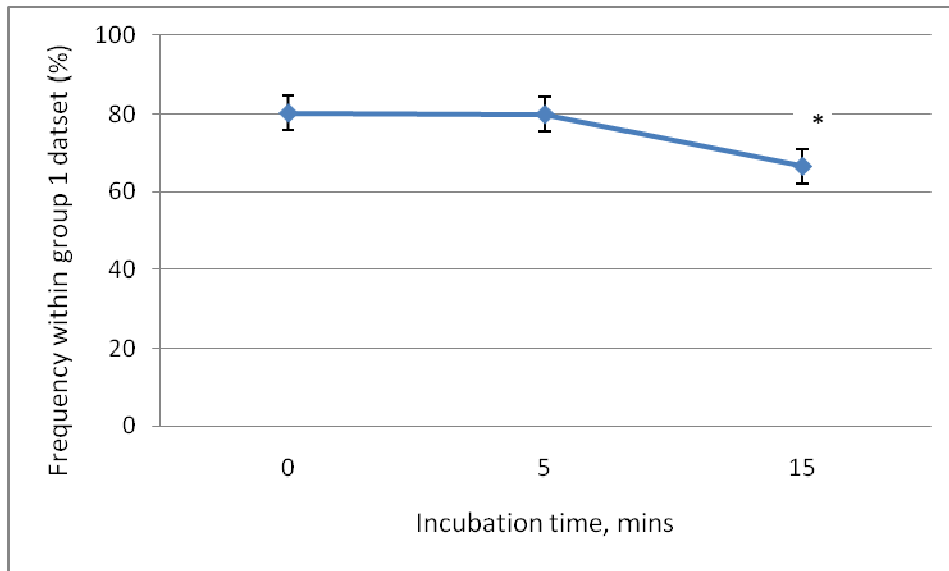


Figure 4.9 Frequency (percentage) of fish spermatozoa in data set classified as group 1 in relation to the period of pre exposure to rotenone; vertical bars denote 95% C.I. ;*P<0.05

Inspection of the interaction plot of rotenone treatment against incubation suggested that rotenone reduced the proportion of fast linear spermatozoa. This was confirmed by statistical analysis where rotenone treatment and incubation render differences for sperm subpopulation group as evident by factorial ANOVA ($F_{8,60} = 4.184$, $P=0.000$). These results support the hypothesis that pre exposure of rotenone act upon a particular subset of spermatozoa and altered their trajectories. The results also indicate that a small proportion of fast linear spermatozoa change their motility behavior in the presence of rotenone. Furthermore, factorial ANOVA demonstrated, that the sperm subpopulation are significantly affected by rotenone dosage with variation due to duration of the treatment, with the evidence of dose-duration interaction.

4.3.6 Effect of rotenone on sperm morphology

No significant difference was observed in terms of sperm morphology ($p>0.05$).

4.4 Discussion

The Hobson Sperm tracker automatically analyzes 14 different parameters of motility, but for simplicity only VSL, VCL and LIN are referred to in this chapter. These sperm trajectories variables were selected as they are correlated with fertility (Moore and Akhondi, 1996; Rurangwa et al., 2001) in the first 5- 20 sec, and provide the most useful data (Kime et al., 2001). In an attempt to establish the effect of water-based pollutants on sperm trajectories, VSL, VCL and LIN data obtained from sperm tracker analysis were subjected to multivariate analysis using PATN software. In this study, we applied a similar multivariate statistical analysis to the one used by Abaigar et al. (1999). The resultant number of groups depends upon the value of an initial association threshold (Bray and Curtis association index) in parallel with the Sequential Agglomerative Hierarchical-Combinatorial (SAHN) flexible unweighted pair groups median average (UPGMA) algorithm. In the present study the value was set at 0.3 to yield a small number of groups. Upon completion of the analysis, each individual spermatozoon was categorized as belonging to one of a small number of groups, or subpopulations. In this study, the groups were distinguished on the basis of multivariate combinations of sperm trajectory descriptors previously attained from Hobson sperm tracker and the descriptive interpretation of the sperm motion behavior that each group represents. PATN analyses clearly demonstrated the existence of a heterogeneous sperm population within sperm samples of tilapia. In most previous analyses of sperm motility in fish species, the occurrence of the sperm subpopulations has been neglected, hindering the rigorous evaluation of sperm quality. In mammalian species, the coexistence of sperm subpopulations has been demonstrated in swine where heterogeneous responses are seen both within single semen samples and between

individual samples (Harrison and Holt 2000, Holt and Harrison 2002) and in stallion ejaculates (Quintero-Moreno et al., 2003).

Our results show that in samples exposed to cadmium, two sperm subpopulation or group were identified. Group 1 represented the slow and non-linear sperm. On the other hand, group 2 represented the fast and linear sperm. In the presence of cadmium, less proportion of fast linear spermatozoa were seen when compared to control. This result could be due to the spermatozoa in group 2 alter their motility behaviors when exposed to cadmium. Similarly when the sperm were pre exposed to cadmium, less number of spermatozoa in group 2 was observed, which may due to the changing of motility behavior to group 1. The dose response effect was evident at 0.44 μM .

Cadmium is known to compete with calcium for entry *via* calcium channels. In *Chlamydomonas*, calcium affects the waveform of flagellar motility through regulation of dynein-driven microtubule sliding (Smith, 2002). In addition, it has been demonstrated that a similar mechanism occurs in tilapia sperm (Morita et al., 2006). Heavy metal ions also bind with the thiol groups of the channel and other proteins. Calcium channel proteins has been identified in fish sperm and is primarily responsible for activation of sperm motility in marine (Vines et al., 2002) and freshwater teleosts (Tanimoto and Morisawa, 1988; Krasznai et al., 2000). Sperm swimming performance has been shown to be controlled by different environmental factors and also by a variety of pollutants. For cadmium, the typical affected parameter reported was the percentage of motile sperm which decreased at a concentration-dependent rate with a complete inhibition of motility at 100 mg /l (which is equivalent to 43 μM cadmium) (Dietrich et al., 2009). Our results are consistent with the conclusions of previous studies which

demonstrated a reduction in sperm swimming speed following sperm exposure to 1000-100,000 $\mu\text{g/L}$ cadmium in sea urchin (Au et. al., 2000) and African catfish (Kime et al., 1996).

Malathion was chosen because it is widely used, due to the perception that its short half life and relatively lower toxicity to fish than to invertebrates renders it environmentally friendlier. Thus, malathion has low cumulative ability due to short term persistence but high pesticidal action (Svoboda et al., 2001). Our results show that in samples exposed to malathion, two sperm subpopulation or group were identified. Majority of the spermatozoa are classified as group1 which represented the fast and linear sperm. On the other hand, group 2 represented the slow and non-linear sperm.

In the present study, our results show that malathion affects tilapia sperm trajectories at 0.03 μM and reduces the proportion of fast-linear spermatozoa where the effect was evident at 5 mins of the pre exposure period. This result indicates that malathion alters tilapia sperm trajectories and reduce the number of fast-linear spermatozoa group. By comparison a typical 96hour LD50 for water exposure of tilapia fingerlings or other cichlids to malathion has been determined between 6 μM and 15 μM (Mani and Konar, 1984; Shao-nan and De-fang, 1996; Pathiratne and George, 1998), indicating the high sensitivity of sperm.

It is important to point out that malathion, can be detoxified by hydrolysis of the carboxyl ester linkage by carboxylesterase enzymes. The drug is known to lack a direct effect on anticholinesterase activity, but has to be metabolized to malaaxon in order to act as a cholinesterase inhibitor (Hayes, 1989). In mammals, carboxylesterases catalyze

rapid degradation of malathion to monoacid and diacid derivatives (Kutz et al., 1992; USEPA, 2000). This detoxification reaction efficiently competes with the cytochrome P450-catalyzed formation of the toxic malathion metabolite, phosphate triester malaoxon. Malaoxon in turn can be degraded by carboxylesterase to dimethylthiophosphate. In general, the major pathway of malathion metabolism is the cytochrome P450-catalyzed bioactivation, resulting in a dramatic increase in the production of malaoxon. Malaoxon inhibits acetylcholinesterase activity and thus inhibits nervous transmission, causing paralysis and eventually death in exposed organisms. Clearly the inhibition of nervous transmission is unlikely to be a relevant process in sperm which lack neurons. However, in mammals the occurrence of acetylcholinesterase in spermatozoa has been demonstrated. For instance, in earlier studies, acetylcholinesterase activity was reported in head, midpiece and tail fractions of bull spermatozoa (Nelson, 1964). It is suggested that acetylcholinesterase regulates some process associated with sperm motility (Nelson, 1972). Alternatively, some data imply that the organophosphorus compounds can produce oxidative stress through production of lipid peroxidation in spermatozoa (Pina-Guzman et al., 2006), which may account for toxicity in sperm, especially since in teleost sperm, most of the phospholipids contain the more susceptible unsaturated fatty acids (Bell et al., 1997). In addition, there is evidence that organophosphorus compounds can act on mitochondria and alter ATP production (Massicotte et al., 2005). If the site of action and toxic effect of malathion in sperm is to inhibit acetylcholinesterase associated with motility, then this suggests that the transformation of malathion to malaoxon is occurring, indicating the presence of cytochrome P450-resembling enzyme in tilapia spermatozoa.

Compared to cadmium and malathion, studies on rotenone sublethal toxicity in fishes are sparse. Its short ecologic half life favours its use as a nonpersistent piscicide compared with other toxic substances. The results show that in samples exposed to rotenone, two sperm subpopulation or group were identified. Majority of the spermatozoa are classified as group1 which represented the fast and linear sperm. On the other hand, group 2 represented the slow and non-linear sperm.

Our results show that rotenone at 0.063 μM alters sperm trajectories of tilapia and reduces the proportion of fast-linear sperm at 15 mins of incubation period. Rotenone is known as a mitochondrial complex I inhibitor that depresses the cellular respiration rate in mitochondria. However, mitochondrial respiration can also function by way of complex II using succinate, consequently bypassing any effect of rotenone on complex I (Garrett and Grisham, 1999), which may be the reason why the effect can only be seen at a longer incubation period (15 mins in the present study).

In the present study, all pollutants affect sperm trajectories within the fast-linear spermatozoa subpopulation of tilapia. Iwamatsu et al. (1997) found that linear swimming velocity is correlated with the ability of spermatozoa to enter the micropyle. The alteration of sperm trajectories by pollutants as demonstrated in our study implies that the chance of fertilization success is reduced. Similarly, as the velocity of fish sperm decreases rapidly with time, the duration of progressive movement will also have a significant influence on the ability of sperm to enter the egg. Hence, a slow and nonlinear spermatozoa would probably be less efficient at achieving fertilization, which if occurring in the environment, as certainly indicated by the sub micromolar effects of

malathion, would have potentially serious consequences for fish populations in polluted environments.

Reduced sperm swimming velocity are often linked to abnormal sperm morphology. For example, sperm exhibited swelling of the midpiece, mitochondrial damage, and breakage of the flagella when exposed to more than 5 mg/L cadmium (Au et al., 2000) and in goldfish, exposure to 100 mg/L mercury reduced sperm flagellar length (Van Look and Kime, 2003). In the present study, the reduction in sperm swimming speed observed in this study occurred at much lower concentrations of toxicants and no abnormal morphologies were observed. Therefore, the possibility that the impaired sperm function in tilapia sperm after exposure to pollutants as described here may be due to morphological damage, can be excluded.

Finally, our study based on data obtained with the CASA system demonstrated that the motile fraction of tilapia sperm can be statistically separated into subpopulations characterized by groupings of motility parameters. However, the significance of the existence of sperm subpopulations in tilapia as regards to fertilizing potential are not fully understood. The results from the present study also suggest that the estimation of motile spermatozoa and the kinematic properties of sperm motion may not given a correct interpretation particularly if the effects investigated are related to sperm motion. Nevertheless, this finding calls for further in-depth analysis of the sperm subpopulation exposed to pollutants or environmental stressors. In addition, tilapia spermatozoa trajectories appear to be sensitive to very low concentrations of cadmium, malathion and rotenone but not morphological damage. In conclusion, CASA and subpopulation analysis would allow a better understanding of the physiology of tilapia sperm exposed

to pollutants, and may provide novel, sensitive, ethically acceptable and ecologically relevant methods for the assessment of the risk posed by chemical contaminants in the environment.

CHAPTER 5 GENERAL CONCLUSIONS

Herein is presented an integrative conclusion describing the key results obtained in the present work.

The first aim of the present study was to identify the extracellular factors and seminal fluid characteristics that trigger movement and morphological changes in *O. niloticus* sperm.

We found that (i) sperm were immotile in seminal plasma and remained quiescent in electrolyte or nonelectrolyte solutions isotonic to seminal plasma; (ii) sperm movement was initiated when the sperm were exposed to hypoosmotic electrolyte or hypoosmotic nonelectrolyte solutions. In general, the highest percentage of motile spermatozoa and highest spermatozoa velocity were observed between 0 to 200 mOsmol/kg. Thus, our results suggest that osmolality regardless of ion play important role for sperm motility in this species. We also found that sperm show optimal activity at pH range of 6-8 which depicts that the effect of pH on sperm motility is negligible.

As opposed to previous reports on freshwater species, such as African catfish, *Clarias gariepinus* (Mansour et al., 2002), common carp, *Cyprinus carpio* (Krasznai et al., 2000) and sea lamprey, *Petromyzon marinus* (Ciereszko et al., 2002), demonstrating that electrolytes are necessary to initiate sperm motility, our results indicate that electrolytes are not necessary for sperm motility which is in agreement with those of others (Hu et al., 2009).

PATN analysis was able to distinguish the existence of sperm sub-populations. We showed that lanthanum (calcium channel inhibitor) and flunarizine (sodium-calcium exchanger inhibitor) affected sperm motility of fast linear group of spermatozoa at 25 and 5 μ M, respectively, demonstrating a dose -response relationship. The existence of membrane-bound ion channel has been demonstrated in the mid piece region of herring sperm (Vines et al., 2002). Thus, we suggest that calcium channel and sodium-calcium exchanger play important roles for sperm motility of *Oreochromis niloticus*. In contrast, we were unable to detect effects on sperm sub-populations with compounds which block epithelial sodium channels, sodium/potassium ATPase or voltage gated potassium channels.

General reviews concerning the evolution of sperm morphology (Jamieson, 1991) consistently regard teleost sperm as having round or eliptoid head with large nucleus, no acrosome and a midpiece composed of mitochondria, which together correspond to a 'primitive' morphology. The spermatozoa of *Oreochromis niloticus* were uniflagellate with clearly differentiated oval-shaped head, midpiece and flagellum thus fitted into the primitive type of the sperm classification.

We also observed that as sperm motility was initiated in hypoosmotic solution (distilled water) the profound swelling of the midpiece area induced changes to the cytoplasmic sheath (sleeve). These results were in agreement with an earlier report in carp that low osmolality induced a restructuring of the membrane (Marian et al., 1993).

Heavy metals such as cadmium at low concentration and non-persistent pesticides commonly introduced into aquatic environments may pose a significant threat to aquatic organisms particularly with regard to reproduction. With this concern in mind, the second aim of the present study was to determine the physiological effects on sperm motility parameters associated with *in vitro* sublethal exposure of selected environmentally relevant pollutants i.e cadmium, malathion and rotenone, using a computer-aided sperm analysis (CASA) system.

Our results have shown the presence of a heterogeneous sperm population within sperm samples of Nile tilapia, similar to the one found in mammals (Holt and Harrison, 2002; Quintero-Moreno et al., 2003; Martinez-Pastor et al., 2005). In most previous analyses of sperm motility in fish species, the occurrence of the sperm subpopulations has been neglected, hindering the rigorous evaluation of sperm quality.

Sperm of fish show great promise as a useful indicator of low level toxic effects. This technique is also an uncomplicated, relatively low-cost methodology that could be applied in monitoring programs at various temporal and spatial scales. We have shown that sublethal exposure of cadmium, malathion and rotenone affect sperm trajectories of fast linear spermatozoa subpopulation. The effect of cadmium, malathion and rotenone was evident at 0.44, 0.03 and 0.063 μM , respectively.

Cadmium acted within 5 mins of exposure to sperm and is known as calcium channel inhibitor and competes with calcium for entry into a cell *via* calcium channels. Calcium channel proteins has been identified in fish sperm and is primarily responsible for activation of sperm motility in marine (Vines et al., 2002) and freshwater teleosts

(Tanimoto and Morisawa, 1988; Krasznai et al., 2000). Further this result is supported by the similar actions of calcium channel blockers, as described above. Like cadmium, malathion also acted very quickly on sperm motility. However, according to the classical model of organophosphate action, malathion has to be metabolized to malaoxon by cytochrome P450 in order to act as a cholinesterase inhibitor (Hayes, 1989). At present, no information on acetylcholinesterase in fish sperm is available. However, acetylcholinesterase activity has been reported in mammalian spermatozoa and has been linked to motility (Nelson, 1964). Therefore, the effect of malathion on sperm motility indicates the presence of an enzyme with P450-like activity in sperm of *Oreochromis niloticus*, which should be investigated further. Alternatively it is possible that malathion acts by a different, completely unknown toxic mechanism in sperm. Rotenone is known as a mitochondrial complex I inhibitor that depresses the cellular respiration rate in mitochondria. In contrast to cadmium and malathion, the effect of rotenone occurs at a later stage i.e. 15 min after pre-exposure. This is likely due to the fact that mitochondrial respiration can also function by way of complex II using succinate, consequently bypassing any effect of rotenone on complex I (Garrett and Grisham, 1999).

To conclude, the initiation of motility in spermatozoa of *Oreochromis niloticus* is regulated by osmotic shock with the aid of membrane calcium channel and sodium-calcium exchanger. Hypoosmotic shock alters sperm morphological structure of spermatozoa as observed by electron microscopy. In addition, the alteration of sperm trajectories in fast linear spermatozoa subpopulation by pollutants at submicromolar concentrations as demonstrated in our study implies potentially serious consequences for fish populations in polluted environments.

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