THE RELATIONSHIP BETWEEN "FERTILIZATION ENVIRONMENT" AND STRUCTURE AND PHYSIOLOGY OF SELECTED ANURAN SPERMATOZOA

BRIAN ADAM WILSON

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BY

BRIAN ADAM WILSON MSc (UWC); HDE (UNISA)

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PROMOTER: *PROFESSOR GERHARD VAN DER HORST*

JOINT PROMOTER: *PROFESSOR ALAN CHANNING*

DECEMBER 1993.

DECLARATION

I declare that **"The relationship between the 'fertilization environment' and structure and physiology of selected anuran spermatozoa"** is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

12/'93

Brian Adam Wilson

DEDICATION

This thesis is dedicated to my wife, Jennifer and daughter Tanya for their love, support and understanding and to my parents Andrew and Dinah for their genes and for instilling a thirst for knowledge.

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ABSTRACT

The propagation of species and modes of fertilization of higher vertebrates are aspects which have been extensively researched. Studies on the spermatology of lower vertebrates have not received the same attention. Anurans exhibit modes of fertilization which place them in a category between true aquatic and true internal fertilizers. They occupy fertilization environments ranging from aquatic to internal. This project was undertaken to establish baseline values for selected anuran spermatozoa and to test the hypothesis that fertilization environment plays a role in regulating sperm form and function.

Anuran spermatozoon structure and ultrastructure were investigated and comparisons drawn between sperm from aquatic and terrestrial fertilizers. Measurements of sperm heads, acrosomes and tail complexes were used to discern whether significant differences exist between sperm from aquatic and terrestrial anuran fertilizers.

Techniques were developed to determine baseline values for spermiograms of selected anuran species. These values were also compared to test the effect of selection pressures of the fertilization environment on sperm morphology and physiology. Sperm motion as a parameter to ascertain the viability of sperm was evaluated by using a Computer Assisted Sperm Motility Analysis (CASMA) system viz. the Sperm Motility Quantifier (SMQ). The pattern and vigour of sperm motion were used as additional data to further elucidate the above-

mentioned hypothesis.

Spermatozoa were aspirated from *Xenopus laevis* toads by developing methods to obtain sperm from amplectant males and via an electro-ejaculation technique. The motility patterns were assessed and compared to determine similarities or differences between testicular and ejaculated spermatozoa.

The research indicated that the fertilization environment plays a major role in modulating sperm structure and function but that the effect of phylogeny is as important. The baseline values obtained during this project could possibly be utilised in programmes to protect endangered anuran species e.g. by using these values for the cryopreservation of sperm.

ABSTRAK

Die voortplanting van species en die metodes van bevrugting van hoër vertebrate is aspekte wat omvattend nagevors is. Spermatologiese studies van die laere vertebrate het egter nie dieselfde aandag geniet nie. Die Anura vertoon metodes van bevrugting wat hulle in 'n kategorie tussen ware akwatiese en ware inwendige bevrugters plaas. Hulle beset bevrugtingsomgewings wat varieer vanaf akwaties tot inwendig. Hierdie projek is aangepak om basiese waardes daar te stel vir spermatosoa van geselekteerde Anura species en om die hipotese dat bevrugtingsomgewing 'n rol in die vorm en funksie van sperme speel, te toets.

Die struktuur en ultrastruktuur van spermatosoa van sekere Anura species is ondersoek en vergelykings is getref tussen sperme van akwatiese en terrestriële bevrugters. Afmetings van die spermkoppe, akrosome en stertkomplekse is gebruik om te bepaal of beduidende verskille tussen die sperme van akwatiese en terrestriële bevrugters bestaan.

Tegnieke is ontwikkel om grondwaardes vir spermiogramme van geselekteerde species van die Anura te bepaal. Hierdie waardes was ook vergelyk om die effek van seleksiedruk van die bevrugtingsomgewing op sperm morfologie en fisiologie te ondersoek. Spermbeweging is ook as parameter gebruik om die lewensvatbaarheid van sperme te bepaal deur 'n rekenaar ondersteunde motiliteits analise sisteem nl. die "Sperm Motility Quantifier" (SMQ) te gebruik. Die patroon en lewenskragtigheid van spermbeweging is gebruik as addisionale data om bogenoemde hipotese verder te belig.

Spermatosoa is verkry van *Xenopus laevis* deurdat metodes ontwikkel is om sperme van mannetjies in ampleksus en via 'n elektro-ejakulasie tegniek te bekom. Die motiliteitspatrone is bepaal en vergelyk om ooreenkomste en verskille tussen testikulêre en geëjakuleerde spermatosoa te bepaal.

Die navorsing het getoon dat die bevrugtingsomgewing 'n groot rol speel in die regulering van spermstruktuur en funksie, maar dat die effek van filogenie net so belangrik is. Die grondwaardes verkry tydens hierdie projek kan moontlik gebruik word om bedreigde Anura species te beskerm deur die data byvoorbeeld

te gebruik in studies om sperme van hierdie species te kriopreserveer.

"Thus says the Lord, Let my people go, ... But if you refuse to let them go, behold, I will plague all your country with frogs;..."

Ex. 8 vs 1 & 2.

CHAPTER 1

CHAPTER 1

GENERAL INTRODUCTION

Fertilization and the propagation of species have been researched by many. Studies on reproductive biology of species enhanced our knowledge on diversity of life on earth. Research in the latter decades has been focused on mechanisms to diagnose and treat infertility in human males and devise contraceptive mechanisms in the human male (WHO laboratory manual, 1987). This led to extensive research into the reproductive physiology of especially the mammalian vertebrates (Fawcett, 1973; Seier *et al.*, 1989; Curry *et al.*, 1989 and Van der Horst *et al.*, 1991). Research of mammalian reproduction is important and has been extensive, but investigations into the reproductive biology of submammalian vertebrates have received considerably less attention. Investigations regarding the adaptations of these lower vertebrates and invertebrates in terms of their fertilization environments could give us more insight into biodiversity.

The fertilization environments of species range from aquatic through to an internal environment. The fertilization environment is one of the regulating factors for sperm structure and physiology (Franzén, 1970; Lee and Jamieson, 1992; Jamieson *et al.*, 1993; Mainoya, 1981 and Mizuhira *et al.*, 1986). Interaction between sperm and egg in the variety of fertilization environments should be adapted for effective fertilization and research into the reproduction

of the lower animals could possibly enhance our knowledge on mammalian fertilization.

Although the fertilization environment plays an important role in shaping sperm morphology, phylogeny is equally important (Morrisett, 1974 and Fouquette and Delahoussaye, 1977). The status of frog classification has a long history of controversy (Kluge and Farris, 1969 and Hillis, 1991). There is general consensus that terrestrial biodiversity is much greater than the aquatic. To have overcome all the natural hurdles on land requires structural as well as physiological adaptations. Reproduction is furthermore one of the major areas of adaptation.

Anurans occupy a wide variety of fertilization environments, ranging from completely submerged under water, to terrestrial. The reproductive biology of the anurans has not been fully researched, but most of the frogs and toads are external fertilizers (Passmore and Carruthers, 1979). There are, however, species whose mode of fertilization is internal (Grandison and Ashe, 1983; Elinson, 1987 and Jamieson *et al.*, 1993). The spermatozoa are thus deposited in a wide variety of media, ranging from water to an internal environment. This variety of environments prompts research into the structure and physiology of spermatozoa and egg cells of the anurans. This study was undertaken to investigate the adaptations of the form and function of selected anuran spermatozoa to their particular fertilization environment.

The sperm cell is one of the most complex structures in living organisms. It has

also undergone extensive evolution in the different living organisms (Baccetti and Afzelius, 1976). Other researchers such as Morrisett (1974) and Fouquette and Delahoussaye (1977) investigated the structure of anuran sperm and related it to the phylogeny of the group. It was suggested that a double tail complex be regarded as primitive compared to a single tail that appears to be a secondary modification. In their studies on the sperm of *Heleophryne*, Visser and van der Horst (1987) regarded the double tail complex as a primitive characteristic. Franzén (1970) and Nicander (1970) related the differences in the structure of spermatozoa as adaptations to special fertilization environments. Van der Horst (1979) investigated spermatozoon structure in three anuran species and reached the same conclusions. Garrido, *et al.* (1989) studied the sperm morphology of *Batrachyla* and concluded that there is a correlation between sperm morphology and phylogenetic relationships as well as the mode of fertilization.

Reproduction can be regarded as one of the strongest reflexes in the life of plants and animals. Baccetti and Afzelius (1976) argued that evolution of the reproductive habits should have preceded any advent of a terrestrial mode of life. As such, the reproductive traits of the frogs can perhaps elucidate this phenomenon. In the evolution from the aquatic to the terrestrial environment one of the main problems to overcome, was the watery medium for the embryo to develop in. This "problem" was overcome in a variety of ways; such as the amnion of mammals and the eggs of birds.

The terminology related to fertilization and fertilization environment is confusing.

For this reason some of the terminologies used in the literature are briefly reviewed (Table 1.1).

Table 1.1: Review of terminologies

TERMINOLOGY	DESCRIPTION
Fertilization environment	The environment in which the spermatozoa are released during amplexus.
External fertilization environment	Eggs and spermatozoa are released outside the body. Fertilization takes place externally.
Internal fertilization environment	Spermatozoa are released in the body (cloacal cavity) of the female. Fertilization takes place within the female body.
Micro-fertilization environment	The environment immediately surrounding the egg and thus the area of sperm and egg inter-action. This will include the egg capsules.
Aquatic fertilizer	Toads and frogs who need bodies of water for fertilization. Amplexus normally takes place within the water. Eggs are laid in water and they usually have free-swimming tadpole stages (Passmore and Carruthers, 1979).

TERMINOLOGY	DESCRIPTION
Terrestrial fertilizer	Frogs occupying habitats away from water. Spermatozoa are normally released on or very near to the eggs in burrows or in mossy areas. These species normally construct nests under rocks or in burrows. They are typically direct developers with no free-swimming tadpole stages (Passmore and Carruthers, 1979). Species whose mode of fertilization is internal (Grandison and Ashe, 1983; Jamieson *et al.*, 1993) are also included.
Aquasperm	Sperm released in water and fertilizing the eggs externally in the water. Spermatozoa released in water exhibit typically primitive characteristics such as shorter and stouter head- and midpieces (Franzén, 1970). They are also called "primitive sperm" (Franzén, 1970) or "ect-aquasperm" (Jamieson and Rouse, 1989).

TERMINOLOGY	DESCRIPTION
Introsperm	Spermatozoa released in the cloacal cavity of the female and fertilizing the eggs internally. They have advanced features such as longer head- and midpieces (Jamieson and Rouse, 1989). Also called "modified sperm" (Franzén, 1970).
Niche	The function of an organism and its relationship to abiotic and other biotic factors in its environment (Hickman *et al.*, 1988).
Habitat	The place where an organism or members of its population live (Hickman *et al.*, 1988).

The boundary between the fertilization environment and the micro-fertilization environment is not very clear. For the purposes of this study, therefore, fertilization environment as explained in Table 1.1 will be used as basis for the hypothesis that this environment determine morphology and function of anuran spermatozoa. Although the environment in which the terrestrial fertilizers deposit their sperm could strictly be defined as the micro-fertilization environment, I regarded it as the gross fertilization environment, to distinguish between fertilization within bodies of water and fertilization on land.

The anurans are "typically" aquatic fertilizers. The structure of their spermatozoa

signifies this (Franzén, 1970 and Fouquette and Delahoussaye, 1977). However, within this order of amphibians, a wide variety of adaptations also exists, because of the habitats occupied by the various families of frogs. The fertilization environments of the anurans range from aquatic (eggs laid in the water) to terrestrial (eggs laid in burrows on land, or in a foam nest on a tree branch) to internal. The variety of fertilization environments dictate adaptations in the egg coverings but, more importantly, also evolution of the spermatozoa to penetrate those layers to fertilize the eggs.

Spermatozoa have a motility of their own and have features comparable to those of the Mastigophora (Protozoa). The flagella of these cells seem to indicate this (Baccetti and Afzelius, 1976). The movement of the spermatozoa requires adaptations regarding their energy requirements. Van der Horst (1986) compared the respiratory physiology of fish, mammalian and amphibian sperm and established correlations in terms of the fertilization environment. The swimming characteristics and the motility parameters have also been studied in detail by Samuels and van der Horst (1986). Bernadini *et al.* (1988b) inferred that there is no correlation between the motility status and respiration rate of *Xenopus* sperm, whereas a correlation could be observed in spermatozoa of other species. The patterns and vigour of sperm motility in the different anurans may render valuable information as to the adaptations of the anuran sperm.

The advent of electron microscopy provided methodology to study sperm structure in more detail. Many researchers studied various aspects of the structure

and physiology of spermatozoa. Unfortunately research has largely been restricted to the sperm of higher vertebrates and current research seems to focus on mammalian spermatology. There is merit in the study of higher vertebrate spermatozoa, but to gain an insight into the diversity of life on earth, we need to research the reproductive habits of lower vertebrates and the invertebrates.

Despite the lack of detail on sperm biology of anurans, several reports have appeared on sperm structure of these amphibians. Burgos and Fawcett (1956) studied spermatid differentiation in *Bufo*. Poirier and Spink (1971) published a report on the ultrastructure of the spermatozoa of *Rana*. Picheral (1979) in comparing structural and functional aspects of urodele spermatozoa, suggested some homologies between urodele and mammalian sperm tails. Mainoya (1981) made some observations regarding the ultrastructure of *Chiromantis* spermatids. Microscopic investigations of the head piece of the sperm of *Rhacophorus* was made by Mizuhira *et al.* (1986). Visser and van der Horst (1987) described the sperm structure of the ghost frog (*Heleophryne*). Our knowledge of the structure of spermatozoa has been enhanced by researchers such as Gosálvez *et al.* (1986) who used a silver staining method for visualizing the acrosome. The membranes of the sperm cell have also been detailed by spermatologists such as Bernadini *et al.* (1989).

The biology of fertilization in the anurans were studied by Raisman and Pisanó (1970) who showed that jelly-less eggs of *Bufo arenarum* can be fertilized by high concentrations of sperm. Yoshizaki (1987) compared the fertilizing capacity and

the ultrastructure of the sperm of *Rana japonica* and *Xenopus laevis*. The effect of the egg water of *Bufo* on fertilizing capacity was studied by Díaz Fontdevilla *et al.* (1991). They found a decreased fertilizing capacity of spermatozoa incubated in egg water.

It is clear that curiosity about sex and especially sperm structure have intrigued many researchers. The earlier work on spermatozoa was based on studies pertaining to the structure and ultrastructure of the spermatozoon. This holds especially true for studies done on amphibian sperm. Although valuable work has been done on the physiology of mammalian, and especially human sperm, work on the physiology of amphibian sperm has only been recently documented (Gosálvez *et al.*, 1986; Yoshizaki, 1987 and Bernadini *et al.*, 1989).

Because of the special environments inhabited by the amphibians, it is necessary to also research the adaptations regarding their fertilization. Some researchers such as Fouquette and Delahoussaye (1977) regard sperm structure as a phylogenetic trait, rather than an adaptation to the specific fertilization environment. The micro-fertilization environment can be regarded as that area surrounding the egg and will include the egg layers (Table 1.1). The different habitats occupied by the frogs could dictate the size and composition of the egg coverings and therefore the micro-fertilization environment of a terrestrially breeding frog will differ considerably from that of an aquatic breeding one.

In later years researchers concentrated on parameters pertaining to the motility

of the spermatozoa. Our knowledge regarding the amphibian sperm has been enhanced by these predecessors, but it is clear that in depth studies are required to test the hypothesis that fertilization environments modulate sperm structure and function.

AIM AND SCOPE

The anuran amphibians have a variety of niches and exhibit different fertilization methods. Passmore and Carruthers (1979) described this variety in mating calls, mating, amplexus, egg laying and development (ranging from metamorphic to direct development). Various physiological parameters will be investigated by studying a selection of Southern African anuran species. This work will attempt to answer questions relating to the fertilization environment and the structure and function of selected anuran species. This will include the following:

1 Description of the structure and ultrastructure of the spermatozoa of the species studied and relating that to the fertilization environment utilised by the frogs. Comparisons will be made between the sperm structure of the different species (Chapter 2).

2 Preparation of a typical spermiogram for each species of the anuran amphibians to be studied. Fertilization environments vary from an aquatic to an internal environment. The question to be answered is whether there is any correlation between the spermiogram and the fertilization environment (Chapter 3).

3 Investigation of different parameters regarding the motility of the sperm of the different species. The type of motility will be investigated - whether there is forward progression or star-spin type of movement. The underlying question will be whether there is a correlation between the fertilization environment and the type of movement of the sperm (Chapter 4).

4 Most studies on anuran sperm physiology are concerned with testicular sperm. Anurans do not possess epididymides and previous researchers have assumed that sperm maturation takes place in the testis. Experiments will be done on *Xenopus laevis* to compare motility characteristics of sperm obtained from the testis, the ejaculate and amplectant pairs (Chapter 5).

6 Many anurans feature on the red data list of scarce and endangered species. It will be attempted to establish baseline data for the anurans which might assist in protecting endangered anurans as was done with the breeding project of the Black-footed ferret (Van der Horst *et al.*, 1991). The information gathered during this research should therefore assist in the current measures instituted to protect our biodiversity.

FACILITIES, SUPPLIES AND METHODOLOGY

Facilities used for the study were at the Department of Physiology at the University of the Western Cape, Bellville, South Africa. Materials for study

purposes were supplied by Alan Channing, the Physiology Department and John Visser. Methods and techniques will be discussed in the appropriate chapters.

SPECIES STUDIED

There are nine (9) families of frogs in Southern Africa. It was attempted to include at least one (1) species of each family in this project. Species from the families Arthroleptidae and Heleophrynidae, however, are not included in the list of species examined. Information regarding the structure of *Heleophryne* spermatozoa, however, have been documented (Visser and Van der Horst, 1987).

The following species were examined:

FAMILY : BUFONIDAE : Fertilization in water.

Bufo rangeri

FAMILY : HEMISOTIDAE : Snout burrowers. Eggs laid in burrows.

Hemisus marmoratus

FAMILY : HYPEROLIIDAE

Hyperoliinae : Tree frogs. Eggs laid in slow running shallow water.

Hyperolius horstocki

Kassininae : Fertilization in water.

Semnodactylus wealii

Leptopelinae : Large tree frogs. Fertilization in burrows.

Leptopelis flavomaculatus

FAMILY : MICROHYLIDAE

Brevicipitinae : Burrowing frogs. Direct development. Fertilization in burrows.

Breviceps gibbosus

FAMILY : PIPIDAE

Xenopodinae : Aquatic habitat. Fertilization in flowing water.

Xenopus laevis

FAMILY : RANIDAE

Petropedetinae : Terrestrial habitat. Fertilization on land.

Arthroleptella lightfooti

Raninae : Large river frogs. Fertilization in streams or on fringes of water.

Pyxicephalus adspersus

Rana fuscigula

Strongylopus grayii

Tomopterna delalandii

FAMILY : RHACOPHORIDAE :

Tree frogs. Fertilization in foam nest on tree branch.

Chiromantis xerampelina.

CHAPTER 2

CHAPTER 2

THE STRUCTURE OF ANURAN SPERMATOZOA - A COMPARATIVE STUDY

2.1 Introduction

Sperm structure, phylogeny and the environment in which fertilization takes place have always fascinated researchers. The advent of electron microscopy has enhanced man's knowledge of the ultrastructure of spermatozoa and major advances in understanding the complex sperm structure of many species have been made (Burgos and Fawcett, 1956; Poirier and Spink, 1971; Reed and Stanley, 1972 and Baccetti and Afzelius, 1976).

Amphibians can be regarded as the bridge between true aquatic fertilizers and true terrestrial breeders. Research on the modes of fertilization of these animals could broaden our current knowledge of biodiversity. Although phylogeny plays an important role in determining sperm structure, the selection pressure created by the fertilization environment cannot be disregarded. Many researchers studied the structure and ultrastructure of the spermatozoa of the anuran amphibians. Morrisett (1974) compared the ultrastructure of three families of anurans. Van der Horst (1979) described the sperm structure of three anuran species. Yoshizaki (1987) demonstrated that the anuran sperm suffer no severe damage from the isolating procedure used and could therefore describe the

sperm ultrastructure of two frogs. Bernadini *et al.* (1989) concluded that *Xenopus laevis* sperm differ from other species in that their plasma membranes do not have an array of particles.

In their work on the *Hyla rubra* group, Fouquette and Delahoussaye (1977) regarded sperm structure as a phylogenetic trait, rather than an adaptation to the fertilization environment. Lee and Jamieson (1992) also used phylogeny as a basis for their work on the ultrastructure of myobatrachid frog spermatozoa. In a later work, Jamieson *et al.*, (1993), working on the ultrastructure of *Ascaphus* sperm, took the fertilization patterns into consideration. They deduced that fertilization environment can also be considered as one of the regulating factors of sperm structure. Mainoya (1981) in his observations of the sperm of *Chiromantis xerampelina* explained the modified spermatozoa of frogs on the basis of their unusual fertilization environment. Franzén (1970) also advocated that the nature of the environment in which sperm is released, should be regarded as important in influencing sperm structure.

Sperm tail structure is an important indicator of the fertilization status of a species. Van der Horst *et al.* (1989b) investigated the tail structure of a caecilian (*Typhlonectes*) and an onychophoran (*Peripatopsis*) with reference to their respective fertilization environments. In their joint work on the *Heleophryne* sperm, Visser and Van der Horst (1987) concurred with Fouquette and Delahoussaye (1977) that a double tailed structure is a primitive feature.

Motility of the sperm and the interaction between sperm and oocyte are matters which have been detailed by many researchers. Mizuhira *et al.* (1986) described their observations on the motility patterns of the extraordinary sperm of *Rhacophorus*. Swan *et al.* (1980) described the structure of the undulating membrane of *Bufo* sperm and its function in propelling the sperm. Bernadini *et al.* (1988a) investigated the structure of *Xenopus* spermatozoa and the correlation between shape and motility.

The complex nature of the spermatozoon could give us an insight into the evolution of a species. We should, however, also consider the role of the fertilization patterns and environment of species in regulating the sperm structure.

The Anura in the Southern African subcontinent is divided into nine families. I investigated the structure and ultrastructure of the spermatozoa of at least one species of seven of these families. Throughout this work comparisons were drawn between the structure of the spermatozoa of the different species. In this project the hypothesis is tested whether a correlation exists between "fertilization environment" and sperm morphology and physiology. The emphasis of my research will be on establishing the role of the fertilization environment on modulating sperm structure in the anurans. To date most studies on amphibian sperm were concerned with testicular sperm. Anurans do not possess epididymides and previous investigators such as Reed and Stanley (1972) and Poirier and Spink (1971) have assumed that sperm maturation takes place in the testis.

This study describes and compares the structure and ultrastructure of mature spermatozoa obtained from the testes of the frogs and toads.

2.2 Materials

Frogs were mostly caught at night after a rainy spell at their respective breeding sites. Rain induced calling by the frogs. *Xenopus laevis* toads were obtained from a breeding farm in Cape Town. Only the testicular tissue from the following species were obtained: *Hemisus marmoratus* and *Leptopelis flavomaculatus*. These testes were kept in Sørensen phosphate buffered glutaraldehyde (2,5%). Spermatozoa could be aspirated from these testes for scanning electron microscopy. Table 2.1 contains a list of the species studied, the localities where collected and the number of specimens collected.

The frogs were anaesthetized within 48 hours of capturing and the sperm aspirated.

Table 2.1: Data on species examined

FAMILY	SPECIES	LOCATION	NO.
Bufonidae	*Bufo rangeri*	Wellington, Worcester, Cape Town	10
Hemisotidae	*Hemisus marmoratus*	St. Lucia	1
Hyperoliidae			
Kassininae	*Semnodactylus wealii*	Kleinmond	6
Leptopelinae	*Leptopelis flavomaculatus*	Natal	1
Microhylidae			
Brevicipitinae	*Breviceps gibbosus*	Stellenbosch	5
Pipidae			
Xenopodinae	*Xenopus laevis*	Cape Town	15
Ranidae			
Petropedetinae	*Arthroleptella lightfooti*	Cape Town	4
Raninae	*Rana fuscigula*	Worcester	5
	Strongylopus grayii	Worcester	24
	Tomopterna delalandii	Hermanus	6
Rhacophoridae	*Chiromantis xerampelina*	St. Lucia	4

The following criteria were used to distinguish between aquatic and terrestrial fertilizers:

AQUATIC FERTILIZERS (A):

Those toads and frogs who need bodies of water for fertilization purposes, such as ponds, rivers or streams. These anurans breed in the water or on the fringes of the body of water. The males commonly call from within the water and amplexus takes place in the body of water. The eggs are therefore laid in the water and they usually have free-swimming tadpole stages (Passmore and Carruthers, 1979).

TERRESTRIAL FERTILIZERS (T):

Those frogs who normally occupy habitats away from bodies of water. It must be remembered that most frogs need water for reproduction purposes. These anurans, however, need a minimal amount of water to breed. The males usually call from burrows or from under rocks and bushes. A morning dew, or a few drops of rain can be enough to induce calling and amplexus. The fertilization behaviour of the anurans has not been fully researched, but it is generally accepted that most are external fertilizers. There are, however, species whose mode of fertilization is internal (Grandison and Ashe, 1983 and Jamieson *et al.*, 1993). The terrestrial fertilizers are typically direct developers with no free-swimming tadpole stages (Passmore and Carruthers, 1979). The terrestrial fertilizers used in this study utilise fertilization environments ranging from the fringes of bodies of water to burrows and foam nests.

Table 2.2: Modes of amplexus and fertilization of the different species studied

SPECIES	MODE OF AMPLEXUS	ENVIRONMENT OF AMPLEXUS	F.E. GR.	EGGS
B. rangeri	Often initiated out of water. Axillary clasp.	In streams. Male calls from water's edge.	A	In strings around water plants.
H. marmoratus	Axillary clasp.	In burrows and mud. Male calls from mud.	T	Large and unpigmen-ted. Laid in burrows. Thick jelly layers
S. wealii	Axillary clasp.	In pans and pools. Male calls from bank or submerged po-sitions.	A	Small pig-mented. At-tached to submerged plants.

SPECIES	MODE OF AMPLEXUS	ENVIRONMENT OF AMPLEXUS	F.E. GR.	EGGS
L. flavomaculatus	Axillary clasp.	Out of water in damp places.	T	Large slightly pigmented. Tadpoles wriggle towards water.
B. gibbosus	Adhesion.	Burrows. Male calls from burrow.	T	Large, unpigmented. Thick viscous jelly capsules. Deposited in clutches in damp burrows.

SPECIES	MODE OF AMPLEXUS	ENVIRONMENT OF AMPLEXUS	F.E. GR.	EGGS
X. laevis	Inguinal clasp.	Below surface of water. Male calls from below surface.	A	Small heavily pigmented. deposited in strings attached to submerged objects.
A. lightfooti	Axillary clasp.	Damp vegetation, wet mossy areas. Male calls from cavities in mud or moss.	T	Few large unpigmented eggs individually covered by thick jelly capsule.
R. fuscigula	Axillary clasp.	Shallow water or streams. Male calls from deep water.	A	Small pigmented. Large jelly capsule.

SPECIES	MODE OF AMPLEXUS	ENVIRONMENT OF AMPLEXUS	F.E. GR.	EGGS
S. grayii	Axillary clasp.	On fringes of bodies of water. Male calls from shallow water, well concealed.	A	Small pigmented.
T. delalandii	Axillary clasp.	Edges of dams and bodies of water. Male calls from cavities or under plants.	A	Small and pigmented. Individual jelly layers.
C. xerampelina	Axillary clasp.	In foam nest overhanging water. Male calls from branches. Female secretes gelatinous mucus. Amplectant pairs and other males beat mucus into stiff foam ball.	T	Laid in foam nests. Unpigmented. Tadpole completes cycle in water.

2.3 Methods

2.3.1 Aspiration of sperm

Chiromantis xerampelina and certain *Bufo rangeri* specimens were anaesthetized with chloroform. The other frogs were anaesthetized by immersing them in water containing a few granules of MS222. The latter procedure yielded better results as the muscles of the frogs were more relaxed. The abdominal cavity was dissected and the internal organs exposed. The testes were removed and placed into a sterile petri dish. The blood vessels on the surface of the testes were then carefully removed.

Portions of one testis were placed in Sørensen phosphate buffered glutaraldehyde (2,5%) for transmission electron microscopy (TEM) preparation. The other testis was squashed between two microslides. Two or five μl of testicular fluid were placed in one ml of Sørensen phosphate buffered glutaraldehyde (2,5%). This was later used for scanning electron microscopy (SEM) and sperm density measurements.

2.3.2 Light microscopy

Sperm smears were made from the testicular fluid or by evenly smearing a portion of the testis on a microslide. The smears were stained with Spermac, by immersing the slides for one minute periods in solutions A, B and C. Afterwards they were fixed with Spermac fixative (5 minutes). Spermac is a differential stain which stains the acrosome green, the head blue and the tail red in human spermatozoa.

Sperm extruded from the testis were also mixed with a nigrosin-eosin solution and left for five minutes. Sperm smears were made on microslides and air dried. Afterwards cover slips were mounted, using DPX as mounting medium. Sperm smears were viewed on an Olympus microscope (model CH-B145-T) using oil immersion with the 100x objective lens.

2.3.3 Scanning electron microscopy

Sperm were fixed in Sørensen phosphate buffered glutaraldehyde (2,5%) for at least 48 hours. The method of preparing sperm for SEM described by Van der Horst *et al.* (1989a) was used. Fifteen μl of the suspension were washed with Sørensen phosphate buffer and very slowly injected onto the membrane in the filter holder. Sørensen phosphate buffered osmium tetroxide (1%) was injected into the filter holder and left for one hour. The osmium tetroxide was then replaced with Sørensen phosphate buffer for 15 minutes. The spermatozoa were dehydrated in increasing concentrations of ethanol (70%, 90%, 95% - each for 15 minutes). There were two changes of absolute ethanol for 30 minutes and 1 hour respectively.

The membrane filters containing the sperm samples were then rapidly transferred into a metal basket and placed in the precooled chamber of a Hitachi critical point dryer. Routine critical point drying was performed. The membrane filters were attached to SEM specimen stubs with Press On sticky tags. The material was sputter coated with gold using an Edwards sputter coater model S150B. A Hitachi X-650 Scanning Electron Microanalyzer was used and

operated at 25 kV. Original magnifications ranged from 1000 to 15000 and were enlarged photographically to sizes presented here.

2.3.4 Transmission electron microscopy

Testicular tissue was fixed in Sørensen phosphate buffered glutaraldehyde for at least 48 hours. Testes were cut into 1 mm^3 cubes and rinsed twice with Sørensen phosphate buffer (30 minutes each). The cubes were postfixed in Sørensen phosphate buffered osmium tetroxide (1%) for one hour. After two buffer rinses the cubes were dehydrated in increasing concentrations of acetone (70%, 90%, 95% - each for 15 minutes). There were two changes of 100% acetone (30 minutes each). The material was placed in propylene oxide to clear, for 30 minutes and 1 hour respectively. The material was infiltrated with a 1:1 mixture of propylene oxide and Spurr's resin for 1 hour. Further infiltration was accomplished with Spurr's resin for 1 hour and fresh Spurr's resin overnight. The cubes were then placed at the bottom of a beam capsule filled with Spurr's resin. These beam capsules were placed overnight in an oven at a constant 70° C.

Beam capsules were trimmed and thin sections of an interference colour of grey to silver were cut by glass knives on a Sorvall Ultramicrotome. Sections were picked up on 300 or 400 mesh copper grids and contrasted in 2% uranyl acetate for 15 minutes. They were then washed in distilled water and 0,02 M sodium hydroxide. Afterwards they were contrasted for 20 minutes at room temperature in lead citrate (Reynold's solution) (Dawes 1971). Grids were washed in 0,02 M

sodium hydroxide and in distilled water. These ultrathin sections were viewed on a Hitachi Transmission Electron microscope. The sections of *Chiromantis xerampelina* were viewed on a Philips 450 Electron microscope.

The transmission electron micrographs of *C. xerampelina* were included to elucidate the structure of the components of this highly aberrant sperm. The transmission electron micrographs of the other anuran sperm studied, ratified the work done by previous researchers and TEM was only performed to confirm the main subcomponents of the sperm.

An Image Processing System was used to measure the lengths of the different components of the sperm. The SEM photographs were used to determine the lengths of the heads and tails of the spermatozoa. At least 25 spermatozoa of each species were measured. Other components measured, were the length of the acrosome (where possible) and the head width. A computer mouse was used in conjunction with a Kontron Image Analyzer (Videoplan Release 2.1) to trace and measure the head, tail and acrosome lengths, as well as the head width.

2.3.5 Statistical analyses

Data were reflected in a spreadsheet and imported into the Statgraphics program. Analyses of variance (ANOVA) and T-sample tests were used to determine the source of significant differences. Where tests regarding the homogeneity of variance revealed significant differences in variance, nonparametric analyses namely the Mann-Whitney tests were performed.

Multivariate analyses were also performed using star symbol plot analyses. This method analyzes data by presenting them as rays in a star symbol. The lowest value of a particular parameter is calculated and expressed as a ray 10% the length of the highest value. All the data present in a single set of star symbols are therefore compared. Star symbols of a particular set cannot be compared with symbols from any other set. These star symbols were used as a multivariate test to graphically compare patterns in sperm structure.

2.4 Results: Description of spermatozoa

2.4.1 Family : Bufonidae

Bufo rangeri

THE HEAD AND ACROSOME

The head is typically spear-shaped. The tip of the head is demarcated by the pointed acrosome. The acrosome, 4,1 μm long forms a cap on the attenuated anterior end of the nucleus (Fig. 2.1). The nucleus is surrounded by a thin sheath of cytoplasm. The head, 17,5 μm long (from tip of acrosome to start of tail) and 0,9 μm wide (measured in the centre of the head), is relatively straight and widens slightly posteriorly.

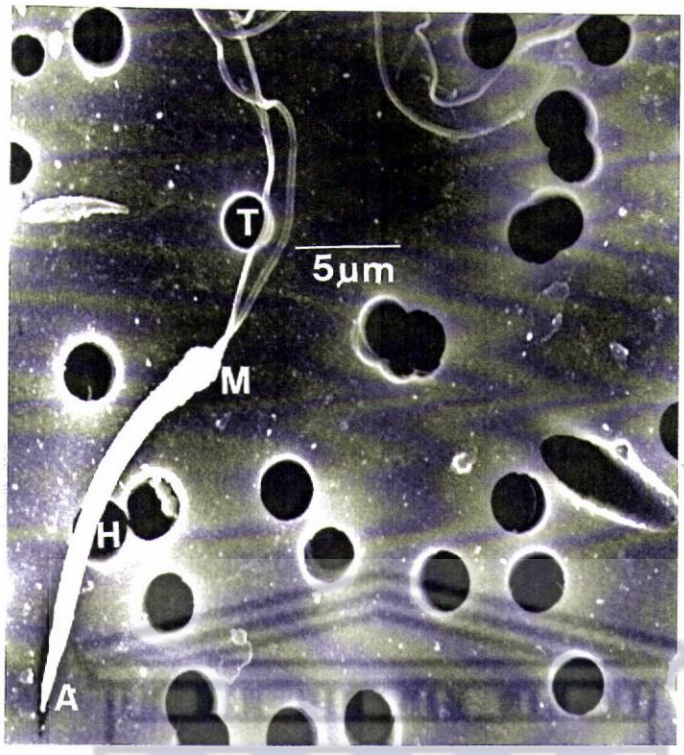


Fig. 2.1 Scanning electron micrograph of *Bufo rangeri* sperm. (A-acrosome; H-head; M-midpiece; T-tail complex)

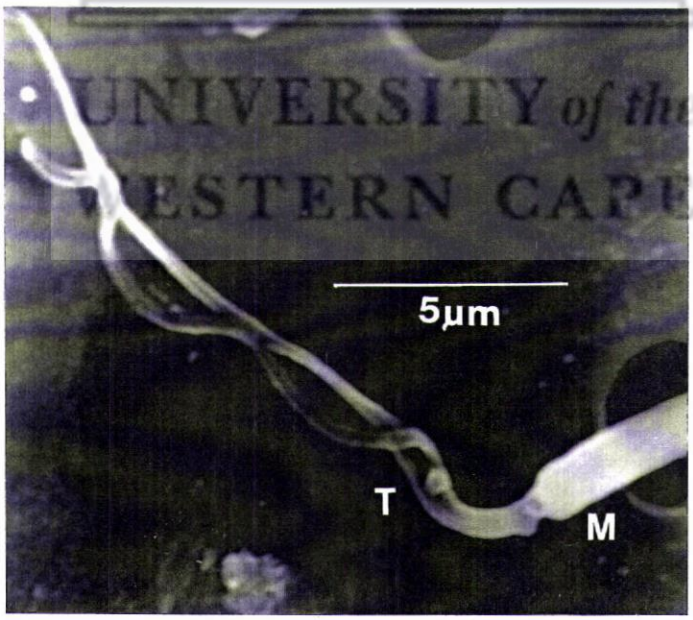


Fig. 2.2 Scanning electron micrograph of the tail complex of *B. rangeri* sperm. (M-midpiece; T-tail)

THE TAIL

The tail complex is approximately 23 μm long and consists of a double structure, namely a 9 + 2 flagellum adjacent to an electron-dense axial rod. The flagellum and the rod are joined with an undulating membrane (Figs. 2.1 and 2.2).

2.4.2 Family : Hemisotidae

Hemisus marmoratus

THE HEAD AND ACROSOME

The head is a long, straight structure 23,9 μm long (Fig. 2.3). It is more or less of uniform width (0,9 μm) throughout, except for the tapering acrosome. The acrosome is 3,3 μm long. The midpiece could not be clearly demarcated.

THE TAIL

The tail comprises a single flagellum, approximately 27 μm long. It is more or less of uniform thickness throughout (Fig.2.4).

2.4.3 Family : Hyperoliidae

Kassininae

Semnodactylus wealii

THE HEAD AND ACROSOME

The torpedo-shaped head is approximately 16 μm long. It is a relatively straight structure and tapers towards the acrosome (Fig. 2.5), which is ±1.9 μm long.

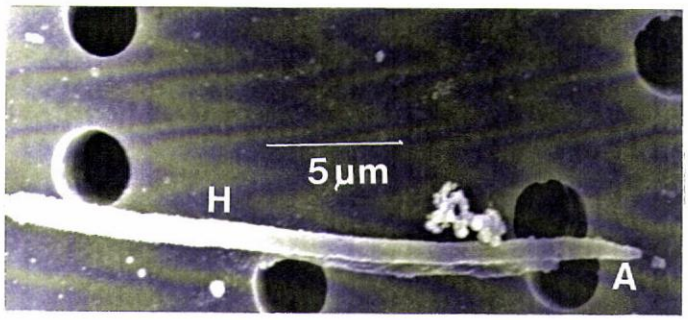


Fig. 2.3 Scanning electron micrograph showing sperm head of *Hemisus marmoratus*. (A-acrosome; H-head)

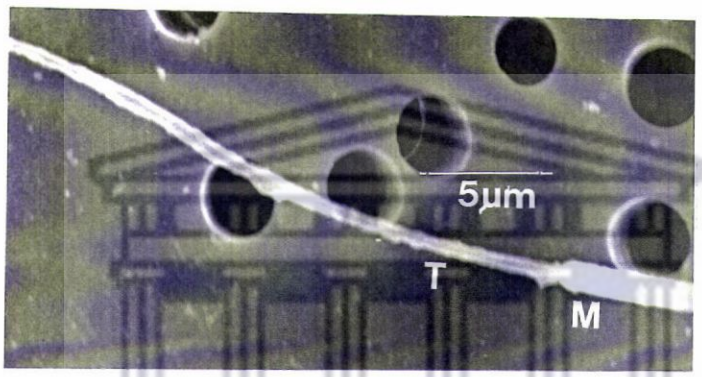


Fig. 2.4 Tail complex of *H. marmoratus*. (M-midpiece; T-tail)

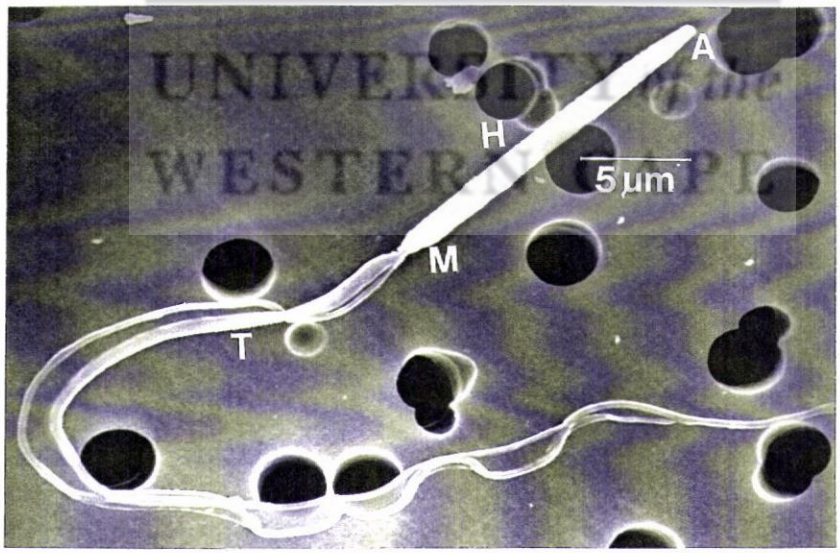


Fig. 2.5 *Semnodactylus wealii* - scanning electron micrograph of sperm. (A-acrosome; H-head; M-midpiece; T-tail)

The head widens slightly posteriorly towards the midpiece. The width of the head, measured at its centre averages 1,2 μm.

THE TAIL

This complex encompasses a flagellum with the 9 + 2 pattern lying next to an axial rod. These two are joined by an undulating membrane (Fig. 2.5). The length of the tail varies from ± 35 to 45 μm.

Leptopelinae

Leptopelis flavomaculatus

THE HEAD AND ACROSOME

The head is a broadly curved spear-shaped structure 23,6 μm in length and of uniform width (1,4 μm) (Fig. 2.6). The acrosome tapers proximally and is approximately 2,6 μm in length.

THE TAIL

The tail complex consists of a thick flagellum connected by an undulating membrane to the axial rod (Fig. 2.7). The length of this complex varies from 18 to 25 μm in length.

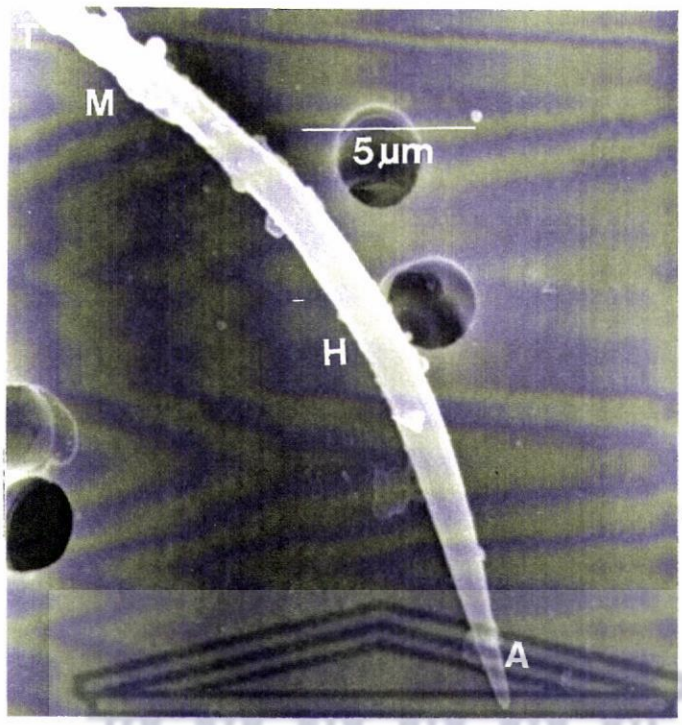


Fig. 2.6 Scanning electron micrograph showing sperm head of *Leptopelis flavomaculatus*. (A-acrosome; H-head; M-midpiece; T-tail)

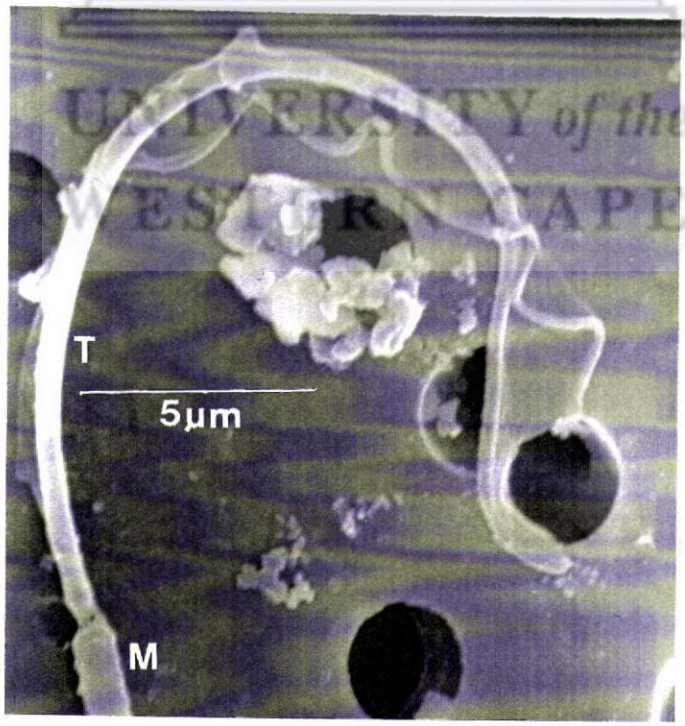


Fig. 2.7 Tail complex of *L. flavomaculatus*. (M-midpiece; T-tail)

2.4.4 Family : Microhylidae

Brevicipitinae

Breviceps gibbosus

THE HEAD AND ACROSOME

The head is a thin, very long, proximally tapering structure, 34,4 μm in length (Fig. 2.8). The acrosome spans approximately 3,8 μm (Fig. 2.9). The head widens slightly posteriorly with an average width of 0,9 μm. The head is a relatively straight structure. The midpiece is approximately 2 μm in length.

THE TAIL

This complex consists of a single flagellum of a length varying from 40 to 46 μm.

2.4.5 Family : Pipidae

Xenopodinae

Xenopus laevis

THE HEAD AND ACROSOME

The sperm head consists of 1½ coils. The vermiform head has a characteristic corkscrew shape (Fig. 2.10) and spans 17,9 μm. It has a width of 1,1 μm and an attenuated acrosome of 3,4 μm. The head helix is tapered at both ends. The midpiece is clearly demarcated and is roughly 2 μm long.

THE TAIL

It is difficult to establish the actual length of the tail as it readily breaks off during SEM preparation. The length, however, varies from 30 to 40 μm. The

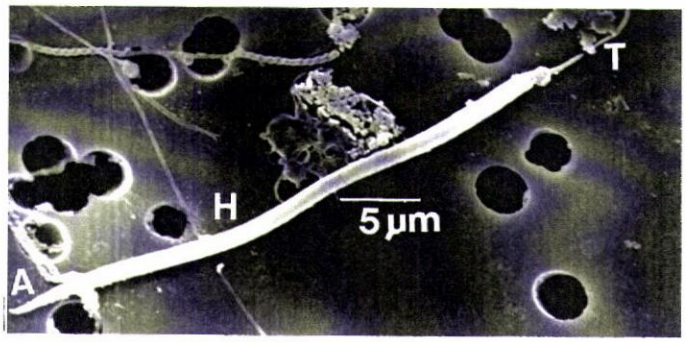


Fig. 2.8 *Breviceps gibbosus* sperm. (A-acrosome; H-head; T-tail)

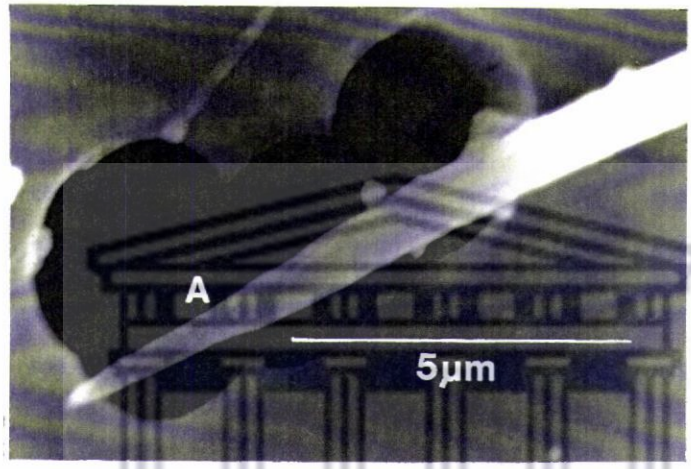


Fig. 2.9 Scanning electron micrograph showing sperm acrosome of *B. gibbosus*. (A-acrosome)

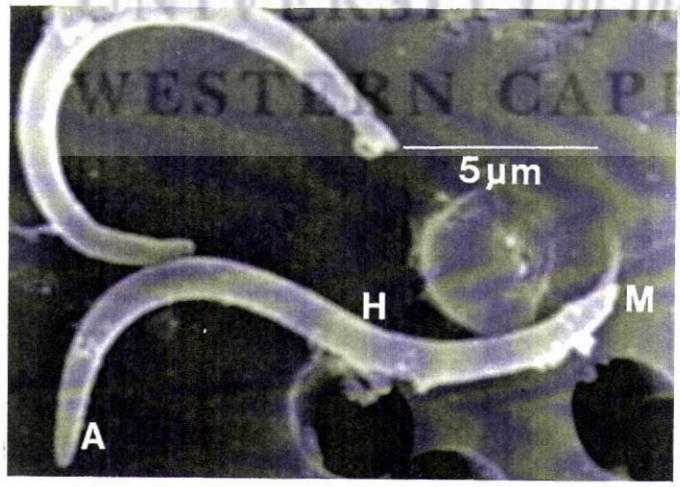


Fig. 2.10 Sperm structure of *Xenopus laevis*. (A-acrosome; H-head; M-midpiece)

complex consists of a single flagellum with a conventional 9 + 2 microtubule array.

2.4.6 Family : Ranidae

Petropedetinae

Arthroleptella lightfooti

THE HEAD AND ACROSOME

The head is an extremely long (41,6 μm), slightly curved structure (Fig. 2.11). It has a diameter of 0,9 μm. The head tapers towards the acrosome and is slightly wider nearer to the tail.

THE TAIL

A single flagellum makes up the tail complex. Its length varies from 35 to 42 μm.

Raninae

Rana fuscigula

THE HEAD AND ACROSOME

The characteristic cigar-shaped head has a length of 12,1 μm (Fig. 2.12). The acrosome (1,1 μm) can be clearly seen. The midpiece spans 2,4 μm.

THE TAIL

The length of the tail varies from 35 to 45 μm. Although the proximal portion of the tail appears in SEM to be a double structure (Fig. 2.12), the complex

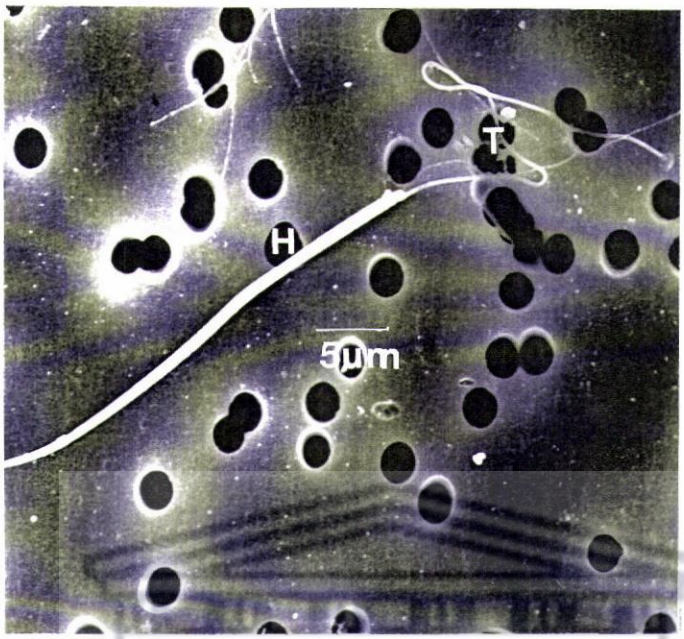


Fig. 2.11 *Arthroleptella lightfooti* - sperm structure. (H-head; T-tail)

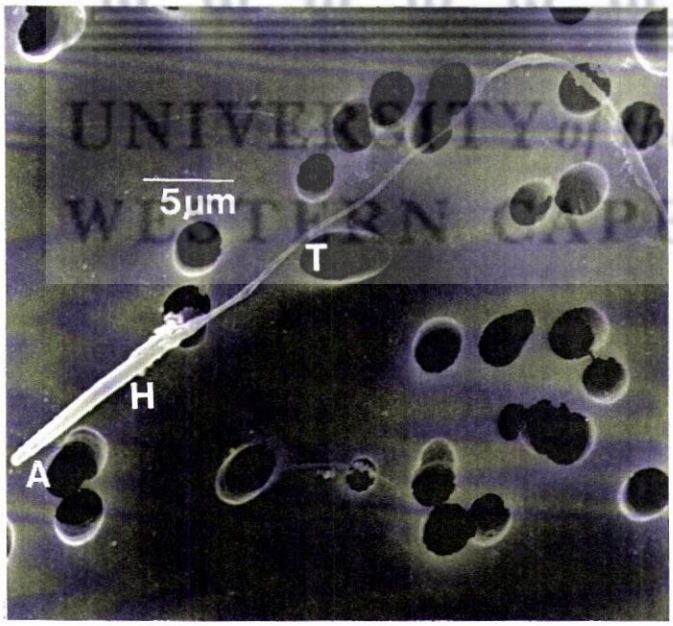


Fig 2.12 Scanning electron micrograph of *Rana fuscigula* sperm. (A-acrosome; H-head; T-tail)

consists of a single flagellum with a 9 + 2 microtubular arrangement.

Raninae

Strongylopus grayii

THE HEAD AND ACROSOME

The sperm head has a characteristic helical structure consisting of 1½ coils. The head tapers proximally and distally (Fig. 2.13) and has a length of 16,4 μm. The acrosome (1,9 μm long) forms a distinctive attenuated cap on the proximal end of the nucleus. The helical shape of the head is regarded as the normal form, but many sickle-shaped heads were evident.

THE TAIL

A single flagellum with the typical 9 + 2 pattern occurs. The complex is ± 37 μm in length.

Raninae

Tomopterna delalandii

THE HEAD AND ACROSOME

The head has the distinctive cigar shape and is 15,8 μm in length (Fig. 2.14). A ridge where the acrosome fits onto the nucleus is visible. The acrosome is 1,6 μm long. The head is a fairly straight structure.

THE TAIL

A single flagellum comprises the tail complex and varies from 20 to 30 μm in

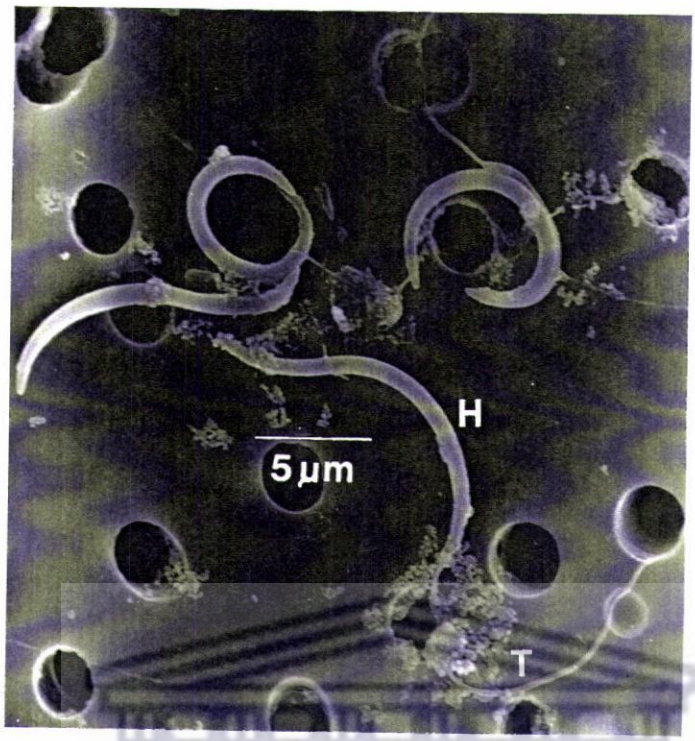


Fig. 2.13 Scanning electron micrographs of *Strongylopus grayii* sperm. (H-head; T-tail).

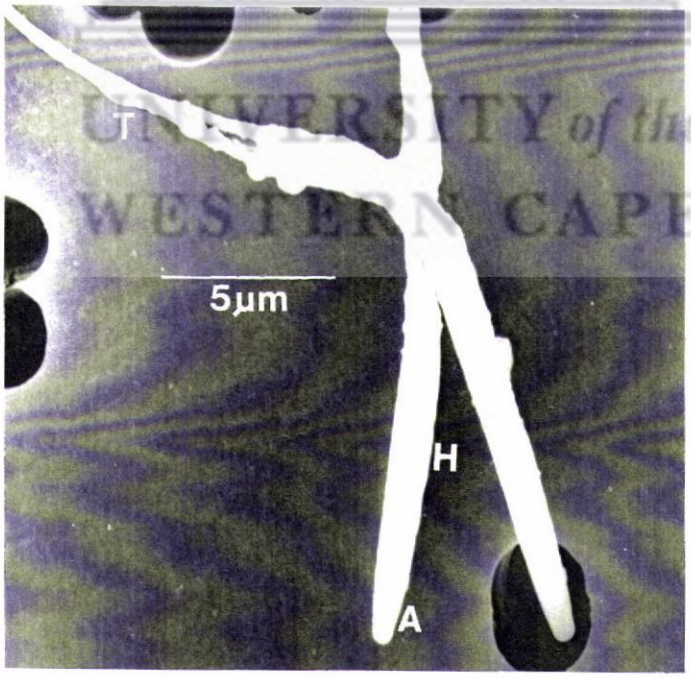


Fig. 2.14 *Tomopterna delalandii* - sperm. (A-acrosome; H-head; T-tail)

length. The SEM (Fig. 2.14) indicates a dual structure at the proximal end of the tail, but TEM revealed this to be a preparation artefact.

2.4.7 Family : Rhacophoridae

Chiromantis xerampelina

THE HEAD AND ACROSOME

The peculiar head is in the shape of a tightly coiled helix (Fig. 2.15). A constant number of eleven coils are present. A curved acrosome, 3,5 μm in length, is present at the proximal end of the coiled head (Fig. 2.16). The length from the acrosome to the start of the tail is approximately 8,5 μm. The diameter of the coils is 2.3 μm whereas the width of the head is 0,5 μm. When the coils are completely straightened, the length of the head could be well over 35 μm. The midpiece comprises the last 1½ coils of the head and is packed with mitochondria (Fig. 2.17). In the testis the spermatozoa are neatly packaged and embedded in Sertoli cells (Fig. 2.18).

THE TAIL

The tail complex comprises a single flagellum consisting of two 9 + 2 patterns (Figs. 2.19). The tail is approximately 44 μm long and frays out distally (Fig. 2.21). The two 9 + 2 microtubular pattern is surrounded by a mass of microtubules (Fig. 2.20). SEM studies indicated a double-tailed structure (Fig. 2.21), but this was shown by TEM to possibly be a preparation artefact.

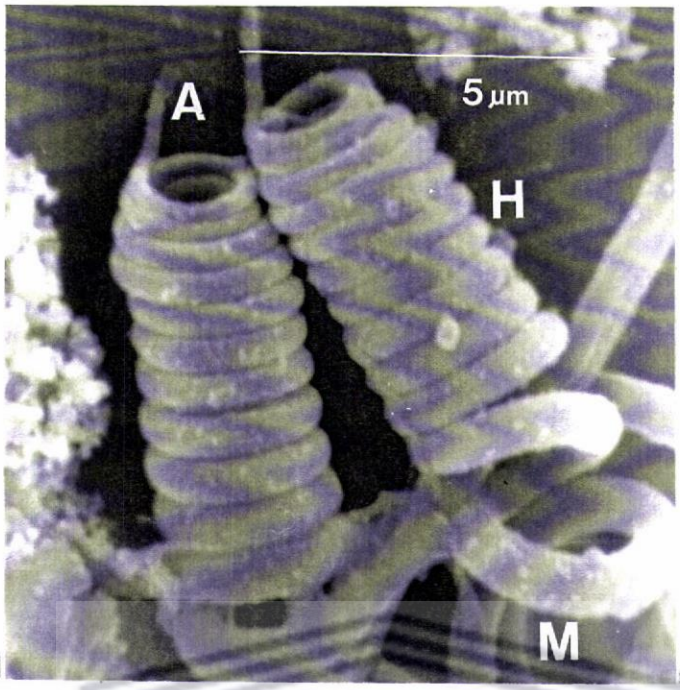


Fig. 2.15 Scanning electron micrograph of *Chiromantis xerampelina* sperm depicting the unusually coiled head. (A-acrosome; H-coiled head; M-midpiece)

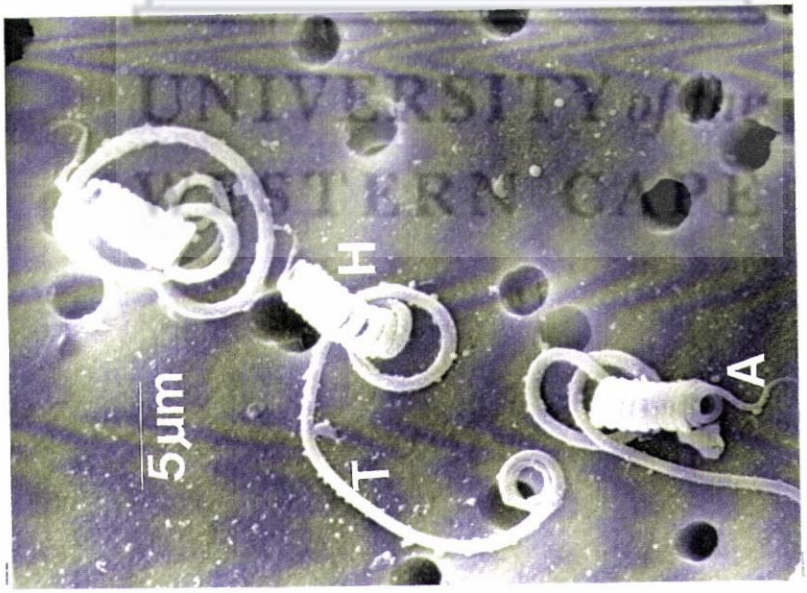


Fig. 2.16 *C. xerampelina* sperm. Micrograph depicts acrosome, head and tail. (A-acrosome; H-head; T-tail)

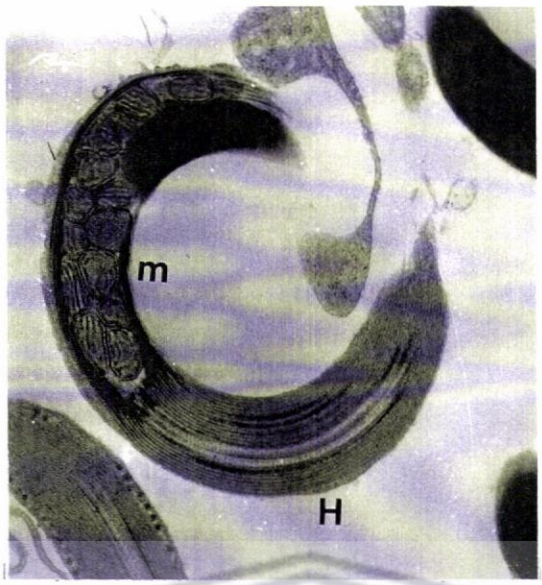


Fig. 2.17 Transmission electron micrographs of midpiece region of *C. xerampelina* sperm. (H-head; m-mitochondria)

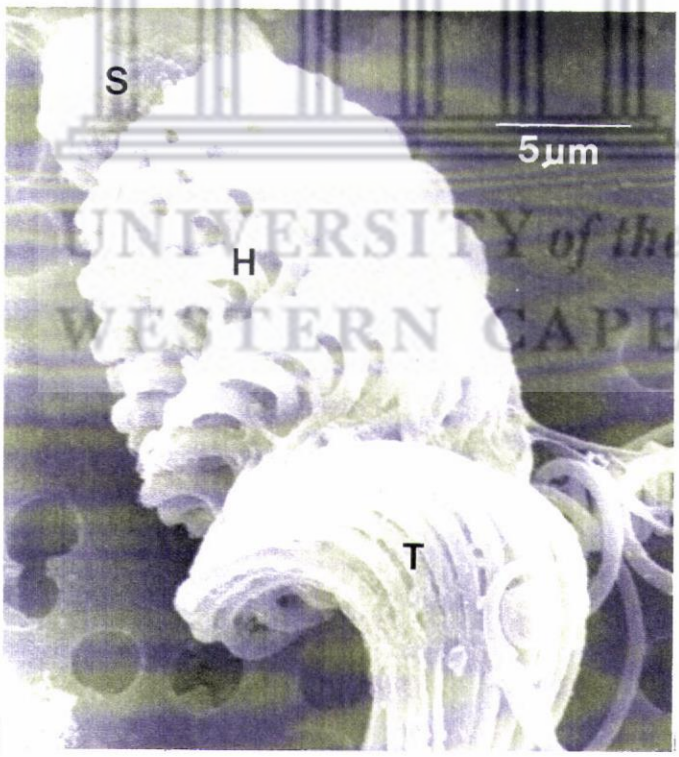


Fig. 2.18 *C. xerampelina* sperm packaged and anteriorly embedded in Sertoli cell. (S-Sertoli cell; H-heads; T-tails)

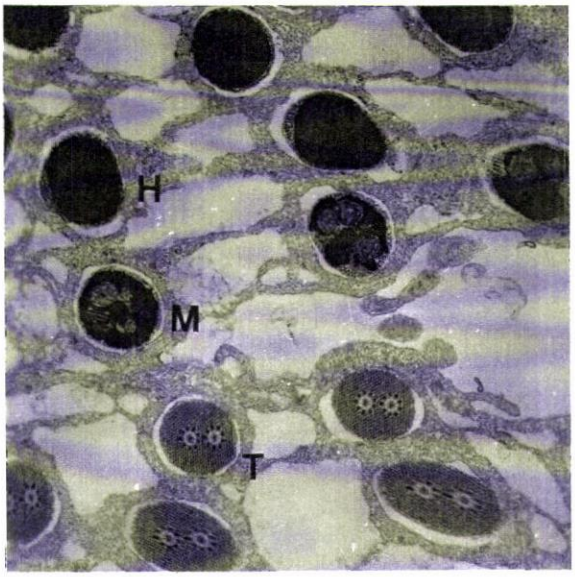


Fig. 2.19 Transmission electron micrograph showing longitudinal section through head, midpiece and tail of *C. xerampelina* sperm. (H-head; M-midpiece; T-tail).

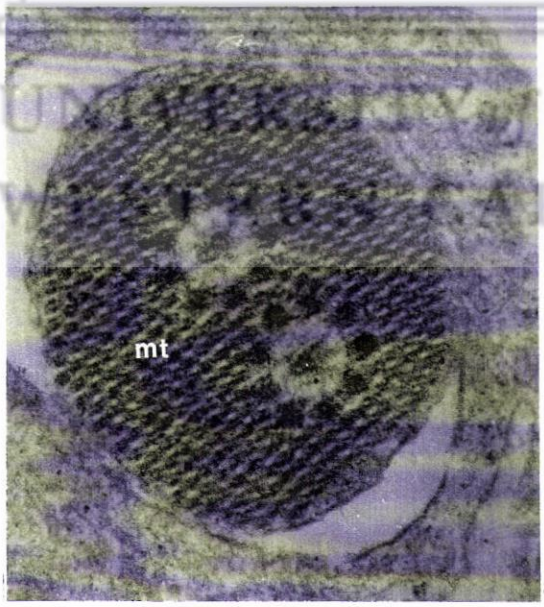


Fig. 2.20 Cross section through tail of *C. xerampelina* sperm showing microtubules (mt) surrounding the two 9+2 microtubular arrangement.

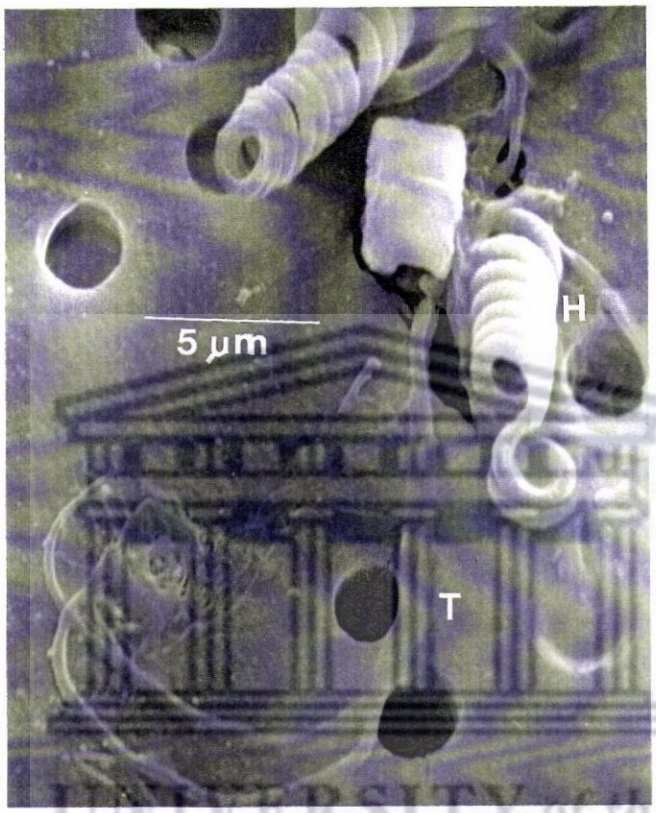


Fig. 2.21 Scanning electron micrograph showing double tail complex of *C. xerampelina* sperm. Tail frays out posteriorly. (H-head; T-tail).

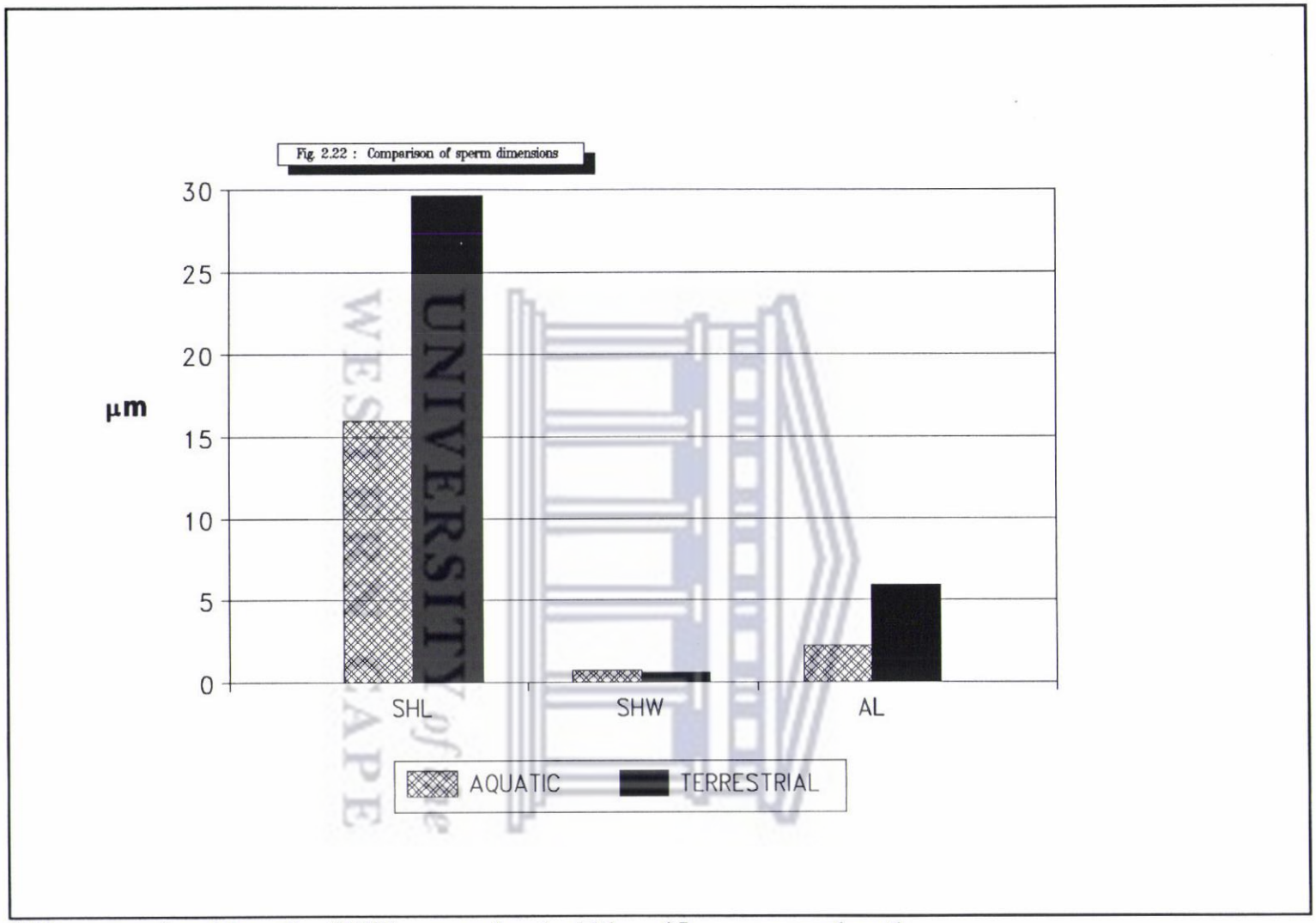
2.4.8 Summary of results

The data and comparisons of the sperm head dimensions of the aquatic and terrestrial fertilizers are depicted in Table 2.3 and Fig. 2.22. Star symbol plot analysis of the dimensions of the two groupings were also done (Fig. 2.23).

Table 2.3 Sperm dimensions of aquatic and terrestrial fertilizers

FERTILIZATION GROUPING	HEAD LENGTH (μm)	HEAD WIDTH (μm)	ACRO-SOME (μm)
AQUATIC	16,0 (±2,2)	0,7 (±0,4)	2,2 (±1,2)
$\bar{x}$(±SD)Range	10,3 - 20,5	0,1 - 1,6	0,7 - 4,6
	*		*
TERRESTRIAL	29,6 (±6,0)	0,6 (±0,3)	5,9 (±1,6)
$\bar{x}$(±SD)Range	21,4 - 45,0	0,1 - 1,1	3,2 - 7,3
	*		*

* : Significant difference ($p < 0,05$)



SHL: sperm head length; SHW: sperm head width; AL: acrosome length

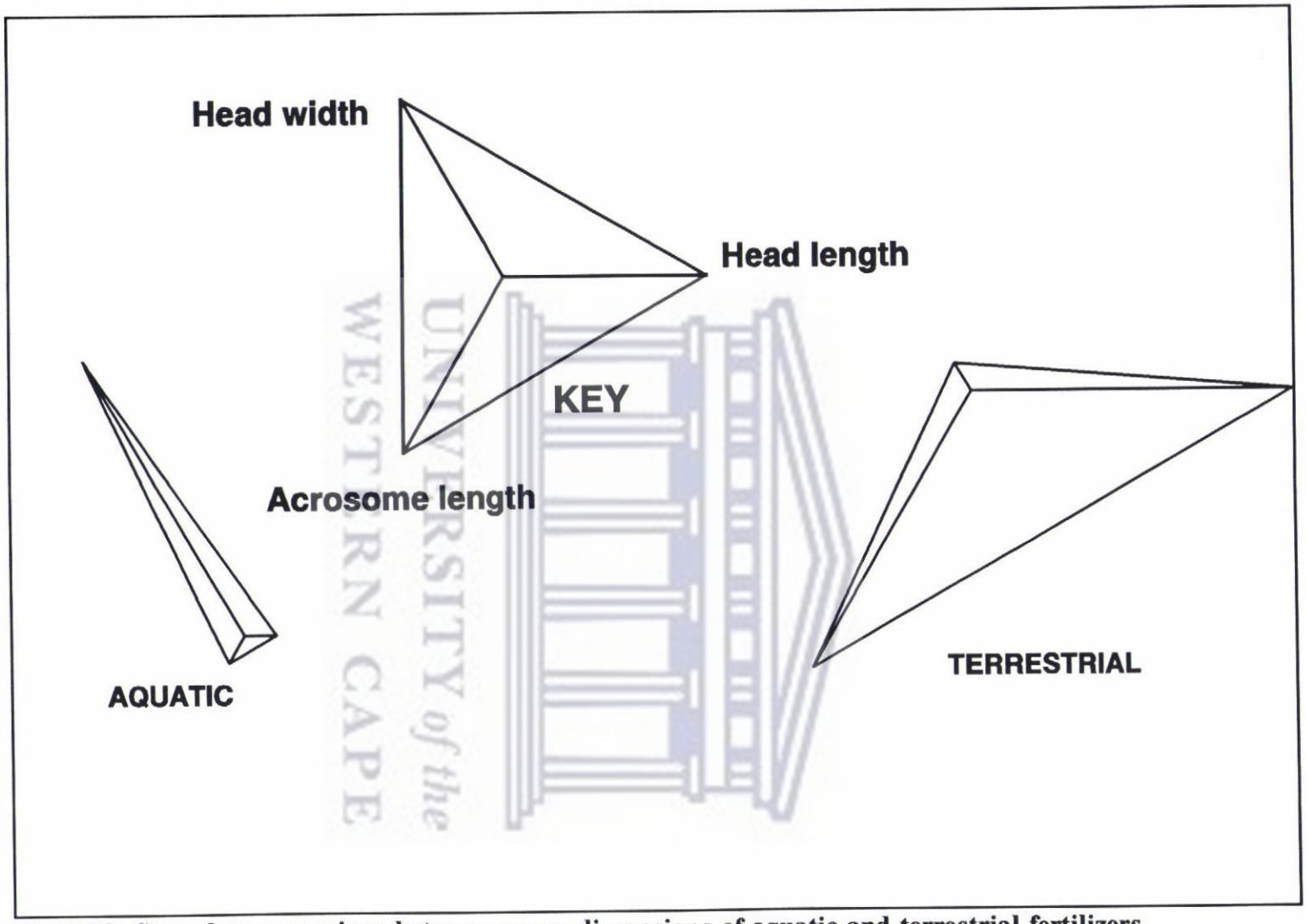


Fig. 2.23 Star plot comparison between sperm dimensions of aquatic and terrestrial fertilizers

2.5 Discussion

Morrisett (1974) in his work on three families of anurans stated that the ultrastructural studies of anuran sperm might contribute to our knowledge of amphibian systematics. Fouquette and Delahoussaye (1977) concurred that the differences in sperm structure reflect phylogenetic differences. It can be argued that anuran sperm morphology could be used for determining phylogenetic traits. The fertilization environment can, however, not be ruled out as a factor determining sperm structure (Franzén, 1970). Mainoya (1981) and Jamieson *et al.* (1993 and pers. comm.) also regard the fertilization environment as important in regulating sperm structure.

It is generally regarded that the double tailed structure of sperm is a primitive feature as postulated by Visser and van der Horst (1987). The sperm head structure of teleosts exhibits primitive features and reflects their aquatic fertilization environment. Mammalian sperm on the other hand represent the most advanced features, reflecting their unique fertilization environment. The terrestrial and aquatic fertilizers have major differences regarding their sperm structure and physiology.

The form and function of the anuran spermatozoa could be exceptional, because of the unique habitat they occupy. It is reasonable to expect that, because of their dependence on water for fertilization, anurans will have rather primitive sperm structures. The Anura exhibit a wide range of fertilization environments. These range from fully submerged to completely terrestrial. Sperm of *Bufo*

rangeri, *Semnodactylus wealii*, *Xenopus laevis*, *Rana fuscigula*, *Strongylopus grayii* and *Tomopterna delalandii* exhibit typical anuran sperm features such as an elongated head, small midpiece and a tail with the 9 + 2 pattern. The lengths of the sperm heads in these species range from 12 μm (*R. fuscigula*) to 18 μm (*B. rangeri* and *X. laevis*). All the species mentioned above are aquatic fertilizers.

The sperm heads of *Hemisus marmoratus*, *Leptopelis flavomaculatus*, *Breviceps gibbosus*, *Arthroleptella lightfooti* and *Chiromantis xerampelina* are very long tapering structures, ranging from 23 μm to over 40 μm in length. These anurans occupy habitats away from the water and are in many cases only dependant on dampness of early morning dew or mist for fertilization purposes and are regarded as terrestrial fertilizers.

This investigation indicated that those species depending on bodies of water for their fertilization, display shorter, stouter sperm heads. It is also significant that species with typical terrestrial fertilization environments, exhibit adaptations to compensate for the absence of water. *A. lightfooti* and *B. gibbosus* have direct development with no free swimming tadpole stages. In *C. xerampelina* fertilization takes place within a gelatinous mucus and the embryos drop down into a stream where the tadpoles complete metamorphosis. Another adaptation of terrestrial fertilizers is that they have very thick egg coverings. This is predictable, since the major environmental hurdle to be overcome is dehydration.

Van der Horst (1979) described the sperm head of *Pyxicephalus* (aquatic

breeder) as "stout and short". Visser and van der Horst (1987) described the sperm of *Heleophryne* (eggs laid in shallow water or out of the water on wet gravel) and found the sperm head to be long (29 μm) and slender. This further reinforces the hypothesis that the anuran aquatic fertilizers have short sperm heads. Table 2.3 demonstrates that there is a significant difference between the head lengths of terrestrial and aquatic fertilizers. I also found the motility patterns (Chapter 4) of species with differing fertilization environments to differ significantly.

Frogs laying their eggs in water have less extensive egg coverings than those with eggs on land (Passmore and Carruthers, 1979) (Table 2.2). Penetration of these coverings involves mechanical as well as chemical mechanisms. The extensive egg coverings of the terrestrial fertilizers, therefore, dictate long sperm heads to penetrate and successfully fertilize. In the case of *Chiromantis* the sperm have to penetrate the foam to reach the egg (Mainoya, 1981). My studies on the sperm of *C. xerampelina* concurred with observations by Mainoya (1981) detailing the aberrant coiled sperm head structure. Mizuhira, *et al.* (1986) described a similar shape in the case of *Rhacophorus*, another rhacophorid. I found the acrosome lengths of the terrestrial and aquatic fertilizers to differ significantly. A larger acrosome may contain more hydrolytic enzymes to cope with the penetration of the thicker egg coverings.

The sperm tail complex of anurans exhibits variation. The sperm tails of some consist of a flagellum with the 9 + 2 microtubule pattern and an axial rod,

connected by an undulating membrane. Such a pattern was observed in *Bufo*, *Semnodactylus* and *Leptopelis*. In most of the other species studied, a single flagellum with the 9 + 2 pattern was discerned. *Chiromantis* spermatozoa have a single tail with two axial filaments each containing a 9 + 2 microtubular pattern. Such a pattern was also found by Mizuhira *et al.* (1986) in their studies on the spermatozoa of *Rhacophorus*. My observations on the sperm tails of *C. xerampelina* revealed similar patterns.

The double tailed structure is regarded as a primitive trait by Fouquette and Delahoussaye (1977) and Visser and van der Horst (1987) as typical internal fertilizers such as mammals, exhibit a single sperm tail structure. In my studies on the anurans, it was clear that both aquatic fertilizers (such as *Bufo*) and terrestrial fertilizers (*Leptopelis*) have double tail complexes. The sperm tail is essential in propelling the sperm towards the eggs. The studies revealed that the sperm tail morphology is less readily influenced by the different fertilization environments as is the case with sperm head morphology. It may well be that the flagellum as propulsive mechanism was a successful adaptation as early as in the Mastigophora (Baccetti and Afzelius, 1976) and has been retained. Retention of successful adaptations is also supported by similarities between arthropod metameres and segmentation centering on rhombomeres of developing brain, branchial arches and cranial nerves in the vertebrates (Gould, 1992).

It is clear that with a change in the biology of fertilization, the sperm structure alone can less readily explain phylogenetic relationships. It can, however, give

us a more complete picture of the phylogeny of a species. The sperm head structure of the anurans shows a clear correlation with changes in the fertilization environment. In species less dependant on water for their fertilization, the tendency is for the sperm head to become more elongated. The acrosomes of terrestrial fertilizers are also longer than those of the aquatic fertilizers. This adaptation seems to be necessary to penetrate the egg coverings and/or to swim through a viscous medium to reach the eggs for effective fertilization. Figs. 2.22 clearly shows the differences between the sperm head and acrosome lengths of the aquatic and terrestrial fertilizers. The star symbol plots in Fig. 2.23 furthermore indicate two distinct patterns and support the aquatic versus the terrestrial model based on sperm morphology.

In the testes, the spermatozoa are neatly packaged and embedded in the Sertoli cells. Shorter, stouter sperm heads are more easily packaged. In the case of *Chiromantis*, the coiled structure could facilitate easier packaging in the testis.

Fouquette and Delahoussaye (1977) indicated that a double tail complex (primitive trait) occurs in aquatic as well as terrestrial fertilizers. Certain anurans with modified sperm heads, however, have primitive tail structures and this validates the hypothesis that phylogeny as well as fertilization environment play important roles in shaping spermatozoon morphology.

CHAPTER 3

CHAPTER 3

ANURAN SPERMATOZOA - A SPERMIOGRAM

3.1 Introduction

Much work has been done on the structure and ultrastructure of sperm (Chapter 2). Fertilization, however, involves more than just the morphological properties of the sperm and egg cells. Spermiograms are routinely prepared to assess male fertility and to detect and remedy male infertility in humans (WHO laboratory manual, 1987). These values are recognised as baseline values for the assessment of male fertility.

Spermiograms in humans involve the following macroscopic and microscopic procedures (WHO laboratory manual, 1987). Macroscopic evaluation of a normal semen sample is done to assess whether the semen sample is homogeneous, has a grey appearrance and if liquefaction occurs within 60 minutes. The volume, consistency and pH of the sample will also be assessed as part of routine preparation of the human spermiogram. Microscopic investigations of the motility, concentration, agglutination, sperm viability, morphology and other procedures in obtaining values for human sperm are described in the WHO laboratory manual (1987).

No protocol exists for the preparation of submammalian spermiograms. Amphi-

bian spermiograms could provide us with valuable baseline values for this group. The fertilization environment plays a role in determining the structure of spermatozoa (Chapter 2). Interaction between the sperm and egg cells involves far more than only the structure of the spermatozoa. Analyses of sperm densities, integrity of the sperm cell membrane, sperm motility and percentage normal morphology could enhance our knowledge in terms of this interaction. Anurans are regarded as external fertilizers (Passmore and Carruthers, 1979). Spermatozoa are released in a foreign aquatic environment. Spermatozoa should therefore be adapted to move and live for periods of time within this aquatic environment. Most anurans are seasonal breeders and spermiograms could give us a better understanding of adaptations of these lower vertebrates. Fundamental data could be extracted from spermiograms. Little or no information on spermiograms, however, exists for sub-mammalian vertebrates and invertebrates. In this study I endeavoured to establish techniques and present basic data on the spermiograms for selected anuran species.

Van der Horst (1976) detailed sperm densities for *Xenopus laevis*. No work was, however, done on other anuran species. The sperm densities of *Xenopus* were also used in a study on the respiratory physiology of spermatozoa (Van der Horst, 1986). Baseline values of spermatozoa obtained for anuran spermiograms might provide a basis for comparing aquatic versus terrestrial fertilizers. Studies pertaining to the gonadal mass, sperm densities and the permeability of the sperm membrane (percentage eosinophilic sperm) were done. The gonadal masses were expressed as percentages of the total body mass. These values are

regarded as the gonadosomatic indices (GSI). Sperm densities of external fertilizers such as teleosts are high (Van der Horst, 1986). This would be expected as the sperm are released in flowing bodies of water. Within this environment it would also be expected that the sperm cell membrane should be less permeable to prevent external substances to penetrate the sperm cell.

Testicular sperm were used in preparing spermiograms as it is difficult to obtain a normal ejaculate in the case of the anurans. The percentage of "normal" spermatozoa may give a indication of the adaptations of the frogs and toads to their fertilization environment. An external fertilization environment might be more hazardous than an internal environment and selection pressures would be for a high percentage of normal spermatozoa. Experiments were done on *Xenopus laevis* to obtain their electro-ejaculate (Chapter 5). The sperm thus obtained were used for motility evaluation and to determine the permeability of the cell membrane.

The motility status of spermatozoa is crucial to the fertilization process. Motility is one of the parameters used in spermiograms (WHO laboratory manual, 1987). As the sperm motion parameters are so diverse, motility will be presented as separate investigations (Chapters 4 and 5). Motility, however, forms an integral part of any spermiogram.

Anurans, because of their wide range of fertilization environments, exhibit differences in structure (Chapter 2) and motility (Chapter 4). It is conceivable

that differences could exist between the spermiogram parameters of aquatic and terrestrial fertilizers. This research will therefore attempt to establish baseline values for spermiograms of the anurans and will include the following:

1 Determination of the sperm densities of the different anurans studied.

2 Assessment of the gonadosomatic indices of the anurans.

3 Evaluation of the integrity of the sperm cell membranes.

4 Preparation of data on the percentage "normal" sperm.

5 The data on the different species will then be statistically compared.

3.2 Materials

Sperm densities, gonadosomatic indices (GSI) and the determination of the permeability of the cell membrane were prepared on the sperm of the following species: (Number of specimens between brackets).

AQUATIC FERTILIZERS:

Bufo rangeri (10), *Semnodactylus wealii* (6), *Xenopus laevis* (10), *Rana fuscigula* (5), *Strongylopus grayii* (15) and *Tomopterna delalandii* (6).

TERRESTRIAL FERTILIZERS:

**Breviceps gibbosus* (5), **Arthroleptella lightfooti* (4) and *Chiromantis xerampelina* (3).

*: Only the GSI's and permeability of the cell membranes were determined as it was difficult to aspirate exact volumes of sperm from the very small testes of these species.

The percentage eosinophilic spermatozoa of *B. rangeri* was determined after the spermatozoa were allowed to swim out for 1¼ hours in a 10 % Ham's F10 solution. Statistical comparisons were drawn between the values obtained from the different species.

Differential counts were prepared on the sperm of the following species: *B. rangeri*, *X. laevis*, *R. fuscigula* and *S. grayii*.

3.3 Methods

3.3.1 Sperm density measurements

Spermatozoa were aspirated as described in Chapter 2 (2.3.1). An exact volume of sperm (2 or 5 µl) was placed in 1 ml of Sørensen phosphate buffered glutaraldehyde (2,5%). Ten µl of this suspension were placed on a Neubauer Improved Bright-Line Haemacytometer and viewed microscopically at 400x magnification. The total number of sperm in the white cell chambers was counted and sperm density was expressed as the number of sperm per ml by applying the following

formula:

$$\frac{\textit{TOTAL SPERM COUNT (4 CHAMBERS) X DILUTION FACTOR X } 10^4}{4}$$

3.3.2 Gonadosomatic Index (GSI)

Frogs and toads were anaesthetized using the methods described in Chapter 2. They were then dried off using Whatman filter paper and weighed on a Sartorius type 1475 toploading balance. After the testes were dissected out and cleared of major blood vessels, their weights were determined. The gonadosomatic index was calculated as the ratio of testicular mass to body mass and expressed as a percentage.

3.3.3 Live/dead staining of the spermatozoa

Two or five μl sperm extruded from the testes, were mixed with 15 μl nigrosin-eosin solution and left for five minutes. A small drop of each suspension was placed on a microslide and smears were made. The sperm smears were then air dried. Slides were mounted, using DPX as mounting medium and viewed on an Olympus microscope (CH-B145-T), using oil immersion with the 100x objective lens.

Pink-stained spermatozoa were regarded as dead sperm, as the nigrosin-eosin solution permeated through the cell membrane (Dott and Foster, 1972).

Spermatozoa with membranes impermeable to nigrosin-eosin were regarded as live sperm since such impermeability demonstrated the integrity of these sperm membranes. The percentage live and dead sperm was calculated by assessing at least 100 spermatozoa for each sample.

3.3.4 Differential count

I performed differential counts to assess the different forms of spermatozoa. One hundred sperm were scored each time per specimen, using an Olympus microscope with 400x magnification and the results expressed as a percentage.

3.4 Results

3.4.1 Sperm density; GSI; Percentage live sperm

The results are presented in Table 3.1.

Table 3.1 Data on the sperm densities, GSI's and percentage live sperm

SPECIES	DENSITY (x 10^8 sperm/ml)	GSI (%)	LIVE (%)
B. rangeri	12,3 (±2,6)	0,3 (±0,05)	87,4 (±2,5)
A	9,8 - 16,4	0,22 - 0,39	84 - 91
S. wealii	5,0 (±0,2)	0,2 (±0,01)	80,4 (±1,6)
B	4,8 - 5,4	0,2 - 0,23	78 - 83

SPECIES	DENSITY (x 10^8 sperm/ml)	GSI (%)	LIVE (%)
X.laevis	6,0 (±0,8)	0,3 (±0,03)	78,2 (±1,8)
C	4,5 - 7,8	0,2 - 0,32	75 - 81
R. fuscigula	12,2 (±0,8)	0,3 (±0,01)	82,3 (±3,2)
D	11,9 - 13,9	0,26 - 0,3	77 - 86
S. grayii	9,3 (±1,1)	0,5 (±0,11)	86,6 (±1,4)
E	7,7 - 11,8	0,41 - 0,76	83 - 88
T. delalandii	6,4 (±0,5)	0,3 (±0,1)	80,5 (±1,9)
F	5,5 - 7,2	0,21 - 0,53	77 - 82
B. gibbosus	no values obtained	0,3 (±0,18)	83,3 (±2,3)
G		0,19 - 0,42	79 - 86
A. lightfooti	no values obtained	0,3 (±0,03)	82,4 (±1,8)
H		0,25 - 0,31	79 - 85
C. xerampelina	1,7 (±0,6)	5,5 (±1,08)	85,5 (±1,4)
I	0,8 - 2,1	3,8 - 7,2	82 - 88

Format: $\overline{x}$ (±SD) Range

3.4.2 Comparison of sperm densities, GSI's and percentage live sperm

ANOVA and nonparametric tests were used to statistically compare the

spermiogram values obtained.

SPERM DENSITY:

A vs B; A vs C; A vs E; A vs F; A vs I; B vs D; B vs E; B vs I; C vs D; C vs E; C vs I; D vs E; D vs F; D vs I; E vs F; E vs I; F vs I

Significant difference ($p < 0,05$)

GSI

A vs B; A vs E; A vs I; B vs C; B vs D; B vs E; B vs F; B vs G; B vs H; B vs I; C vs E; C vs I; D vs E; D vs I; E vs F; E vs G; E vs H; E vs I; F vs I; G vs I; H vs I

Significant difference ($p < 0,05$)

Percentage live sperm

A vs C; A vs H; C vs D; C vs E; C vs G; C vs H; C vs I;

Significant difference ($p < 0,05$)

It was clear that significant differences exist among all the anurans studied. The anurans with the highest sperm densities (*B. rangeri* and *R. fuscigula*) showed no difference. This could possibly be because of their similar fertilization environment, namely within bodies of water. *C. xerampelina* exhibited a very low sperm density and exceptionally high GSI values which differed significantly from values of the other anurans studied.

I could only determine the GSI's and live/dead ratios for *Breviceps gibbosus* and *Arthroleptella lightfooti*. It was not possible to obtain exact volumes of sperm from their small testes for sperm density measurements.

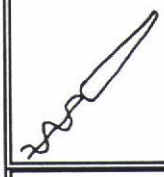
The percentage live *Bufo* sperm after 1¼ hours of swim out in Ham's F10 (10%) was 19,6% which differed significantly from the values obtained from the fresh samples of all the anurans studied.

The comparisons of certain spermiogram parameters are illustrated in the bar graphs (Figs. 3.1 and 3.2) and the star symbol plot (Fig. 3.3). Fig. 3.1 clearly shows the similar sperm density values of *B. rangeri* and *R. fuscigula* and the low value for *C. xerampelina* sperm. The graph comparing the GSI's (Fig. 3.2) indicates the exceptionally high value for *C. xerampelina* testis. The star symbol plot analysis (Fig. 3.3) indicated differences among all the species studied and this, together with the information obtained from Table 3.1 could be indicative of species specificity regarding their spermiograms.

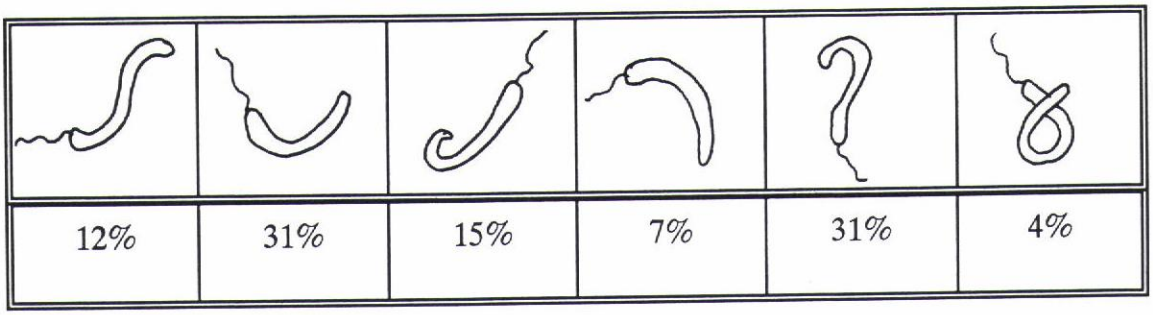
3.4.3 Differential counts

Differential forms of the sperm were drawn and the percentages recorded below.

B. rangeri

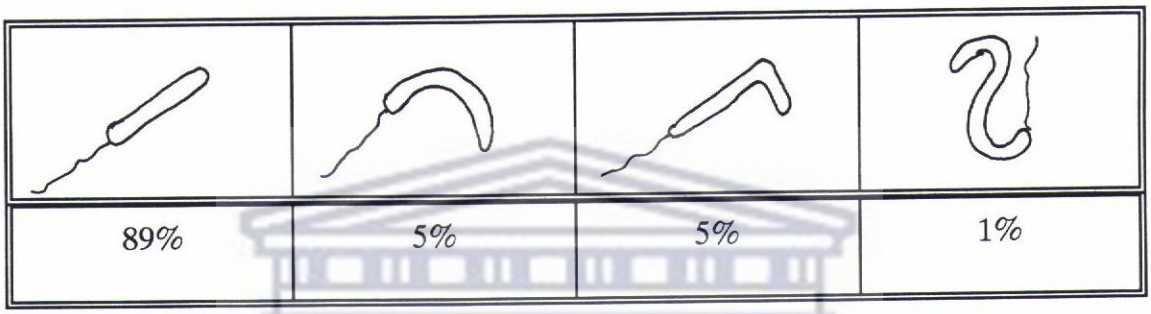
					
82%	4%	8%	4%	1%	1%

X. laevis



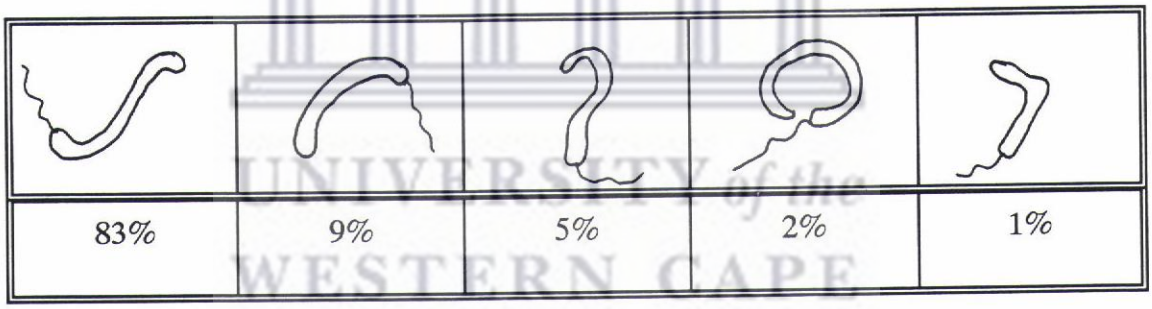
12%	31%	15%	7%	31%	4%

R. fuscigula



89%	5%	5%	1%

S. grayii



83%	9%	5%	2%	1%

Observations of live spermatozoa in different physiological media used to quantify sperm motility, also indicated the above mentioned sperm abnormalities. This supports the fact of differential sperm forms and the abnormalities should therefore not be regarded as preparation artefacts. Abnormal sperm forms also exhibited abnormal swimming patterns.

Fig. 3.1 Comparison of sperm densities of selected anurans

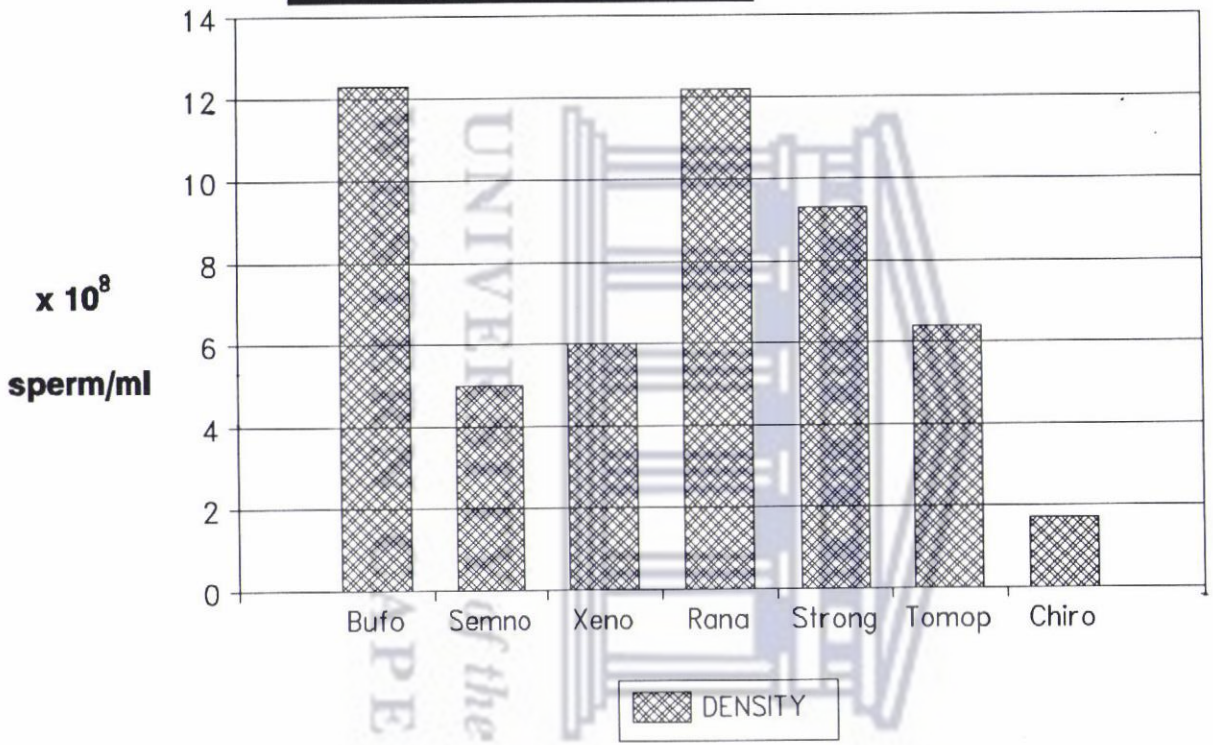


Fig. 3.2 Comparison of the GSI of selected anurans

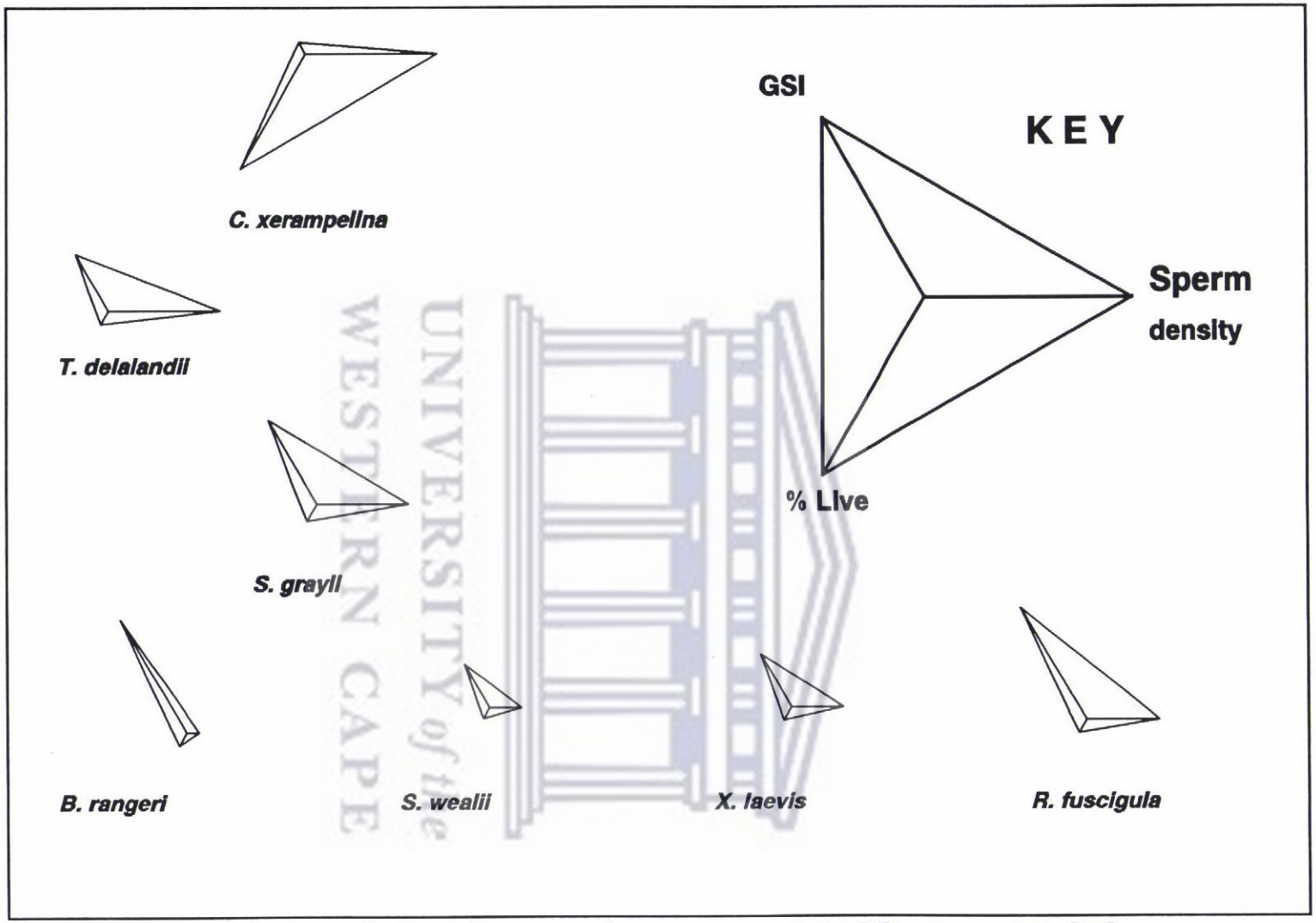


Fig. 3.3 Star symbol plot analysis of three spermiogram parameters of the anurans studied

3.5 Discussion

The determination of sperm densities and abnormalities in the spermatozoon structure is done on a routine basis in the case of human sperm. The WHO laboratory manual (1987) details procedure and techniques for the compilation of spermiograms for humans. Spermiograms for other mammals such as vervet monkeys have been detailed by Seier *et al.* (1989). These spermiograms are compiled to obtain baseline values for spermatozoa of species. Protocols for obtaining spermiograms for submammalian vertebrates have not been reported. Van der Horst (1976) compiled sperm densities for *Xenopus laevis*. No work in this regard on the other anuran species has been done. I attempted with this research to compile baseline values for a selected group of anuran species.

There is a clear indication of a significant difference between the sperm morphology of terrestrial and aquatic fertilizers (Chapter 2). I attempted to use the baseline values of the spermiograms to test the hypothesis that fertilization environment determines form and function of anuran spermatozoa. The terrestrial fertilizers posed a problem in the sense that exact volumes of sperm could not be aspirated from their testes. The testicular mass of *C. xerampelina* constitutes 5,5% of the total body mass. Although the sperm density of this species is significantly lower than that of the other anurans studied, the total volume of sperm per testis could be the same as that in other anurans. *C. xerampelina* is regarded as a typical example of the terrestrial fertilizers (Chapter 2). The significant differences between the sperm densities of *C. xerampelina* and the aquatic fertilizers could not be extrapolated to all other terrestrial

fertilizers because of the lack of data on other terrestrial breeders. My data on sperm density measurements of *X. laevis* are slightly lower than the densities detailed by Van der Horst (1976). The values obtained by me, however, fall within the range of those obtained by Van der Horst (1976) for the same dilutions.

I compared the spermiograms of all the anurans used in this study. The analyses indicated significant differences between the sperm densities and GSI's of the sperm of *Chiromantis* and the aquatic fertilizers (Table 3.1). There were, however, significant differences among the spermiogram data of all the anurans studied. The lack of data from the other terrestrial fertilizers made clear distinctions between the data for aquatic and terrestrial fertilizers difficult. Methods should be devised to aspirate exact volumes of testicular sperm from these anurans so that comparisons can be drawn between aquatic and terrestrial fertilizers.

Throughout this study on spermiograms only testicular sperm was used. Motility data on ejaculate sperm (Chapter 5) indicated differences between testicular and ejaculate spermatozoa. Methodology should be devised to obtain ejaculate sperm from the anurans to compare the spermiograms of ejaculate and testicular sperm. Experiments were done on *X. laevis* to obtain ejaculate sperm (Chapter 5), but during aspiration the ejaculate was mixed with Ham's F10 and could thus not be used to prepare baseline values for sperm density, GSI and eosinophilic sperm.

The percentage live sperm of the anurans was very high (above 80%), except in the case of *X. laevis* which showed significant differences with most of the species studied. The normal values for viability of human sperm are presented as 50% (WHO laboratory manual, 1987) and for vervet monkeys between 53% and 68% (Seier *et al.*, 1989). Anuran spermatozoa are normally released in water for external fertilization. The high percentage of viable sperm in the anurans could be an indication of the effective adaptation of these species to their fertilization environment.

The values for normal sperm morphology is in the region of 50% in the case of humans (WHO laboratory manual, 1987). Vervet monkeys have a low abnormal sperm morphology viz. 3% to 6% (Seier *et al.*, 1989). *X. laevis* had the lowest percentage of normal sperm (12%) of the anurans studied, whereas the percentages of normal sperm in the other species were above 80%. This may be a reason why the percentage motile testicular sperm in the case of *X. laevis* is low (Chapter 4) and higher in the case of all the other anurans studied. Such a low percentage of motile sperm was also observed by Van der Horst (1976). The external fertilization environment of the anurans would select for spermatozoa to be highly viable and able to fertilize the eggs, whereas human spermatozoa are deposited in a less variable fertilization environment.

Data on the other terrestrial fertilizers can enhance our knowledge on the impact of the fertilization environment on the structure and physiology of anuran spermatozoa. Anuran species exhibit a wide range of fertilization environments,

from totally aquatic to internal (Passmore and Carruthers, 1979 and Grandison and Ashe, 1983). The star symbol analysis (Fig. 3.3) showed differences in patterns among all the anurans studied.

The comparisons of the spermiogram values for the anurans studied, indicated differences among all the species. This could be ascribed to species specificity and thus a result of gene expression in species. This study indicated that no clear distinction could be made between aquatic and terrestrial fertilizers, as significant differences exist among the aquatic fertilizers.

Anurans occupy a niche between the true external fertilizers (teleosts) and the true internal fertilizers (mammals). Teleosts have sperm densities to the order of 10^9 spermatozoa per ml (Van der Horst, 1986). Fertile human males present sperm densities to the order of 10^7 spermatozoa per ml. (WHO laboratory manual, 1987). The anurans (sperm densities to the order of 10^8 sperm/ml) fit in between teleosts and humans. This could indicate the intermediate fertilization environment of the anuran species. Although fertilization environment and phylogeny play roles in determining sperm form and function, research into spermiogram parameters of aquatic and terrestrial anuran fertilizers could elucidate the adaptations of anuran spermatozoa to their particular fertilization environment.

CHAPTER 4

CHAPTER 4

COMPARISON OF SPERM MOTILITY PATTERNS OF SELECTED ANURANS

4.1 Introduction

Sperm motility measurements in mammals assist to define the vigour and fertilizing capacity of sperm. In the lower vertebrates there is a need to explore motility parameters to gain insight in the fertilization biology. In this study testicular spermatozoa of mature breeding adults were aspirated and their swimming patterns compared.

Earlier researchers used a subjective way of quantifying sperm motion. This is still evident in the description given in the manual of the World Health Organisation for the examination of human semen (1987). Human sperm motion is divided into four categories viz. rapid and linear progressive (excellent progression); sluggish linear or non-linear progression (moderate progression); non-progressive motility and immotile spermatozoa. Effective motility is crucial for fertilization. Studies pertaining to the motility of spermatozoa are not reliable because they are subjective and therefore objective quantification of sperm motion is required.

The analysis of sperm movement of species created a need to find means to

accurately describe and quantify patterns of sperm motion. Brokaw (1968) used high-resolution photomicrographs to assess sperm flagellar movement. Our knowledge on mammalian sperm movement has been enhanced by Phillips and Olson (1973) who related structure to the functional motion of the spermatozoa. They used high speed cinematography to relate the beat form of the spermatozoa to structure. Woolley (1973) used a rapid freezing technique to halt sperm motility in the rat. He then examined the fine structure of the spermatozoa microscopically so as to relate it to movement in the flagella. Katz and Dott (1975) utilised computers to devise methods to measure the velocities of spermatozoa. Overstreet *et al.* (1979) and Katz and Overstreet (1981) assessed motion characteristics of human spermatozoa by using computerized video-micrography. Frame lapse videography and computer analysis were used by Samuels and van der Horst (1986) to evaluate sperm velocity of human, bull and white mussel spermatozoa. The Cellsoft image analysis system (Cryo Resources Ltd., New York, NY) was utilised by Working and Hurtt (1987) to analyze rat sperm motility.

It is clear that computer technology inevitably led to a more objective means of quantifying sperm motility parameters. Most image analysis systems were developed to analyze mammal and specifically human sperm motion. In this study the swimming patterns of the anuran spermatozoa were analyzed using the Sperm Motility Quantifier (SMQ) as image analysis system.

Yanagimachi (1988) described the motility of sperm in the vicinity of eggs. This

"hyperactivation" involves a vigorous action of the spermatozoa. The spermatozoa exhibit non-linear motion in the vicinity of the eggs. This "dancing" manner of motion is interrupted by short periods of linear progression ("dashing"). Star-spin type of motility could possibly be a mechanical means for the sperm to penetrate the outer egg layers, as it generates enough forward thrusting. The visual effect of hyperactivation is that the spermatozoa seem to thrash about. Yanagimachi (1988) described this particular type of motion in mammalian sperm but it was clear from my studies that anuran spermatozoa exhibit the same non-progressive vigour in the vicinity of the eggs (Chapter 5).

Sperm motility studies on the invertebrates and lower vertebrates have not elicited the same amount of research as in the case of mammals. Flower (1967) in his research on fluid dynamic drag and the effect of sperm tail shape of onychophorans and insects found no correlation between the shape and effective propulsion of the sperm. Brokaw *et al.* (1970) investigated the sperm motility patterns of marine invertebrates and contended that sperm movement studies be regarded as important in comparative spermatology.

Earlier studies on the motility of anuran spermatozoa relied mostly on light microscopical observations. A survey of the literature reveals subjective descriptions of the motility patterns of the anuran spermatozoa. The work of these researchers, however, laid the basis for the work done in this study. Del Rio (1979) showed that *Bufo arenarum* sperm sustained motility under aerobic as well as anaerobic conditions. His observations were done on a microslide

under high magnification and estimated visually. Swan *et al.* (1980) using cinematography, concluded that in *Bufo marinus* the axoneme moved actively whereas the axial fibre played a less important role in the flagellar propulsion of the sperm. Sperm motility alone, however, is not sufficient for fertilization in *Leptodactylus chaquensis* spermatozoa according to Raisman *et al.* (1980). Other physiological parameters and the interaction of the sperm with the egg coverings play consequential roles. Mizuhira *et al.* (1986) described an active corkscrew motion of the sperm of the tree frog, *Rhacophorus*. Van der Horst (1986) described *Xenopus* sperm motion as slowly rotating at an approximate velocity of 15 μm/s. In their description of *Xenopus* sperm movement, Bernadini *et al.* (1988a) concurred with Inoda and Morisawa (1987) that effective sperm motion of these anurans is initiated by low osmolalities.

Comparative spermatology should be utilised to ascertain the extent of the diversity of life on earth. It can also be used in the search for effective contraceptive methods in humans. Research on spermatology of the invertebrates and lower vertebrates may reveal techniques and data which can be applied to the higher vertebrates. Sperm motility as an important barometer of fertilizing capacity should be analyzed quantitatively and subjective methods should be discontinued.

In this study the main purpose was to quantitatively analyze sperm motility characteristics of selected anuran species. The research will attempt to answer questions relating to the fertilization environment of the different species. This

will include the following:

1 A description of the methods using a computer aided sperm motility system (SMQ) will be given, with special reference to the parameters used in this study.

2 The sperm motility parameters of the different species used will be compared to establish whether the sperm motility patterns of the aquatic fertilizers differ from the terrestrial ones.

3 Fertilization environments differ between the various species. This study was done to differentiate between the motility characteristics of selected anurans.

4 The patterns of sperm motion of the selected anurans will be described.

5 Star plot analyses (cf. Chapter 2) will be used as a multivariate test to compare swimming patterns and velocities of the spermatozoa of the anurans.

4.2 Materials

All anurans were caught at their breeding sites, except for *Xenopus laevis* specimens which were obtained from a breeding farm. The sperm of mature breeding adults of species listed in Table 4.1 were used for motility studies.

Table 4.1: Anuran species used for sperm motility measurements

SPECIES	F.E. GR.	NUMBER
Bufo rangeri	A	8
Xenopus laevis	A	6
Strongylopus grayii	A	12
Pyxicephalus adspersus	A	3
Semnodactylus wealii	A	3
Hyperolius horstocki	A	2
Arthroleptella lightfooti	T	3
Breviceps gibbosus	T	3
Chiromantis xerampelina	T	3

F.E. GR. : Fertilization Environment Grouping

A : Aquatic fertilizers; T : Terrestrial fertilizers

4.2 Methods

4.2.1 Aspiration and preparation of spermatozoa

The spermatozoa were aspirated as described in Chapter 2. Fifteen μl of sperm were suspended in a 10% Ham's F10 solution in a petri dish. The spermatozoa were placed in the centre of the petri dish and allowed to swim towards the periphery of the dish. This is called the swim out technique. One ml of

suspension was collected from the periphery of the petri dish with a Gilson pipetman pipette and placed in a sperm chamber (Fig. 4.1).

Spermatozoa were placed in different concentrations of Ham's F10 solution to ascertain the osmotic environment best suited for the effective motility of the spermatozoa. The 10% Ham's F10 solution with an osmolality of 28 mOsm proved to be ideal.

Toads Zwartkops Solution (TZS) with an osmotic concentration of 30 mOsm, devised by Van der Horst (1986) was also used as a suspension for *B. rangeri* sperm.

4.2.2 Assessment of the motility patterns

The sperm chamber was placed on an inverted Zeiss microscope (ICM 405) using the 16X phase contrast objective. Every 30 minutes for up to 5 hours the swimming patterns of the spermatozoa were videotaped. The swimming patterns of at least 75 spermatozoa of each specimen were videotaped at the different time intervals. Davis and Katz (1989) proposed that between 50 to 200 spermatozoa be used for computer-aided sperm analysis. Analysis of the sperm swimming patterns was done with the Sperm Motility Quantifier (Wirsam Scientific, Pty LTD).

4.2.3 Sperm Motility Quantifier (SMQ)

The SMQ is a Computer Assisted Sperm Motility Analysis (CASMA) system

developed by Van der Horst (1992). Seventeen motility parameters as well as sperm density measurements can be assessed by this system in both the automated and manual modes. The videotaped swimming patterns of the spermatozoa were played back via a VCR through the SMQ card in the 486 computer, to a TV monitor.

Video images were captured at 3,125 Hz (Frameskip 4). This means that the image was captured every tenth of a second. Sperm motion was taped for eight seconds at a time. Automatic evaluation of the motility parameters were then performed. Thirteen sperm motility parameters were studied. Table 4.2 and Fig. 4.2 explain and indicate the different parameters. Data on the motility patterns were stored as text files (.DXP and .PXP) and imported to a spreadsheet for further analysis.

ANOVA tests were used and in cases where there was a significant difference in variance, Mann-Whitney analyses were performed to statistically compare data on the motility parameters.

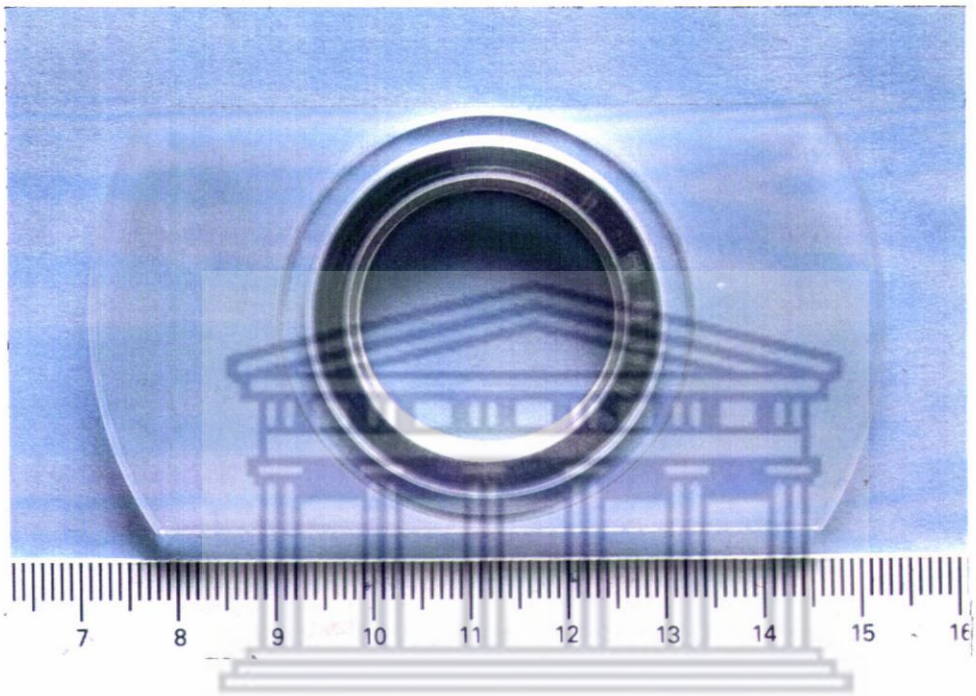


Fig. 4.1 Sperm chamber.

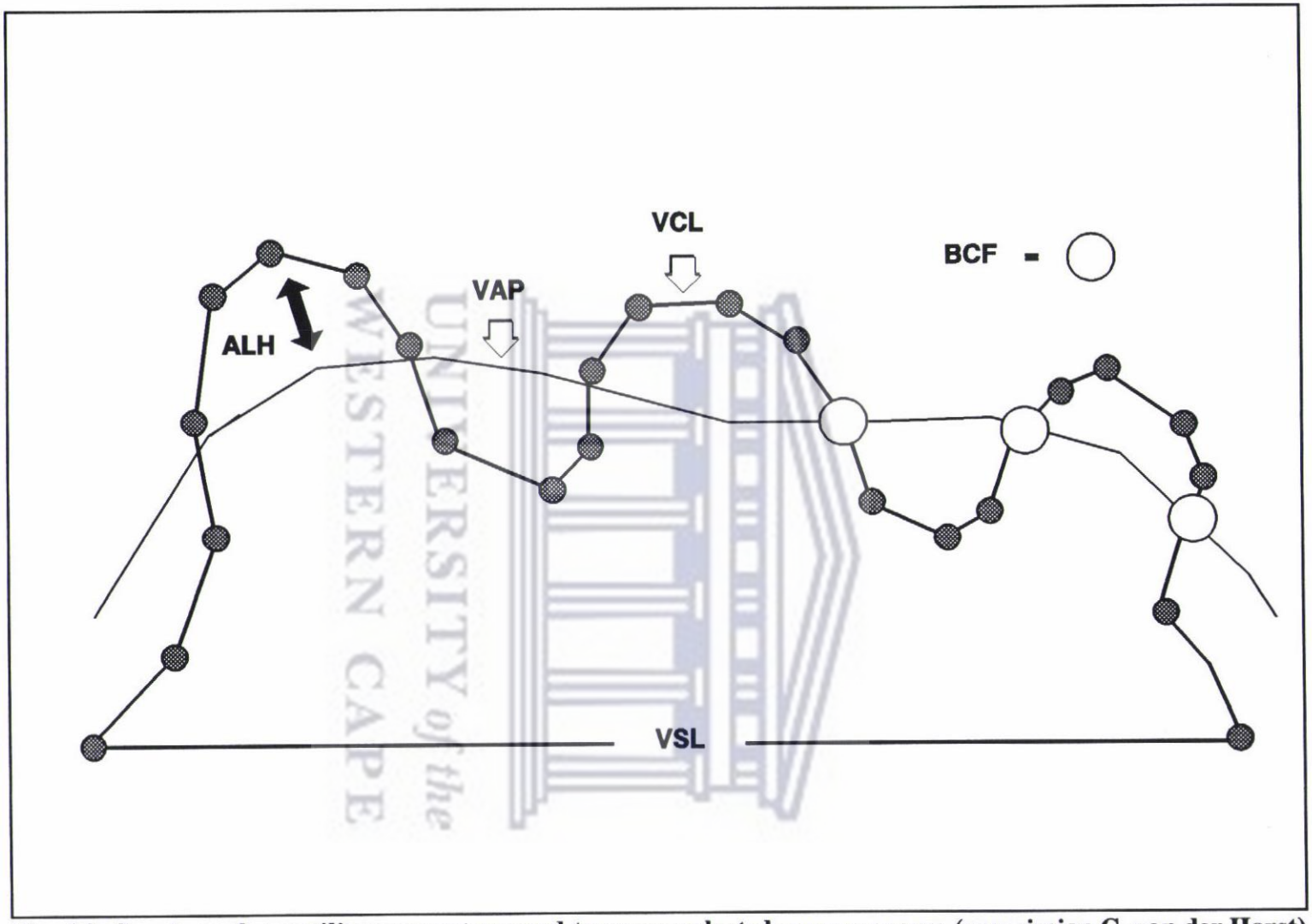


Fig. 4.2 Graph on the motility parameters used to assess selected anuran sperm (permission G. van der Horst)

The sperm motility parameters used for this study can be divided into two functional groups, viz.:

Vigour of motility : VCL; VSL; VAP and BCF

Pattern of motion : LIN; mnALH; mxALH; DNC; DNCmn; STR; WOB and CURV.

Table 4.2: Interpretation of the sperm motility parameters (Van der Horst, 1992)

PARAMETER	ABB.	EXPLANATION
Curvilinear velocity	VCL	The time average velocity of the sperm head on its actual (precise) path. Expressed in μm/s.
Straightline velocity	VSL	The time average velocity of the sperm head projected along the straight line between its first and final detected positions. Expressed in μm/s.
Average Path velocity	VAP	The time average velocity of the sperm head projected along its spatial average trajectory. Expressed in μm/s.

PARAMETER	ABB.	EXPLANATION
Beat Cross Frequency	BCF	The time average rate at which the curvilinear path crosses its average path. Expressed as Hz.
Linearity	LIN	Ratio of projected length to total length of the curvilinear trajectory. Expressed as %.
Mean Amplitude of Lateral Head Displacement	mnALH	Average amplitude of lateral distances of the actual sperm head trajectory about its spatial average path. Expressed as μm.
Maximum Amplitude of Lateral Head Displacement	mxALH	Maximum amplitude of lateral distances. Expressed as μm.
Dance	DNC	Space occupied by the sperm head path during 1 sec. Product of VCL and mnALH. Expressed in $\mu m^2/s$.
Dancemean	DNCmn	Product of mnALH and the ratio of VCL and VSL. Expressed in μm.
Straightness	STR	Ratio of VSL to VAP. Expression of the straightness of the average path.

PARAMETER	ABB.	EXPLANATION
Wobble	WOB	Ratio of VAP to VCL. Expression of degree of oscillation of curvilinear path about its spatial path.
Percentage Motile Sperm	PM	Percent motility of sperm population. Represents all forms of motility.
Curvature	CURV	Progressiveness of movement. A smaller curve indicates a straighter sperm path and higher progressive-ness.

ABB. : Abbreviation

4.4 Results

4.4.1 Suspensions used to assess sperm motility

The motility patterns of *B. rangeri* spermatozoa in Ham's F10 (10%) were compared to the sperm motility patterns of the same specimens in TZS. There was no significant difference between the vigour of motility and pattern of motion between the spermatozoa in the different media. There was, however, a significant difference between the percentage motile sperm in Ham's F10 (10%) and TZS as demonstrated in Table 4.3. As a result of these differences it was decided to use Ham's F10 (10%) as the medium in which to study the

motility patterns.

Table 4.3: Comparison of percentage motile sperm in Ham's F10 and TZS

SPECIES		HAM'S F10	TZS
B. rangeri	$\bar{x} \pm SD$	56,8% ± 13,9 *	22,8% ± 5,9 *

* Significant difference ($p < 0,05$)

Different dilutions of Ham's F10 were used to test the effect of osmotic concentrations on the motility of the spermatozoa. The vigour of sperm motility and percentage motile sperm decreased rapidly in suspensions with an osmolality higher than 50 mOsm/kg and no sperm motion could be detected in suspensions higher than 200 mOsm/kg.

In the Ham's F10 (10%) solution the spermatozoa could swim for up to 5 hours (*B. rangeri*) with a resulting decrease in motility. At the end of this time period the motile spermatozoa were still swimming with more or less the same vigour as at 30 minutes. Different time intervals were used to ascertain the best time frame in which to analyze the motility parameters. The percentage motile sperm of *B. rangeri* was assessed at time intervals of 30 minutes, 3 hours and 5 hours. Table 4.4 shows the decrease of percentage motile sperm with time. As a result of this, it was decided to evaluate the sperm motility patterns of the different anurans videotaped at between 15 to 30 minutes after aspiration.

Table 4.4: Percentage motile sperm of *B. rangeri* at different time intervals

SPECIES		30 MINUTES	3 HOURS	5 HOURS
B. rangeri	$\bar{x} \pm$ SD	56,8% ± 13,9*	34,8% ± 6,3*	17,3% ± 3,2*

* Significant difference ($p < 0,05$)

4.4.2 Assessment of sperm motility parameters

Table 4.5 illustrates the data obtained for the different species.

Table 4.5 Sperm motility parameters for different anuran species ($\bar{x} \pm$SD)

PARA-METERS	A- *B. rangeri*	B- *S. wealii*	C- *S. grayii*	D- *P. adspersus*	E- *X. laevis*	F- *H. horstocki*
VCL (μm/s)	21,8 ±4,8	19,9 ±8,4	27,2 ±6,0	29,5 ±7,4	21,7 ±7,7	31,1 ±9,7
VSL (μm/s)	14,9 ±5,6	9,5 ±3,9	11,3 ±3,4	11,4 ±4,9	8,6 ±3,5	23,0 ±9,1
VAP (μm/s)	16,7 ±5,7	12,0 ±4,5	13,8 ±4,3	13,6 ±4,4	12,2 ±3,7	27,6 ±10,4
BCF (Hz)	4,7 ±3,7	2,6 ±1,8	3,7 ±1,9	3,9 ±3,1	3,0 ±1,7	6,1 ±3,8

PARA-METERS	**A-** *B. rangeri*	**B-** *S. wealii*	**C-** *S. grayii*	**D-** *P. adspersus*	**E-** *X. laevis*	**F-** *H. horstocki*
LIN (%)	67,6 ±16,6	49,3 ±15,4	42,4 ±11,5	40,0 ±17,1	41,3 ±14,3	72,7 ±16,5
mnALH (μm)	1,6 ±0,7	1,9 ±1,3	3,2 ±1,3	3,6 ±1,4	2,5 ±1,3	1,8 ±1,0
mxALH (μm)	4,7 ±1,3	5,3 ±2,3	7,5 ±3,4	7,2 ±2,5	5,9 ±1,9	5,1 ±1,9
DNC ($\mu m^2/s$)	37,3 ±20,4	47,3 ±55,8	91,2 ±51,6	113,6 ±67,5	62,4 ±63,5	59,6 ±43,5
DNCmn (μm)	2,9 ±2,4	4,7 ±4,6	8,3 ±4,6	11,9 ±8,9	7,3 ±6,0	2,8 ±2,2
STR	0,9 ±0,1	0,8 ±0,1	0,9 ±0,4	0,8 ±0,2	0,7 ±0,2	0,8 ±0,1
WOB	0,8 ±0,2	0,6 ±0,2	0,5 ±0,2	0,5 ±0,1	0,6 ±0,2	0,9 ±0,1
CURV	0,4 ±0,3	0,7 ±0,4	0,6 ±0,4	0,6 ±0,4	0,7 ±0,4	0,5 ±0,4
PM (%)	56,8 ±13,9	37,6 ±23,3	31,8 ±21,5	38,4 ±11,6	12,9 ±4,6	36,6 ±12,2

4.4.3 Comparison of motility parameters

VCL: A vs C; A vs D; A vs F; B vs C; B vs D; B vs F; C vs E; D vs E; E vs F;

significant difference ($p < 0,05$)

VSL: A vs B; A vs C; A vs D; A vs E; A vs F; B vs F; C vs E; C vs F; D vs E;

D vs F; E vs F

significant difference ($p < 0,05$)

VAP: A vs B; A vs C; A vs D; A vs E; A vs F; B vs F; C vs F; D vs F; E vs F

significant difference ($p < 0,05$)

BCF: A vs B; A vs E; B vs C; B vs F; C vs F; D vs F; E vs F

significant difference ($p < 0,05$)

LIN: A vs B; A vs C; A vs D; A vs E; B vs D; B vs E; B vs F; C vs F; D vs F;

E vs F

significant difference ($p < 0,05$)

mnALH: A vs C; A vs D; A vs E; B vs C; B vs D; B vs E; C vs E; C vs F

D vs E; D vs F; E vs F

significant difference ($p < 0,05$)

mxALH: A vs C; A vs D; A vs E; B vs C; B vs D; C vs E; C vs F;

D vs E; D vs F

significant difference ($p < 0,05$)

DNC: A vs C; A vs D; A vs E; A vs F; B vs C; B vs D; B vs E; C vs F

D vs E; D vs F

significant difference ($p < 0,05$)

DNCmn: A vs C; A vs D; A vs E; B vs C; B vs D; B vs E; B vs F;

C vs F; D vs E; D vs F; E vs F

significant difference ($p < 0,05$)

STR: A vs B; A vs E; C vs E; D vs E; E vs F

significant difference ($p < 0,05$)

WOB: A vs B; A vs C; A vs D; A vs E; A vs F; B vs C; B vs D;

B vs F; C vs F; D vs E; D vs F; E vs F

significant difference ($p < 0,05$)

CURV: A vs B; A vs D; A vs E; E vs F

significant difference ($p < 0,05$)

PM: A vs B; A vs C; A vs D; A vs E; A vs F; B vs E; C vs E; D vs E; E vs F

significant difference ($p < 0,05$)

Sperm motion of the terrestrial breeders (*A. lightfooti* and *B. gibbosus*) were assessed in Ham's F10 (10%) and TZS. Different dilutions of Ham's F10 were used, but the spermatozoa remained immotile in the suspensions. *C. xerampelina* spermatozoa were suspended in Frog Ringer's, distilled water, tap water and pond water. A jerky star-spin motion (thrashing about of the sperm) was evident and the coiled heads of these spermatozoa uncoiled, but no forward progression was observed. No motility data could therefore be recorded via the SMQ from the spermatozoa of the terrestrial breeders examined.

4.4.4 Qualitative description of the swimming motion of the anuran spermatozoa

Bufo rangeri

The sperm head undulated from side to side with a simultaneous rolling motion

along its longitudinal axis. The sperm tail complex undulated laterally and rolled three-dimensionally.

Semnodactylus wealii

An undulation of the sperm head accompanied by a slow rolling motion was observed.

Strongylopus grayii

The helical sperm head progressed spirally and the single tail followed the same trajectory. A pronounced longitudinal rolling of the sperm head was observed.

Pyxicephalus adspersus

The short, stout sperm head undulated from side to side, but there was less longitudinal rolling of the sperm head compared to the sperm of the other species.

Xenopus laevis

A spirally rolling type of motion of the helical sperm head was observed. Immediately after suspension in Ham's F10 (10%) the spermatozoa seemed immotile. After approximately 30 seconds the spermatozoa slowly began to move until they reached their maximum velocity after 2 minutes.

Hyperolius horstocki

An undulation of the sperm head with a longitudinal rolling along the axis was

evident. The curvilinear velocities of these spermatozoa were higher than those of the other anuran spermatozoa examined.

4.4.5 Comparisons of pattern of motion

Fig. 4.3 shows the differences in the vigour of sperm motion between the different anurans studied. The velocities of the spermatozoa of the species are reflected in the bar graph. *H. horstocki* spermatozoa display the highest values for all the velocity measurements. Microscopic assessment of the vigour of sperm motion indicated that sperm of this species swim faster and more linearly than sperm of the other species. The high VSL and VAP of *H. horstocki* sperm reflected in Fig. 4.3 is an indication of this phenomenon. *X. laevis* spermatozoa swim the slowest. The motion of the *X. laevis* sperm was microscopically observed to be sluggish in relation to the sperm of the other anurans studied. The similarities in the VSL and VAP of the sperm of *S. wealii*, *S. grayii* and *P. adspersus* are indicated in the graph. There was, however, a significant difference between VCL of *S. wealii* and *S. grayii*, as well as between the VCL of *S. wealii* and *P. adspersus*. There was no significant differences between the parameters of vigour of *S. grayii* and *P. adspersus*. These two species are closely related and their "fertilization environments" are basically the same (on the fringes of bodies of water). Fig. 4.3 further shows differences of sperm velocity parameters among all the other anurans studied. The high values of VSL and VAP of *B. rangeri* sperm (although not as high as in the case of *H. horstocki*) is an indication of fairly linear progression of the sperm which was microscopically observed.

The percentage motile sperm and the linearity of the sperm motion are shown in Fig. 4.4. The high LIN of *H. horstocki* sperm was again an indication of their high linear progression. *B. rangeri* spermatozoa are shaped for effective motion in water (Chapter 2) and their linear progression was shown by the high LIN value. The highest percentage motile sperm was observed in the case of *B. rangeri* sperm and the lowest value recorded was that of *X. laevis* spermatozoa. *S. grayii* and *P. adspersus* have very similar values for LIN and PM.

The pattern of motion of the spermatozoa is depicted in Fig. 4.5. BCF, ALH, CURV, WOB and STR constitute some of the characteristics of the observed swimming patterns of the spermatozoa. The similarities between the values obtained for *S. grayii* and *P. adspersus* are again evident. The high BCF value for *H. horstocki* sperm indicated their vigorous mode of progression.

Certain parameters of the motility characteristics of the anuran spermatozoa were used to compile star symbol plots (Fig. 4.6). These star plots reflect similarities and differences between the swimming attributes of the anuran spermatozoa. Patterns of progression emerged from these star plots. The similar appearance of the star plots of *S. grayii* and *P. adspersus* reflected similar patterns of motion and was an indication of comparable fertilization environments. The relatively high values for velocities as exhibited by *H. horstocki* is evident in the star plot for this species. The star plots furthermore indicated differences in the motility patterns of *X. laevis*, *B. rangeri* and *S. wealii*. The relatively small star plot of *S. wealii* indicated the relatively lower values in these anurans.

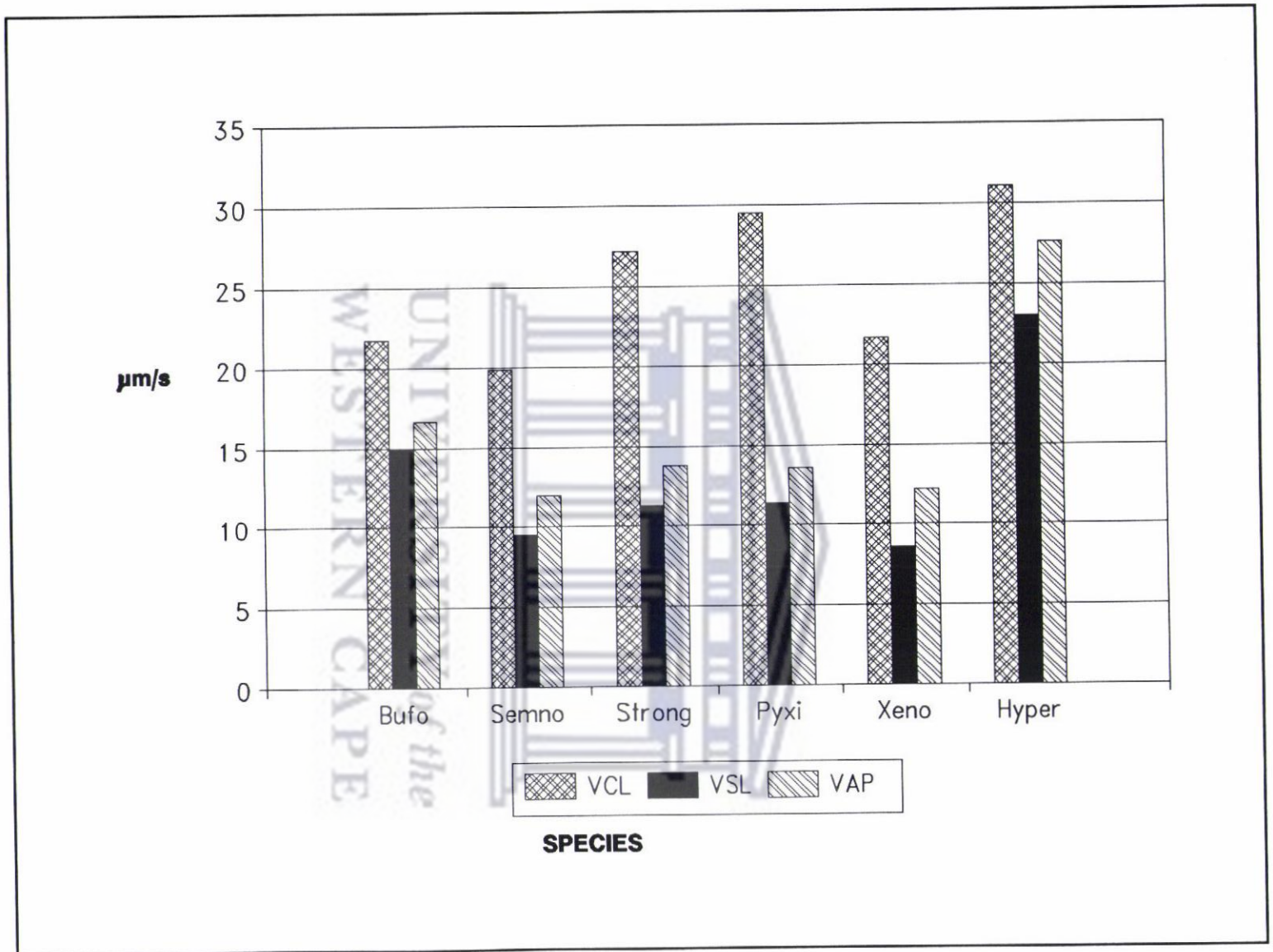


Fig. 4.3 Comparison of the vigour of sperm motion

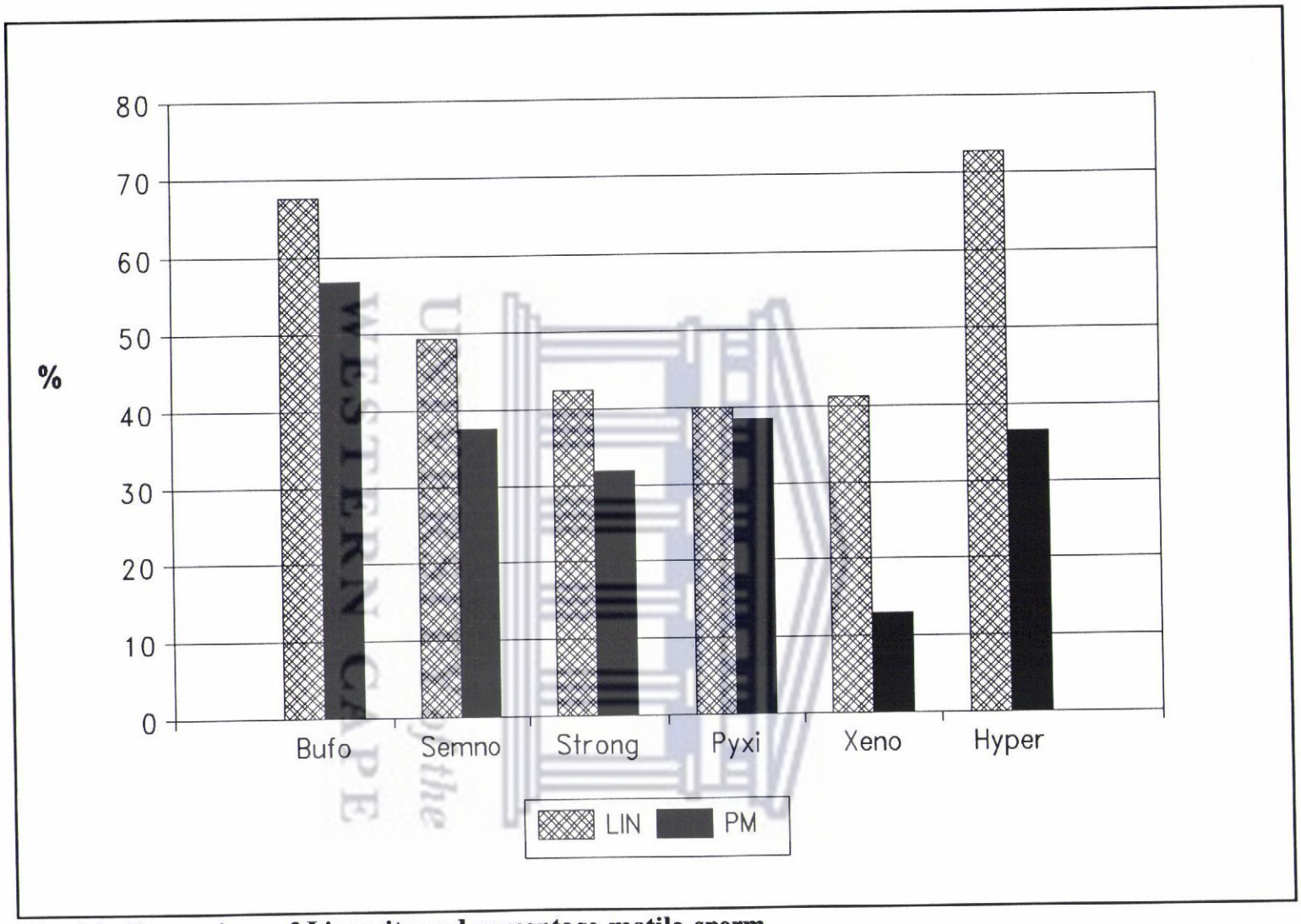


Fig. 4.4 Comparison of Linearity and percentage motile sperm

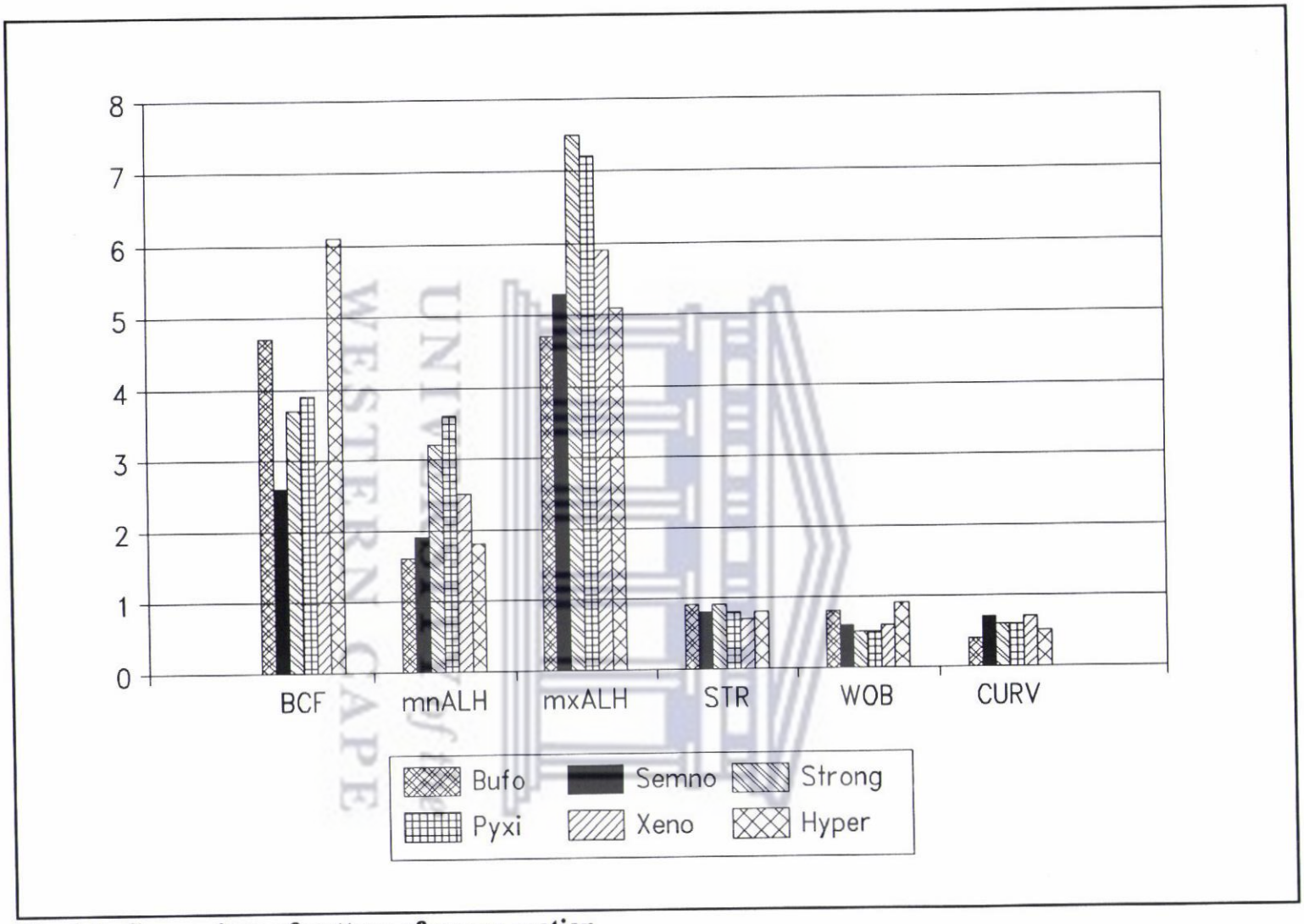


Fig. 4.5 Comparison of pattern of sperm motion

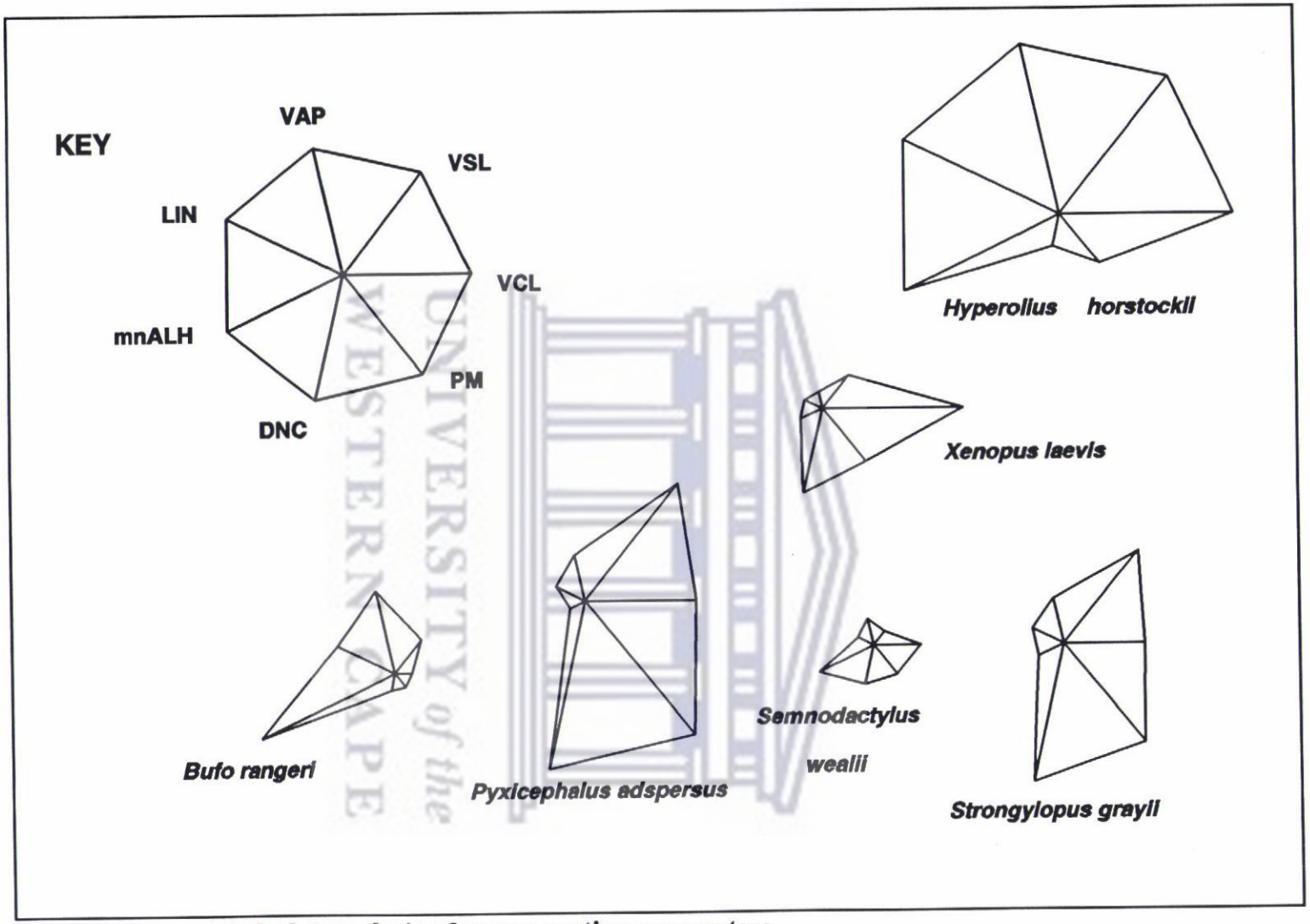


Fig. 4.6 Star symbol plot analysis of sperm motion parameters

4.5 Discussion

Spermatozoon motility has been an invaluable tool in assessing fertilizing capacity in domestic mammals and humans. Spermatozoa require considerable energy supplies from ATP to reach the eggs, but their fertilizing capacity is diminished before total loss of motility (Śliwa, 1983). Although sperm motility cannot be regarded as the only factor essential in fertilization (Raisman *et al.*, 1980), it presents more detailed information regarding the fertility status of an organism. Comparative spermatology is necessary to broaden our knowledge of biodiversity and sperm motion could be an invaluable tool in this regard.

Fertilization environments of anuran species range from totally aquatic to an internal environment. This could have changed their biology of reproduction. The swimming characteristics of the spermatozoa released in the various environments may therefore be adapted for effective fertilization. The anurans can be regarded as aquatic fertilizers (Passmore and Carruthers, 1979). Within this group of amphibians, however, a wide range of fertilization environments exists, from completely submerged to internal (Grandison and Ashe, 1983). The fertilization environments of the aquatic fertilizers studied, are so diverse (from completely submerged to the fringes of bodies of water - Chapter 2) that differences in the sperm motility patterns of the aquatic fertilizers are evident.

Bernadini *et al.* (1988a) in their study on the *Xenopus* spermatozoon found sperm motion duration to be less than 10 minutes. I found spermatozoa to be motile for up to 5 hours in the Ham's F10 (10%) solution (Table 4.3). The buffered

saline solution used by these researchers possibly only contained phosphates and NaCl, whereas Ham's F10 contains balanced salts and amino acids. It is conceivable that the micro-environment surrounding the egg capsules contain amino acids and salts necessary for sperm-egg interaction and Ham's F10 (10%) solution could therefore be the ideal nutritive medium in which to evaluate sperm motion. A low osmolality in their aquatic environment is necessary for the anuran spermatozoa to be effectively motile (Inoda and Morisawa, 1987) and the motility of anuran sperm in the low osmolality of Ham's F10 (10%) confirmed this.

The fact that no sperm motion could be elicited from the spermatozoa of the terrestrial fertilizers (*B. gibbosus* and *A. lightfooti*) in the Ham's F10 solution can possibly be ascribed to their special fertilization environments (in burrows). It is nonetheless necessary that more research be done on especially their micro-fertilization environment. It is peculiar that their spermatozoa remained immotile even though they were subjected to suspensions of varying osmolalities. The spermatozoa of these anurans are released on the eggs in the burrows and they do not need to swim through water towards the eggs. They only need to penetrate the jelly capsules, presumably via a rapid star-spin motion. It is conceivable that this hyperactivated motion could be completed within the first few seconds after ejaculation, so that the spermatozoa use their energy only to penetrate and fertilize the egg. This would be in line with the inference of Anderson and Personne, (1973) that fertilization is swift when the spermatozoa are deposited on or near the eggs. I suspended spermatozoa of terrestrial

fertilizers in various dilutions of Ham's F10 to determine the effect of osmolality on the sperm motion. It was evident that no sperm motion could be elicited from the terrestrial anurans. It should be stated that the same methods of measuring sperm motion was used for both aquatic and terrestrial fertilizers. In all the media and dilutions used, the spermatozoa remained immotile even after being microscopically observed within one minute after aspiration.

Mizuhira *et al.* (1986) described the pattern of sperm motion in the *Rhacophorus* species examined. They observed very active beating of the flagella, propelling the sperm forward in a counterclockwise motion. This forward motion is necessary to thrust the spermatozoa through the gelatinous mucus towards the eggs. The foam nest frog (*C. xerampelina*) lay their eggs in the same type of gelatinous nest. It is likely that the sperm of this rhacophorid could be propelled in the same manner as that of *Rhacophorus*. The pattern of motility observed in this study on *C. xerampelina* was a rapid star-spin motion and the uncoiling of the sperm head within the first 15 seconds after dilution in water. The motility of the spermatozoa was then lost. Uncoiling of the head coils, together with the star-spin activity could be forceful enough to thrust the sperm head through the egg capsules for fertilization.

The fact that the spermatozoa of the aquatic fertilizers exhibit motility for lengthy periods, could be an adaptation because they are released in a predominantly aqueous environment. These spermatozoa have to swim through the water (albeit a short distance) towards the eggs. The physiology of the

spermatozoa should therefore be adapted to sustain effective motility to enable the sperm to reach the eggs. Spermatozoa aspirated from amplectant *X. laevis* males exhibited patterns of hyperactivation (star-spin) which could enable them to penetrate the egg capsules (Chapter 5).

Aquatic fertilizers release their spermatozoa in water during amplexus. This is in contrast with the environment in which the spermatozoa of terrestrial fertilizers are released. Eggs of the anuran terrestrial fertilizers are surrounded by mucous and body fluids, such as egg water and fluids released by the male during ejaculation, whereas the eggs of aquatic fertilizers are surrounded in addition by water from the external environment. It should be borne in mind that fertilization does not take place in a completely dry environment, but that dampness (from whatever source) is necessary for sperm-egg interaction.

Comparisons of the vigour of sperm motility of the aquatic fertilizers revealed differences among the species examined (Table 4.5 and Fig. 4.3). The anurans with a fertilization environment completely submerged, probably have less extensive egg coverings than those on the fringes or outside the water (Passmore and Carruthers, 1979). Sperm motion of these anurans should be geared towards reaching the eggs in the streams. Spermatozoa should therefore be able to swim for long periods in order to reach the eggs. This was evident from the observations on the sperm of *Bufo*, *Xenopus* and *Semnodactylus*. The absence of significant differences among the curvilinear velocities of *B. rangeri*, *S. wealii* and *X. laevis* apparently reflects similarities in their fertilization environments.

Spermatozoa of these anurans possibly do not need to penetrate thick jelly coverings as is the case in the terrestrial fertilizers and the patterns of sperm motion support this assumption. Passmore and Carruthers (1979) described jelly coats of different anurans and it is clear that the terrestrial fertilizers have thicker coverings. *H. horstocki* spermatozoa exhibit the highest VSL which is an indication of a fairly linear swimming pattern. The high VAP of this species is also an indication of its adaptation to its fertilization environment - the spermatozoa being deposited in flowing water. The high linearity of *H. horstocki* and *B. rangeri* sperm indicated high forward progression of the spermatozoa and this would be necessary in the aquatic fertilization environments of these two species. No significant difference was evident between the LIN of *B. rangeri* and *H. horstocki* spermatozoa (Table 4.5 and Fig. 4.4).

The fertilization environments of *S. grayii* and *P. adspersus* are much alike and the similarities in sperm motion characteristics support this view. The star plot analysis (Fig. 4.6) revealed a similar pattern between the sperm motility parameters of these two species. There are, however, significant differences between the sperm motion characteristics of *B. rangeri* and *X. laevis*, which have more or less the same fertilization environments. Investigations into the micro-fertilization environment of the anurans might give an indication of the differences.

Sperm structure influences the pattern of motility (Bishop, 1962). The sperm of the anurans with cigar or spear-shaped sperm head structures display more or

less similar types of progression. *S. grayii* and *X. laevis* spermatozoa have similar helical head structures and as a result their patterns of motility are alike (Fig. 4.5), therefore sperm morphology might affect the aqua-dynamics of sperm motion.

It could be expected that anurans, because of their typical external mode of fertilization, would have a high percentage of motile spermatozoa. This was indeed the case in almost all the species studied. *X. laevis*, however, has an extremely low percentage motile sperm compared to the other anurans. Although the fertilization environment pressures will be for a high percentage motility, exceptions could be ascribed to somatic adaptations.

To have a more complete knowledge of biodiversity, research could be aimed at comparative studies. Comparative spermatology is one such discipline to be considered. The fertility status of species could be examined and assessment of sperm motility characteristics is necessary. Subjective criteria for assessing sperm motion should be phased out and objective analyses such as computerized videography should be utilised. Methods for quantifying progressive motility such as prescribed by the World Health Organisation (1987) cannot effectively evaluate sperm parameters. In this study I found the SMQ to be a useful method for objectively quantifying sperm motility.

It was clear from my investigations that significant differences exist among the sperm motility parameters of aquatic fertilizers. Because of their similar

"fertilization environments" one would expect many similarities in their sperm motion characteristics. The fertilization environments are very diverse and this may possibly explain the differences among the sperm motion characteristics of the aquatic anurans. Where species are closely related (*S. grayii* and *P. adspersus*) sperm motion characteristics exhibited similar patterns. This could strengthen the inference of Friend (1936) that adaptations of gametes be regarded as secondary to gross somatic adaptations. Sperm morphology, integrity of the cell membrane and cloacal secretions are some of the factors to be regarded in the total evaluation of fertilizing capacity of anuran spermatozoa. Research into the reproductive biology of anurans should therefore encompass several factors related to fertilization.

Sperm motion characteristics can, however, be used to assess viability of sperm to reach the eggs for fertilization. The absence of detected sperm motility in the terrestrial fertilizers was an indication that the gross fertilization environment does play a role in modulating form and function of the anuran sperm. Motility of sperm in the aquatic fertilizers is crucial for reaching the eggs, whereas in the terrestrial fertilizers the spermatozoa are released on the eggs. The star-spin motion of the sperm is necessary for penetration of the egg layers and research into hyperactivation of anuran sperm may elucidate anuran fertilization. The micro-fertilization environment could possibly play a more significant role in shaping sperm form and function than the gross fertilization environment. Research into the environment of sperm-egg interaction could yield results to further validate the hypothesis that sperm structure is determined by fertilization

environment.

The restraints of only using testicular spermatozoa of anurans are obvious. Amplexus stimulates vigourous sperm motion and patterns (Chapter 5). This indicated that ejaculate spermatozoa could be more viable with regard to penetration and fertilization of the eggs than testicular sperm. This necessitates more research into the comparative spermatology of the anuran ejaculate and sperm motility parameters could be used as a basis for comparing all possible factors influencing fertilization.

The evaluation of the anuran sperm movement patterns indicates that the spermatozoa are adapted to their respective fertilization environments. The spermatozoa of anurans with an aquatic fertilization environment are adapted for sustained motility for long periods of time to reach the eggs. In the vicinity of the eggs, the sperm probably exhibit hyperactivated motion to effectively penetrate the egg layers. On the other hand, the spermatozoa of terrestrial fertilizers are released on the eggs and need only to penetrate and fertilize the eggs. The fertilization will probably be swift (Anderson and Personne, 1973) and sustained motility would not be needed. Although the fertilization environment partly determines the structure and motion physiology of anuran spermatozoa, adaptations of the sperm as secondary genetic expressions of the soma (Friend, 1936) are of equal importance.

CHAPTER 5

CHAPTER 5

Xenopus laevis - COMPARISON OF MOTILITY PATTERNS OF SPERM ASPIRATED FROM AMPLECTANT PAIRS, BY ELECTRO-EJACULATION AND FROM THE TESTES

5.1 Introduction

Most studies regarding amphibian sperm physiology are concerned with testicular sperm. Anurans do not possess epididymides and previous researchers such as Poirier and Spink (1971) and Reed and Stanley (1972) have always assumed that sperm maturation is completed in the testes. Evaluation of the sperm motion characteristics indicated that the anuran spermatozoa are adapted for fertilization in their respective fertilization environments (Chapter 4). Studies pertaining to the fertilizing capacity and motility of anuran spermatozoa should be undertaken on the ejaculate sperm so as to effectively evaluate the motility parameters.

There are obvious restraints in using testicular spermatozoa for these studies. In most animal species the spermatozoa are immotile in the testes and in the anurans it was found that the spermatozoa become motile when released in suspensions with low osmolalities (Inoda and Morisawa, 1987). It is therefore necessary to investigate the motility characteristics of ejaculate spermatozoa of the anurans and to verify differences between testicular and ejaculate spermato-

zoa. This study was undertaken to determine the feasibility of obtaining sperm from amplectant pairs and from males using an electro-ejaculation technique. The swimming characteristics of testicular, ejaculate and sperm from amplectant pairs were then compared. It is clear from the literature that most studies on submammalian spermatozoa have been done on sperm aspirated from the testes. My studies indicated that motility studies can be done on anuran testicular sperm (Chapter 4). It is, however, necessary to ascertain the validity of a motility model based on testicular sperm and therefore this research on obtaining ejaculate sperm from *X. laevis* was undertaken.

Xenopus laevis is widely used as a laboratory model for anatomical and physiological studies and earlier researchers laid the basis for studies of the reproductive biology. Wolf and Hedrick (1971) used *X. laevis* to investigate the viability and *in vitro* fertilization of the gametes. Grey *et al.* (1974) enhanced our knowledge on fertilization in *Xenopus* by investigating the fertilization envelope. Wolf (1974) developed a method to recover cortical granules which prevent poly-spermic fertilization from *Xenopus* eggs. Spannhof *et al.* (1976) investigated osmotic and ionic regulation in *X. laevis*. A low osmolality is necessary for the initiation of sperm motility in *X. laevis* (Inoda and Morisawa, 1987).

Experimental research has been done on a variety of other frogs and toads. The structure and physiology of the egg coatings were extensively researched (Elinson 1971; Uchiyama *et al.*, 1971; Katagiri, 1973; Katagiri *et al.*, 1982 and Ishihara *et al.*, 1984). Miceli *et al.* (1987) pretreated coelomic oocytes with an extract from

oviducal pars recta and found the frequency of fertilization to increase. The lectin binding sites in *Bufo arenarum* oocytes were examined by del Pino and Cabada (1987). Díaz Fontdevila *et al.* (1988) explored the effect of different lipids on the fertilizing capacity of *B. arenarum* spermatozoa. The effect of egg water extracts on the fertilizing capacity of *B. arenarum* spermatozoa was examined by Díaz Fontdevila *et al.* (1991). They reported an inhibiting effect of the egg water on the fertilizing capacity of the spermatozoa depending on the osmolarity of the medium used.

The foundation laid by researchers on the reproductive biology of especially *Xenopus*, the availability of the species and the existing research protocol on *Xenopus*, prompted me to use *X. laevis* as the anuran species to research. *X. laevis* is regarded as one of the more primitive aquatic breeders (Passmore and Carruthers, 1979) and the data assembled from these toads can be used in research of more advanced anurans.

Anuran reproductive tracts undergo characteristic seasonal changes with regard to the size of the testes and reproductive accessories (Mann and Lutwak-Mann, 1963). In this study I attempted to simulate these changes via steroid injections with human chorionic gonadotrophin (hCG). *X. laevis* toads were injected and allowed to go into amplexus and the ejaculate sperm collected. An electro-ejaculation technique was also used to collect sperm from the cloacae of the males.

The pH and osmotic concentrations of the fertilization environment were determined. These values can be used as basis for further studies on the reproductive biology of the anurans.

The purpose of my study was therefore to develop techniques and present data pertaining to the motility characteristics of ejaculate *Xenopus* spermatozoa. The research will include the following:

1 Devise techniques to aspirate and collect spermatozoa from amplectant males. The motility characteristics of the spermatozoa will then be evaluated.

2 Devise an electro-ejaculation technique to aspirate spermatozoa from males. Evaluate the sperm motion characteristics.

3 Aspirate testicular spermatozoa from males.

4 The swimming characteristics and percentage motility of the spermatozoa obtained via the various techniques will then be compared.

5 Determine the osmolality and pH of the medium in which the spermatozoa are ejaculated. Osmolalities of the cloacal water and sperm suspensions will also be determined.

5.2 Materials

5.2.1 Experimental animals

Xenopus laevis toads were obtained from a breeding farm in Cape Town. Twelve females and fifteen males were used in the course of this study. They were kept in a steel tank at temperatures ranging from 18° to 25°C. The water in the tank was changed every second day.

5.2.2 Steroid injections

The hormone Pregnyl (hCG) was used to induce reproductive changes. A disposable syringe with a bent needle, was used to administer the Pregnyl.

5.2.3 Electro-ejaculation

A Harvard stimulator was used to stimulate the testes so as to induce sperm release.

5.2.4 Evaluation of motility parameters

The image analysis program SMQ (Chapter 4) was used to quantify sperm motility characteristics. Statistical comparisons were done using the t-sample test analysis. Where ANOVA tests indicated significant differences of variance, nonparametric (Mann-Whitney) analyses were done.

5.2.5 Osmolality and pH measurements

Osmolality measurements were made on a Wescor 5500 Vapor Pressure Osmometer using Wescor sample discs type SS-033 and only required a sample

of 7 to 8 μl.

The pH readings were done on a Beckman Zeromatic IV pH meter and on a pH M80 portable pH meter No. 64R53N46.

5.3 Methods

5.3.1 Aspiration of sperm from amplectant pairs

Male and female toads were kept apart in separate fish tanks with the water at a constant 23°C for 24 hours prior to the injections. The technique described by Brown (1970) was used. Three pairs of toads were used each time for sperm aspiration. The female toads were given a primer injection of 250 i.u. of Pregnyl. The males were given a primer of 150 i.u. Males and females were kept apart at a constant temperature and after 48 hours the booster injections were administered. Females were given a booster of 300 i.u. and males 200 i.u. The injections were administered subcutaneously in the dorsal lymph sacs. The toads were then randomly paired off and placed in the two fish tanks.

Different dosages of pregnyl were also compared. Higher dosages in females induced them to deposit eggs within an hour after the primer injections. It was found that with dosages as high as 300 i.u. (booster injection) in males, amplexus was induced, but throughout amplexus no sperm was ejaculated.

5.3.1.1 Collection of sperm from amplectant males in the tanks

Males started their mating calls from the water approximately one hour after the

primer injections. The nuptial pads on the hands of the males could be discerned after approximately five hours after the primer. Within an hour after the booster injections the toads went into amplexus. *Xenopus* has the primitive inguinal clasp and the toads could stay in amplexus for up to 30 hours.

During the time of amplexus the toads were closely observed for changes in their movements. The females exhibit a jerky swimming movement when they are ready to deposit their eggs. The male bends his back so as to bring his cloaca nearer to that of the female. The distance from the cloaca of the male to that of the female normally ranges from 19,6 to 27,4 mm. When the female goes into her egg-laying swimming routine, the male brings his cloaca to within 5 mm of that of the female. The eggs were released by the female, passed along the abdomen of the male and induced the male to ejaculate. This ejaculate was collected with a Gilson pipetman pipette.

I attached Eppendorf tubes to the cloacal region of the males after the booster injections were administered. This was done to collect ejaculate after the toads went into amplexus. This method caused stress to the males and some of them did not even go into amplexus. As a result this method was discontinued.

5.3.1.2 Collection of sperm from amplectant males in chamber or bag

I devised a chamber, called the Lewis chamber, to house an amplectant pair of toads. This chamber (Fig. 5.1) was designed so that the sperm and eggs could run down and be collected at the bottom. This was done to collect pure

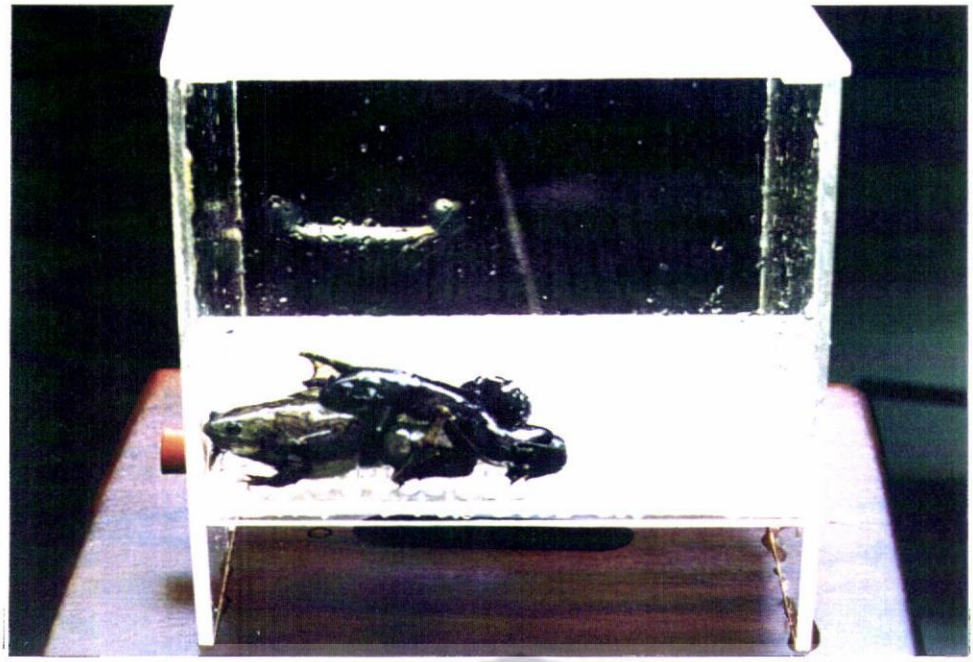


Fig. 5.1 Amplectant toads in Lewis chamber

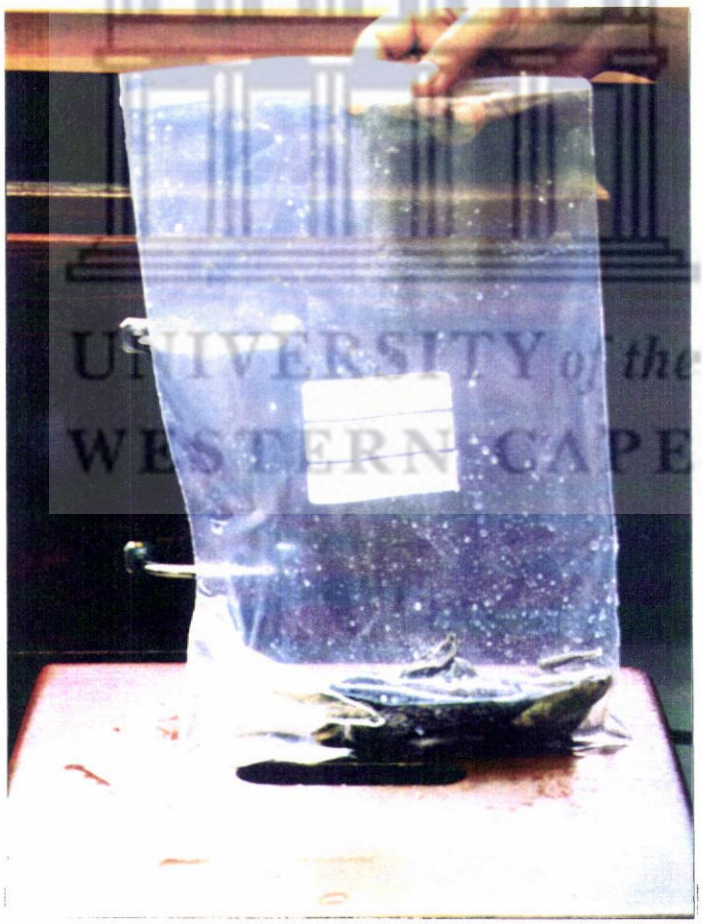


Fig. 5.2 Amplectant toads in plastic bag

ejaculate without the water as collected in the fish tank. The Lewis chamber yielded poor results as the holes in the casing caused the chamber to have the same effects as a desiccator, dehydrating the toads.

Another method used to obtain pure ejaculate, was to put the amplectant pairs in plastic bags (Fig. 5.2). This method yielded better results than the Lewis chamber. The ejaculate obtained by this method, however, contained mucous from the toads.

5.3.2 Electro-ejaculation

Male toads were injected with 250 i.u. Pregnyl 12 to 24 hours before sperm collection. They were then anaesthetized with MS222 and the abdominal cavity exposed. A stimulating electrode was connected to a Harvard stimulator using multiple pulses (50 Hz at 6 to 8 Volts) (Fig. 5.3). The testes and Wolffian ducts were stimulated. The best results were obtained when the testes were stimulated. Spermatozoa were subsequently collected with a Gilson pipetman pipette from the cloaca. All the spermatozoa sampled, were suspended in a 10% Ham's F10 solution and microscopically observed in a sperm chamber. Motility studies were performed using the protocol described in Chapter 4.

After spermatozoa were collected by electro-ejaculation, the testes were removed and spermatozoa aspirated from them by squashing them between microslides. The motility of the testicular sperm was evaluated by means of the SMQ system (Chapter 4) to assess the effect of pregnyl on the motility of the testicular sperm.

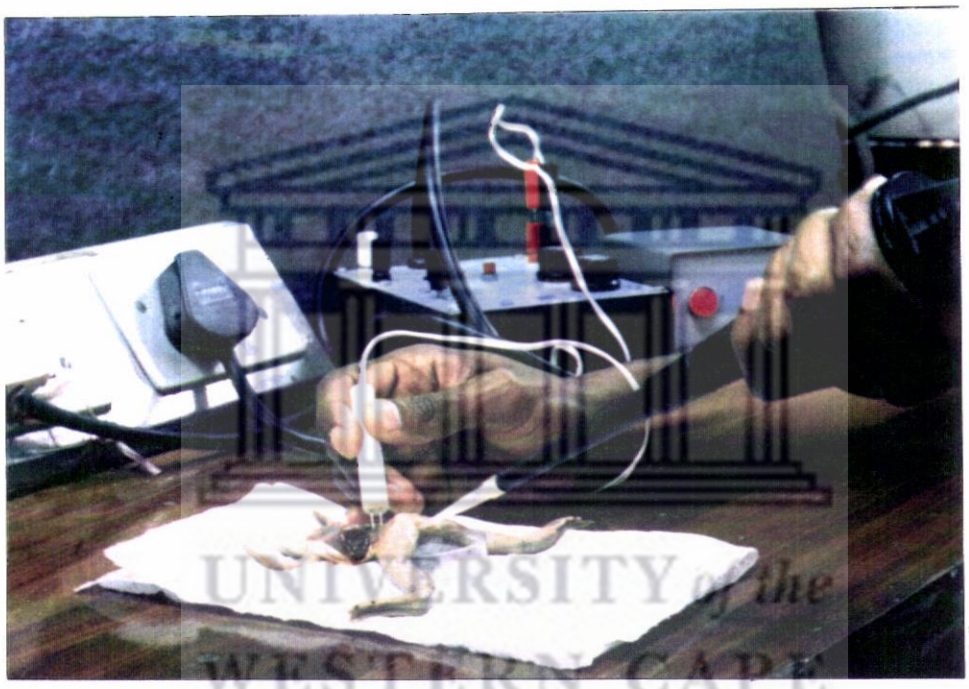


Fig. 5.3 Aspiration of sperm from male using an electro-ejaculation technique. A stimulating electrode was connected to a Harvard stimulator and the testes stimulated.

5.3.3 Osmolality and pH

Osmolality and pH measurements were done on the following suspensions:

Eggs and mucous in the plastic bag

Fluid extracted from the cloaca of the female

Eggs and tank water

Tank water

Eggs and fluid in the Lewis chamber

5.4 Results

The motility characteristics of the spermatozoa aspirated by the methods described in 5.3 were analyzed and statistically compared. For the purposes of this study the following parameters were statistically compared: VCL; VSL; LIN; VAP; DNC; and PM (Abbreviations as described in Chapter 4).

Table 5.1 Data on the motility parameters of sperm aspirated via different methods

	A	T	E	P
VCL (μm/s)	33,2 ± 12,6	21,7 ± 7,7	24,6 ± 10,2	24,8 ± 6,6
VSL (μm/s)	18,3 ± 13,2	8,6 ± 3,5	15,7 ± 9,9	17,9 ± 6,7
LIN (%)	49,7 ± 21,3	41,3 ± 14,3	60,6 ± 19,4	70,7 ± 16,5
VAP (μm/s)	20,8 ± 12,7	12,2 ± 3,7	18,0 ± 9,8	19,8 ± 6,1

	A	T	E	P
DNC ($\mu m^2/s$)	61,8 ± 40,8	62,4 ± 63,5	30,0 ± 23,4	28,9 ± 16,7
PM (%)	27,5 ± 2,3	12,9 ± 4,6	48,3 ± 3,4	15,1 ± 2,8

Format: $\bar{x} \pm SD$

A: Sperm obtained from amplectant males

T: Testicular sperm (toads not subjected to Pregnyl injections)

E: Sperm obtained via electro-ejaculation technique

P: Sperm obtained from testes after Pregnyl injections and electro-ejaculation

5.4.1 Comparison of the motility parameters evaluated

VCL: A vs T; A vs E; A vs P; T vs P; significant difference ($p < 0,01$)

VSL: A vs T; T vs E; T vs P; significant difference ($p < 0,01$)

LIN: A vs E; A vs P; T vs E; T vs P; E vs P; significant difference ($p < 0,01$)

VAP: A vs T; T vs E; T vs P; significant difference ($p < 0,01$)

DNC: A vs E; A vs P; T vs E; T vs P; significant difference ($p < 0,01$)

PM : A vs T; A vs E; A vs P; T vs E; E vs P; significant difference ($p < 0,01$)

5.4.2 Osmolality and pH

The osmolalities of the different solutions and pH values are recorded in Table 5.2 below.

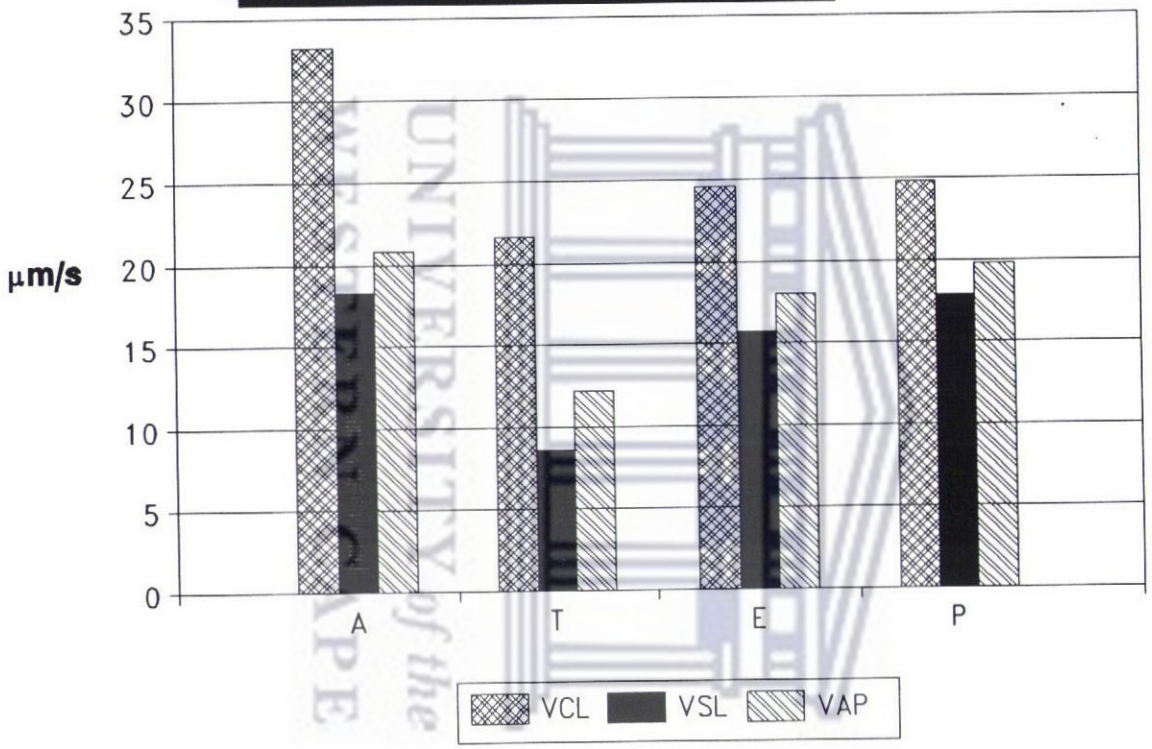
Table 5.2 Data on the osmolalities and pH values of the suspensions

SUSPENSION	OSMOLALITY (mOsm)	pH
Tank water	40 (±0,63) 39 - 41	7,85 (±0,13) 7,62 - 7,96
Eggs and tank water	44,58 (±5,87) 41 - 63	7,15 (±0,06) 7,07 - 7,23
Fluid in plastic bag	112,3 (±6,37) 99 - 126	7,94 (±0,19) 7,72 - 8,21
Fluid in Lewis chamber	72,17 (±0,75) 71 - 73	7,10 (±0,08) 7,01 - 7,22
Female cloacal fluid	49,33 (±7,16) 38 - 56	7,94 (±0,07) 7,82 - 7,99

Format: $\bar{x}$ (±SD) Range

The graphs detailed in Figs. 5.4 through 5.6 illustrate the differences and similarities of the motility parameters of the spermatozoa collected by the different means. The star symbol plot depicted in Fig. 5.7 further elucidate these difference and similarities between the sperm motion characteristics.

Fig. 5.4: Comparison of velocities of the sperm aspirated via diffent methods



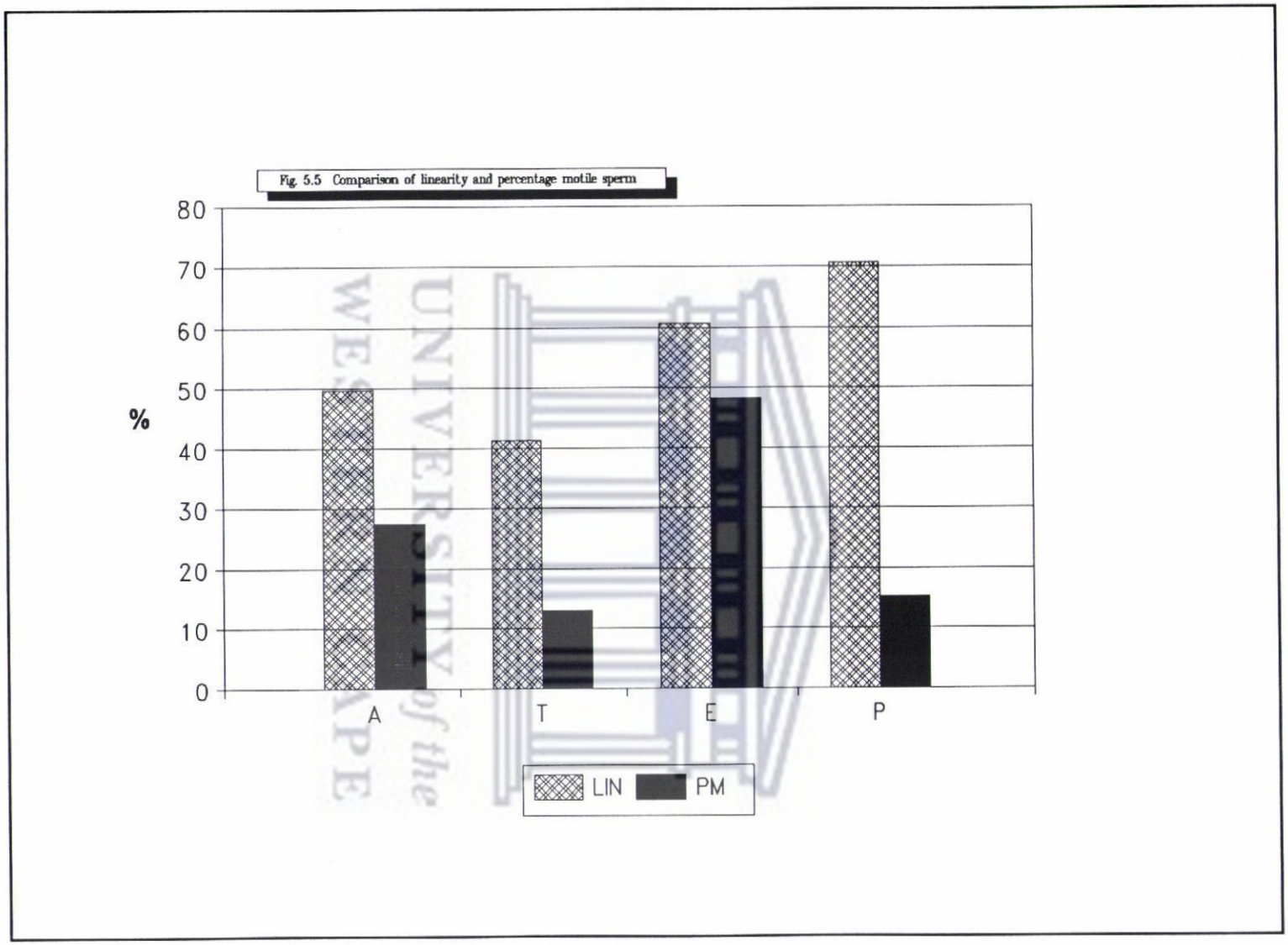
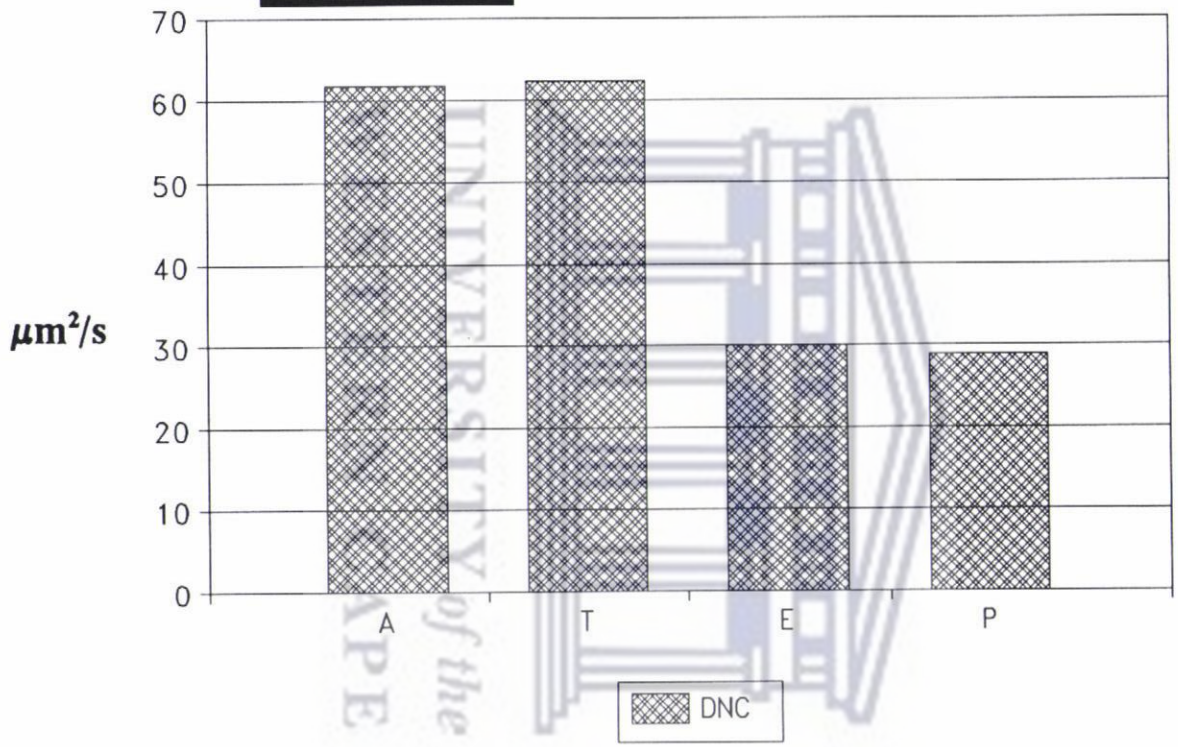


Fig. 5.6: Comparison of dance



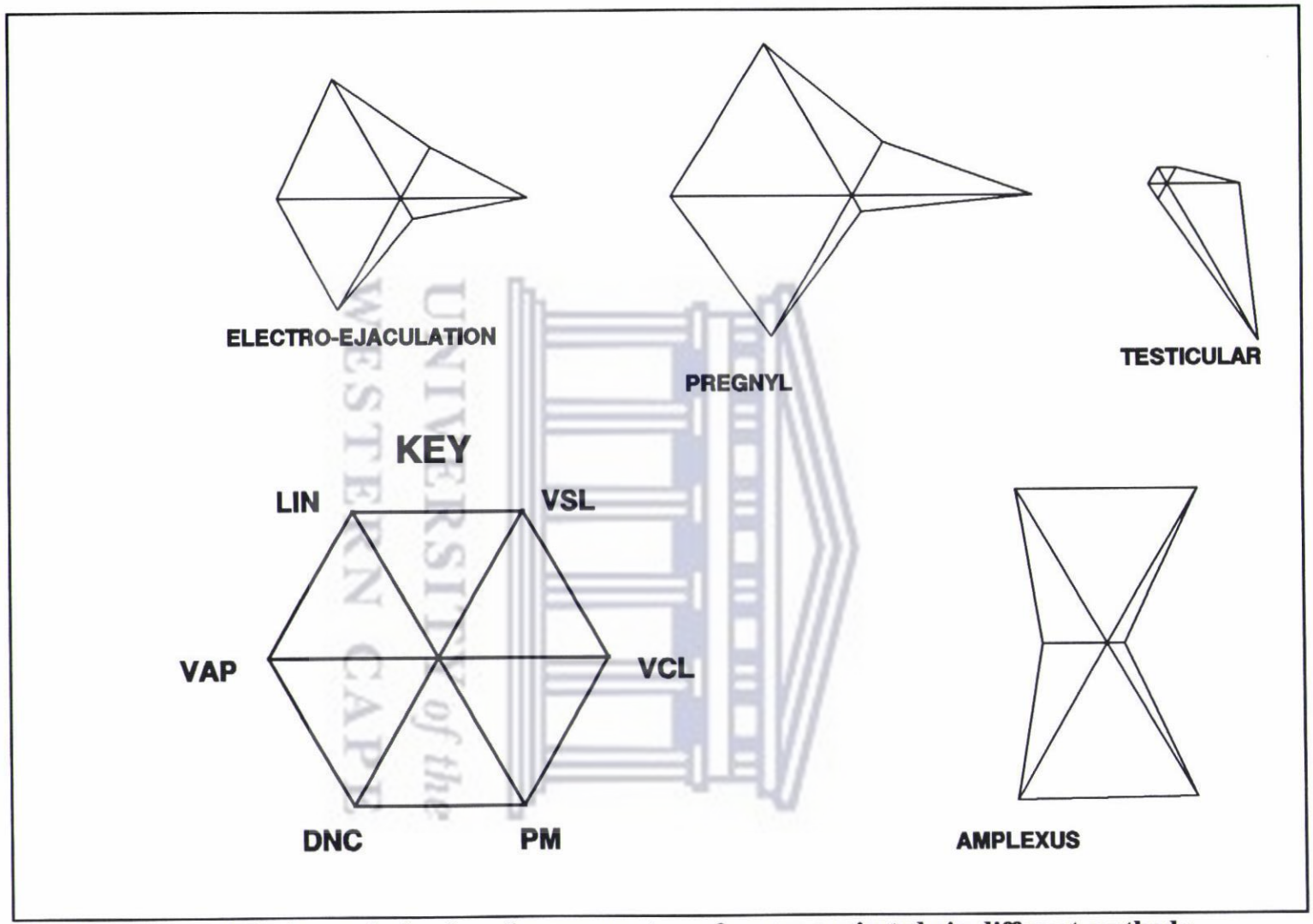


Fig. 5.7 Star symbol plot analysis of motion parameters of sperm aspirated via different methods

5.4.3 Summary of comparison of motility parameters

Fig. 5.4 indicates the high velocities of sperm from the amplectant males. It is clear from the graph that these values differ significantly from values obtained from testicular sperm. The velocities of the sperm aspirated via electro-ejaculation and sperm from the testes after electro-ejaculation, are more or less similar. The LIN of sperm from the testes after steroid injections and electro-ejaculation is the highest (Fig. 5.5) and this could possibly be as a result of the steroid injections administered to the males before electro-ejaculation. The testicular sperm (no pregnyl) and sperm from amplectant males exhibited the highest DNC values (Fig. 5.6).

The star symbol plots (Fig. 5.7) indicate similar patterns of the electro-ejaculate and testicular sperm after steroid (pregnyl) injections. The star symbol plot reflecting the values for sperm from amplectant males displays the high values of these spermatozoa, whilst the plot for testicular sperm reflects their low overall values.

5.5 Discussion

It is problematic to obtain an ejaculate from submammalian vertebrates and invertebrates. Anuran spermatozoa are thought to mature in the testes (Poirier and Spink, 1971 and Reed and Stanley, 1972) because of the lack of epididymides. To date reproductive biology of the frogs and toads primarily involved investigations on testicular sperm. This study was undertaken to determine whether viable sperm can be obtained by using different techniques to aspirate

sperm via an ejaculate. Sperm motility parameters were used as criteria to ascertain the viability of ejaculate sperm.

Ham's F10 as medium to assess sperm motility was found to be ideal (Chapter 4). Bernadini *et al.* (1988a) found the duration of *Xenopus* sperm motility to be less than 10 minutes. I still found motile sperm in the 10% Ham's F10 suspension after 5 hours of swim out (Chapter 4).

Xenopus has a very low percentage of motile testicular sperm. This is in line with the findings of Van der Horst (1976). The percentage motile sperm aspirated from the testes after pregnyl injections and electro-ejaculation, showed no significant difference with "normally aspirated" testicular sperm. The higher percentages of motile sperm in the electro-ejaculate and ejaculate from the amplectant males (Table 5.1 and Fig. 5.5) differed significantly from testicular sperm. These differences may indicate that maturation changes take place in the reproductive ducts during ejaculation. In the testes, the spermatozoa are neatly packaged and embedded in Sertoli cells (Chapter 2). Maturation is not yet completed and this may possibly be one of the reasons for the low percentage of motile testicular spermatozoa. Another factor may be the low percentage of normal spermatozoa in the testis of the toad (Chapter 3). The abnormally shaped spermatozoa are not as aqua-dynamic as cigar-shaped sperm and move in a circular, rather than a linearly progressive manner. This may also be an indication that maturation takes place immediately before, or during ejaculation and that the abnormal spermatozoa be regarded as immature spermatozoa.

More significant, however, were the differences in the motility parameters. Analysis of these parameters indicated that the spermatozoa obtained from the amplectant pairs had significantly higher velocities (VCL, VSL and VAP) than sperm obtained from the testes. It appears that the stimulation of amplexus influences the secretion of substances in the Wolffian duct necessary for effective propulsion towards the eggs and subsequent fertilization. Bernadini *et al.* (1988b) concluded that the respiration rate of *Xenopus* spermatozoa is not affected by their status of motility. They used testicular spermatozoa and a comparative study of spermatozoa obtained from males in amplexus would be extremely valuable.

It is clear from Table 5.1 and Fig. 5.4 that the spermatozoa obtained from the amplectant males differed significantly from those obtained from the testes for most of the motion parameters. A high value of DNC may be an indication of the viability of the sperm to fertilize. Penetration of the jelly layers surrounding the eggs involves chemical and mechanical means. Spermatozoa normally exhibit a star-spin movement (lashing about of the sperm heads) in the vicinity of the eggs (Yanagimachi, 1988). This is a mechanical movement which helps with the penetration of the egg capsules. A high DNC value could be indicative of this hyperactivation. Normally aspirated testicular spermatozoa and spermatozoa from amplectant males exhibited the highest DNC values (Table 5.1 and Fig. 5.6). The high DNC value of the spermatozoa from the amplectant males could be explained in terms of the mechanisms necessary to penetrate the egg capsules. Testicular spermatozoa are still immature and the DNC value could be high

because of the low percentage motile spermatozoa where the sperm heads lash about instead of moving progressively forward.

Spermatozoa obtained via electro-ejaculation and from the testes of the electro-ejaculated toads displayed significantly higher LIN than those obtained via other means. The stimulation of the testes may be instrumental in the sperm having a high LIN. It did not seem that the pregnyl injections played a role in the spermatozoon linearity as the amplectant males were given pregnyl injections as well. The linearity of testicular spermatozoa and spermatozoa aspirated from amplectant males differed significantly from those of the electro-ejaculated sperm and sperm from testes after pregnyl injections and electro-ejaculation.

The bar graphs (Figs. 4.5 through 4.6) depict these differences and similarities of the motion parameters of the spermatozoa obtained via different techniques. It was clear that the motility parameters of the electro-ejaculate spermatozoa and spermatozoa from testes after pregnyl injections, exhibited more similarities. Their pattern of motion also showed similarities. This is portrayed in the star symbol plot (Fig. 5.7). This could be ascribed to the fact that the spermatozoa analyzed were obtained from the same specimens. The small star symbol representing motility parameters of testicular sperm is an indication that these parameters were almost consistently lower than those of the others.

Inoda and Morisawa (1987) in comparing freshwater fishes and anurans to reflect the environment in which reproduction takes place, found that hypo-osmolality

stimulated sperm motility in *Xenopus*. Bernadini *et al.* (1988a) found anuran sperm motility to be inhibited at osmolarities higher than 200 mOsm/l. The results as reflected in Table 5.2 indicate the low osmolality of the aqueous environment in which the spermatozoa are released. Suspensions with a low osmolality, such as Ham's F10 (10%) and Toad Zwartkops solutions (Chapter 4) were found to be ideal solutions in which to initiate sperm motility.

A pH ranging from 7,1 to 7,9 is crucial for fertilization in anurans (Miceli *et al.*, 1987; Bernadini *et al.*, 1988a and Díaz Fontdevila *et al.*, 1991). I found the pH values of the different media in which the spermatozoa were released, to fall within this range.

The results of my study indicated that motile sperm can be obtained from testes, electro-ejaculate and from amplectant males. Differences in the various sperm motility parameters may reflect physiological differences between sperm from the testes and the ejaculate. It may be an indication that the Wolffian ducts and cloacal secretions play a part in the final maturation phases of the spermatozoa. The higher velocities of the sperm from amplectant males suggested that amplexus is a major stimulus in bringing the spermatozoa to maximum fertilizing capacity. Further analyses regarding the physiology of ejaculate sperm in the anurans are required in order to ascertain the functions of the reproductive tracts and cloacal secretions in the maturation of spermatozoa.

CHAPTER 6

CHAPTER 6

GENERAL DISCUSSION AND SUMMARY

Sex and the propagation of species have always fascinated man. Research on reproductive biology is geared towards contraception and fertility studies. This has inevitably led to extensive research on the higher vertebrates with a resulting shift in emphasis away from the lower vertebrates and invertebrates. It was attempted with this study to establish techniques and present data on the lower vertebrates with particular reference to the frogs and toads. Reproductive studies on the lower vertebrates can possibly give us insight into the evolutionary development of sexual reproduction.

Anuran reproductive biology can be used as a tool to explain fertilization patterns in the higher vertebrates. The anurans exhibit fertilization environments ranging from fully aquatic to terrestrial (Chapter 2). Although being regarded as external fertilizers (Passmore and Carruthers, 1979) anurans display modes of fertilization ranging from external to internal (Grandison and Ashe, 1983). This wide variety of environments and modes of fertilization make research into the reproductive biology of the anurans an ideal tool for testing the hypothesis that fertilization environment plays an important role in shaping sperm structure and physiology.

Franzén (1970) contended that sperm morphology in the Metazoa is related to the mode of fertilization. In the external fertilizers the spermatozoa should be adapted to swimming some distance to the eggs, whereas in internal fertilizers the distances to the eggs are insignificant. Most aquatic spermatozoa are regarded as having a primitive morphology (short, roundish head with a long flagellum and a short midpiece). There are, however, suggestions that the sperm of many aquatic fertilizers are secondarily simplified (Jamieson *et al.*, 1993). The more advanced sperm morphology of the internal fertilizers involves longer head- and midpieces with long flagellae (Franzén, 1970). From his work it is clear that the fertilization environment is correlated with sperm morphology. Other researchers who regarded the fertilization environment as a factor in shaping sperm morphology were Lee and Jamieson (1992) in their work on myobatrachid frogs. Jamieson *et al.* (1993) on the sperm of *Ascaphus truei* suggested functional correlation between an internal fertilization environment and the ultrastructure of gametes as the spermatozoa of this internally fertilizing anuran show changes compared to spermatozoa of aquatic fertilizers. They also suggested that the external mode of fertilization in the anurans be regarded as a secondary reversion compared to primitive internal fertilization in lissamphibians. Mainoya (1981) on the spermatozoa of *Chiromantis xerampelina* and Mizuhira *et al.* (1986) on the spermatozoa of *Rhacophorus*, inferred fertilization environment as a factor influencing sperm structure. The large elongated mitochondria of *Rhacophorus* and *Ascaphus* are derived characteristics and arose independently in these two anurans, possibly as a result of their unique fertilization environments (Jamieson *et al.*, 1993).

My studies indicated a general tendency for the sperm head structure to become more elongated and attenuated in anuran species utilising a terrestrial fertilization environment. An elongation of the acrosomal complex of the terrestrial anuran spermatozoa was also evident. Aquatic anuran spermatozoa exhibit shorter, stouter heads. The internal fertilizer *Typhlonectes natans* has an elongated tapering sperm head (Van der Horst *et al.*, 1991) and this strengthens the hypothesis that an elongated sperm head structure should be regarded as a secondary feature. Morrisett (1974) and Fouquette and Delahoussaye (1977) did not regard adaptations to fertilization environments as structuring sperm morphology. In explaining the sperm morphology of three families of anurans, Morrisett (1974) used phylogeny and Fouquette and Delahoussaye (1977) regarded sperm structure as a phylogenetic trait in their work on the *Hyla rubra* group. Visser and van der Horst (1987) researched *Heleophryne* sperm structure and argued that the double tailed flagellum be regarded as a basic feature of anuran sperm. This concurred with the findings of Fouquette and Delahoussaye (1977) that a two filament tail be regarded as primitive.

Sperm tail structure can be explained on the basis of phylogeny, as there was no clear distinction between the flagellar complexes of aquatic and terrestrial anuran fertilizers. It was clear that species from both the aquatic and terrestrial fertilizers exhibit double tail complexes. A double tailed sperm structure should be regarded as a primitive trait (Fouquette and Delahoussaye, 1977 and Visser and van der Horst, 1987) compared to the single tailed structure exhibited by mammalian sperm.

Franzén (1970) regarded the transformation of the midpiece and the lengthening of the sperm head as the primary steps in the modification of spermatozoa. External fertilization involves swimming of the spermatozoa to the eggs and this necessitates adaptations with regard to the mitochondria for energy requirements. The studies on motility of different anuran species (Chapter 4) indicated differences between aquatic and terrestrial fertilizers. Spermatozoa of terrestrial fertilizers could not be induced to swim in the same media as used for the aquatic fertilizers, even when a variety of concentrations of the media were used. Spermatozoa of aquatic fertilizers need to swim through water to reach the eggs, whereas spermatozoa are deposited in fluids on or very close to the eggs in the terrestrial fertilizers. Sperm of the terrestrial fertilizers possibly only need to penetrate the jelly layers of the eggs and the hyperactivated star-spin motion could be enough to propel the sperm through these layers.

The aquatic fertilizers exhibit fertilization environments ranging from deep water to the fringes of bodies of water (Chapter 2). In comparing sperm motion, I found that those anurans with similar environments presented similar patterns and vigour of motility. These studies indicated that fertilization environment may play an important role in determining the motility status of the spermatozoa.

Reproductive biology of the anurans chiefly involves research on testicular spermatozoa. Researchers assumed maturation of anuran spermatozoa to occur in the testes (Poirier and Spink, 1971 and Reed and Stanley, 1972). The study regarding the aspiration of sperm by various means (Chapter 5) indicated that

motility patterns of sperm aspirated via electro-ejaculation and from amplectant males differed significantly from those obtained from the testes. This is an indication that maturation changes occur in the ducts leading to the cloaca. It was also evident that the sperm obtained from the amplectant males exhibited more vigorous patterns of motility than sperm otherwise obtained. It is possible that stimulation of amplexus can further stimulate secretions in the Wolffian ducts to enhance the vigour of motility so as to penetrate the egg capsules. The results of this study indicated that methods be devised to analyze ejaculate spermatozoa and compare their fertilizing capacity with that of testicular sperm.

The intermediate position of the anurans is further elucidated by the studies on the sperm densities and gonadosomatic indices of the species (Chapter 3). The anurans exhibited sperm densities midway between the typical external fertilizers such as teleosts and internal fertilizers such as mammals. The high gonadosomatic indices displayed by *Chiromantis xerampelina* can be regarded as an adaptation to compensate for their relatively low sperm density (Chapter 3). Further investigations regarding spermiograms of anurans are required to enhance our knowledge of the physiology of anuran spermatozoa.

Studies of the reproductive biology of lower vertebrates can only broaden the scope of knowledge to be put to use in studies on mammals. It is suggested that selection pressures in the variety of fertilization environments of the anurans may have favoured changes of shape and motility of the spermatozoa for effective penetration of the egg capsules. Evolution of the gametes was regarded by

Friend (1936) as a by-product of somatic evolution. Franzén (1970), however, argued that the changes in the sperm morphology are necessary for effective fertilization in the variety of fertilization environments utilised by the anurans. A distinction can furthermore be made between "primitive" sperm for aqueous fertilization environment and "modified" sperm for an internal fertilization environment (Anderson and Personne, 1973). Phylogeny plays an important role in shaping the gross morphology of a species and Fouquette and Delahoussaye (1977) inferred phylogeny as shaping sperm structure. It was suggested that because of the distinctive morphology of sperm, that sperm head shape is genetically controlled (Fawcett *et al.*, 1971).

My studies on the spermatozoa of the anurans indicated that a crucial factor in determining sperm morphology and physiology might be the selection pressure of the fertilization environment. The role of phylogeny, however, is as important in determining morphology of spermatozoon structure. Although my studies indicated that fertilization environment influences the form and function of the anuran spermatozoa, the influence of somatic adaptation on shaping the sperm morphology could not be ruled out. The influence of fertilization environment in shaping sperm morphology, as indicated in my studies, contradicts the inference of Friend (1936) that gamete evolution is secondary to somatic evolution. Further research into the spermatology of the anurans and techniques to effectively evaluate parameters relating to the ejaculate spermatozoa, is necessary to establish baseline values for the anurans. The joint importance of phylogeny and fertilization environment in determining sperm form and function,

should be part of further research into anuran spermatology.

The results of this study indicated that fertilization environment plays an important role in shaping sperm structure and that significant differences exist between aquatic and terrestrial fertilizers (Chapter 2). Baseline values regarding spermiograms for selected anurans were obtained (Chapter 3) and differences were evident among all the anurans studied which may be an indication of species specificity. Studies regarding the motility of selected anuran spermatozoa reflected differences between aquatic and terrestrial fertilizers (Chapter 4). It was clear from the studies on ejaculate sperm of *X. laevis* (Chapter 5) that research of the secretions of the cloacae and reproductive tracts are needed to evaluate the sperm motion of ejaculated spermatozoa. The baseline values obtained during this project could possibly be used in programmes to protect endangered anurans. These values could for example be applied in studies pertaining to the cryopreservation of sperm of endangered anurans.

Summary and conclusions

1 Amphibians are regarded as the bridge between true external fertilizers such as teleosts and true internal fertilizers such as mammals. Development may be direct or include larval stages.

2 The Anura can be subdivided into those with an aquatic fertilization environment and those with a terrestrial environment (from within

streams to burrows on land). Their mode of fertilization ranges from external to internal.

3 The aquatic anuran fertilizers exhibit a wide range of environments -- from completely submerged (*Xenopus laevis*) to the fringes of bodies of water (*Strongylopus grayii*). Terrestrial anuran fertilizers occupy environments ranging from burrows (*Breviceps gibbosus*) where a only a thin film of water is necessary for fertilization, to foam nests in tree branches overhanging a stream (*Chiromantis xerampelina*).

4 Spermatozoa are deposited on or very near to the eggs during amplexus. Modes of amplexus vary from an inguinal clasp (*X. laevis*), axillary clasp (*Bufo rangeri*) and adhesion (*B. gibbosus*). *C. xerampelina* females secrete a mucous which is formed into a stiff ball of foam by activity of amplectant pairs and unpaired males. Spermatozoa are then deposited in this foam.

5 Spermatozoa of typical aquatic fertilizers such as teleosts (aquasperm) have a simple sperm head and midpiece structure, whereas mammalian spermatozoa (introsperm) exhibit advanced characteristics. Anuran spermatozoa exhibit advanced features such as elongated sperm heads and modified midpieces with mostly primitive sperm tail features.

6 Anurans with an aquatic mode of fertilization feature shorter, stouter

sperm heads. Those with a terrestrial mode have more elongated and attenuated sperm head structures. *Chiromantis xerampelina* exhibits a unique corkscrew sperm head structure with a single tail flagellum made up of two 9 + 2 microtubular arrangements surrounded by many additional microtubules.

7 The sperm head is adapted to the particular fertilization environment. Sperm of terrestrial fertilizers need to penetrate extensive jelly capsules, whereas the eggs of aquatic anurans have less extensive coverings.

8 Anurans exhibit a basic (plesiomorphic) sperm tail structure relative to true internal fertilizers such as mammals.

9 Motility patterns of sperm from aquatic anurans differed markedly from those of terrestrial breeders. Spermatozoa of terrestrial anurans could not be induced to swim in the same media as those of aquatic anurans or in media with osmotic concentrations of up to 300 mOsm/kg. *Chiromantis xerampelina* exhibited a star-spin motion and an uncoiling of the sperm head for a few seconds before becoming immotile. Spermatozoa of aquatic fertilizers could swim in a 10% Ham's F10 solution and Toad Zwartskops solutions for extensive periods.

10 The difference in motility patterns indicated adaptations to the varying fertilization environments. Spermatozoa of aquatic anurans need to swim

through water to reach the eggs whereas the distance covered by spermatozoa of terrestrial species is negligible. Spermatozoa of terrestrial anurans probably only need to penetrate the egg capsules and the hyper-activated motion of star-spin is necessary for this process.

11 The spermiograms of the anurans are indications of their intermediate position. The anuran sperm densities fall midway between those of external fertilizers such as teleosts and internal fertilizers such as mammals.

12 Fertilization environment is an important factor in shaping sperm morphology and physiology. Studies pertaining to the biology of reproduction in the anurans could give a better insight into the systematics of the amphibians. These studies will also enhance our knowledge on biodiversity.

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* **Not seen in the original.**