THE CRYOPRESERVATION POTENTIAL AND ULTRASTRUCTURE OF AGULHAS SOLE *AUSTROGLOSSUS PECTORALIS* SPERMATOZOA

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requirements for the degree of

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by

Michael Zeljan Markovina

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Abstract

As the estimated market demand for the Agulhas sole *Austroglossus pectoralis* exceeds the annual catch from trawlers, this species is a potential aquaculture candidate. Broodstock conditioning and gamete preservation is part of research and development aiming at establishing a breeding protocol for a new aquaculture species. Based on a literature review of the morphology of pleuronectiform spermatozoa, this study was designed firstly, to contribute to the field of spermatozoan morphology by describing the ultrastructure of *A. pectoralis* spermatozoa. This was followed by an experiment to cryopreserve mature spermatozoa to provide baseline data for future studies on this and related species.

The testis of *A. pectoralis* was a paired structure encased in a membrane, the *tunica albuginea*. The primary testis was located on the dorsal surface of the rib cage and the secondary testis on the ventral side. The testis was of an unrestricted spermatogonial type, based upon observations of spermatogonia along the entire length of the lobule. Mature spermatozoa of *A. pectoralis* had an acrosome-free ovoid head 1.68 ± 1.6µm in length and 1.7 ± 1.6µm in diameter, a short mid-piece of 0.5 ± 0.1µm in length, containing 7 irregularly shaped mitochondria forming a ring-like structure at the base of the nucleus. The flagellae were 47.4 ± 4.8µm in length, most with two plasma membrane lateral fin-like projections. However, some flagellae had either zero or three lateral fin projections. Cross-sections of the flagellae showed an axenome with a 9+2 microtubule configuration. The proximal and distal centriols were coaxal, situated deep within the *nuclear fossa*. The structure of *A. pectoralis* spermatozoa conformed to the type 1 ect-aquasperm, also found in externally fertilizing species. This type has been suggested to be the plesiomorphic form in Neopterigians.

Finally, this study contributed to a cryopreservation protocol for *A. pectoralis* spermatozoa by testing the two cryoprotectants dimethyl sulphoxide (DMSO) and glycerol. Glycerol, at a concentration of 10%, offered better cryoprotection than DMSO. This was established using flow cytometry analysis of post-thaw nuclear membrane integrity after 64 days of storage in liquid nitrogen. The toxicity of DMSO to isolated cellular proteins may have resulted in DMSO-treated sperm having the highest percent (35.2% ± 3.2%) of non-viable cells compared with 23.0% ± 2.5% and 27.8% ± 3.4% for glycerol and the control, respectively. The presence of sucrose in the Modified Mounib Medium extender solution may explain why 45.5% ± 5% of the sperm cells were potentially viable in the control treatment.

Initially, the white margined sole *Dagatichthys marginatus* (Soleidae) was selected as the most suitable candidate for flatfish aquaculture in South Africa. Thus, the aim of this study was to investigate the cryogenic potential and ultrastructure of *D. marginatus* spermatozoa. However, due to a skewed sex ratio, there were not enough males available to study this species. A skewed sex ratio is common amongst soleids, thus, the need to develop effective cryopreservation methods and to develop an understanding of sperm morphology so that the best time for cryopreservation can be chosen.

In conclusion, this first description of spermatozan morphology of *A. pectoralis* contributed to our understanding of soleid sperm ultrastructure. In addition, a comparison of testis appearance between fish sampled just prior to spawning season and fish with mature sperm provided information on the spawning season of this species. The findings from the cryopreservation experiment suggested that glycerol was a feasible cryoprotectant for this species when sperm was prepared under field conditions.

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Chapter 1 - General Introduction

Aquaculture is the controlled production of aquatic species for human consumption and industrial use. It is the fastest growing food-producing sector and is perceived as having the greatest potential in meeting the growing demand for aquatic food (FAO 2006). It is estimated that an increase in production of 40 million tons is required by 2030 to maintain the current per capita consumption of aquatic species (FAO 2006). Commercialization originates in China, Europe, Asia and North America and is spreading rapidly to South Africa and Chile. Total world production in the 1950's was less than 1 million tons, whereas in 2004, production had expanded to approximately 59.4 million tons with an estimated market value of US$70.3 billion (FAO 2006). Chinese production accounted for 69.6% compared with 21.9% by the rest of Asia and the Pacific region, 3.5% by western European countries, 1.3% by North America, and 0.9% and 0.2% by North and Sub-Saharan Africa, respectively (FAO 2006).

Aquaculture diversifies by culturing new species of high market value and demand (Rideout *et al*. 2003). Between 1980, and 1990 approximately 9 new species have been developed each year worldwide. The production of cyprinids reached 18.2 million tons in 2004, with an estimated market value of US$16.3 billion (FAO 2004), making them the most important family by quantity and value. Salmonids and cyprinids dominated research and development efforts regarding finfish aquaculture. More recently, pleuronectiform species such as yellowtail flounder *Pleuronectes ferrugineus* (Richardson *et al*. 1999), southern flounder *Paralichthys lethostigma* (Smith *et al*. 1999), summer flounder *P.s dentatus* (Bengtson 1999), Caribbean flounder *P. tropicus* (Rosas *et al*. 1999), Senegal sole *Solea senegalensis* (Anguis and Cañavate 2005), common sole *S. solea* (Howell 1997), turbot *Scophthalmus maximus* (Alvial and Manriquez 1999), and Atlantic halibut *Hippoglossus hippoglossus* (Olsen *et al*. 1999) have been experimentally and commercially cultured due to their good flesh quality and adaptability to culture conditions. In 2002, the production of *S. solea* and *S. senegalensis* in Spain and Portugal was estimated at 23 tons per year, and Atlantic halibut *H. hippoglossus* production in Iceland was approximately 35 tons per year (Thompson 2004). Turbot *S. maximus* production in Europe was estimated at 5217 tons per year in 2004, with Spain contributing 75% (3847 tons per year) to world production (FAO 2004).

Pleuronectiform species have consistently attracted high prices on the European market (Howell 1997, Brown 2002) and are considered to adapt well to intensive culture conditions (Thompson 2004).

There are seven flatfish families found in the southeast Atlantic. These include Psettodidae, Citharidae, Bothidae, Achiropsettidae, Paralichthyidae, Soleidae and Cynoglossidae (Millner *et al*. 2005) however only soleids, bothids and some cynoglossids are of commercial importance in this region.

Along the South African coastline, 56 flatfish species have been identified (Smith and Heemstra 1986) of which two soleid species *Austroglossus pectoralis* and *A. microlepis* are commercially important (Millner *et al*. 2005). Although soleids account for a small percentage of the annual South African trawl fishery catch, they are the most valuable fish per unit mass (Millner *et al*. 2005). *A. pectoralis*, which is commercially the most important flatfish species in South Africa is targeted by small scale inshore trawlers along the south coast from Port Elizabeth (33º57'S; 25º38'E) to Mossel Bay (34º08'S; 22º08'E). The fishery is managed on a precautionary basis, with a total allowable catch of 800 MT per annum (Thompson 2004), as little is known about the harvesting potential of remaining stocks. This is considered sustainable (Britz *et al*. 2001) however; the average size of sole is decreasing. According to Thompson (2004) the current demand for sole in South Africa is in excess of 1 500 tons per year. This is not achievable through the existing fishery and, hence, there are investment opportunities into research and development of flatfish aquaculture in South Africa.

Initially, this study aimed at describing the spermatozoan ultrastructure and cryogenic potential for the white-margined sole *Dagatichthys marginatus* (Soleidae). *D. marginatus* was highlighted by Thompson (2004) as the most likely candidate for flatfish aquaculture in South Africa. However, the species has a skewed sex ratio of 5:1:1 (females to male) (Thompson 2004), and this highlights sperm availability as a potentially limiting factor to the successful spawning of this species. An understanding of ultrastructure and cryogenic potential of *D. marginatus* spermatozoa would assist in future research regarding artificial propagation and culture of this species. During a seven month sampling period, from November 2005 to May 2006, only 94 *D. marginatus* were captured, comprising only six males. Thus, due to the low number of *D. marginatus* males, this species was not used in this research.. Therefore, this

study focused on South Africa's most important commercial flatfish species, the Agulhas sole *A. pectoralis*.

A. pectoralis occurs within a depth range of 50 to 300 m (Smith and Heemstra 1986); however specimens have been collected in water of 20 meter depth (Edwards *pers. comm.*). Based on its depth range and the perceived inability to acquire adequate broodstock, *A. pectoralis* had been excluded as a potential culture candidate in South Africa by Thompson (2004) and the species had yet to be tested as an aquaculture candidate. A well established market defined by the current fishery, combined with the future uncertainty of their harvest capacity, suggests the importance in the development of techniques associated with their potential culture.

The diversification and intensification of aquaculture requires the development of new technologies. Cryopreservation of fish spermatozoa plays an important role in aquaculture development and has been the subject of investigations (Lahnsteiner *et al*. 1992; Tanaka *et al*. 2002). Since the discovery of glycerol (Pogle *et al*. 1949) and dimethyl sulphoxide (Lovelock and Bishop 1959) as permeating cryoprotective agents (CPA), many cell and tissue types have been cryopreserved. Most cryogenic studies focused on salmonids of aquaculture importance, while less research was conducted on the cryopreservation of marine fish gametes (Gwo 2000).

The first reported successful marine cryogenic trial was conducted by Blaxter (1953) on herring *Clupea harengus* spermatozoa. This was followed by the development of cryogenic protocols for sperm of over 40 marine species (Gwo 2000). These include representatives of the order Clupeiformes, Gadiformes, Perciformes, Pleuronectiformes and Tetraodontiformes (Gwo 2000). Cryopreservation research of pleuronectiform spermatozoa was conducted on Atlantic halibut *H. hippoglossus* (Bolla *et al.* 1987), yellowtail flounder *P. ferrugineus* (Richardson *et al*. 1999), turbot *S. maximus* (Chen *et al*. 2004; Dreanno *et al*. 1997), ocean pout *Macrozoarces americanus* (Yao *et al.* 2000) and winter flounder *Pseudopleuronectes americanus* (Rideout *et al*. 2003).

Cryopreservation of fish gametes is essential for both fisheries' conservation and aquaculture. The number of threatened or endangered species is increasing (Knapp 2000) and cryopreservation offers opportunities for storing genetic material and conserving genetic

diversity. In aquaculture, cryopreserved gametes could be used for maintaining genetic potential of captive broodstock. Cryopreservation of spermatozoa could enhance the economic utilization of males by reducing captive male broodstock size (Richardson *et al.* 1999). Furthermore, cryopreserved gametes would ensure synchronicity in the availability of male and female gametes, provide a simple means for gamete transport and increase the number of offspring from selected males (Rideout *et al.* 2003, Carolsfeld *et al.* 2003)

Cryopreservation comprises the freezing, cryogenic storage and thawing of living material (Tiersch and Mazik 2000). Cell destruction associated with cooling and thawing can be counteracted to some degree by the addition of non-permeating and permeating organic cryoprotective compounds (Bromage and Roberts 1995). Permeating cryoprotectants must reduce ice crystal formation during freezing by dehydration. The choice of cryoprotective agents (CPA) is based on permeability, water solubility and cell toxicity (Chao and Liao 2001). Thus, both cryoprotective efficiency and the toxicity tolerance of cell types to the CPA must be taken into account when evaluating cryoprotectants (Chao and Liao 2001). Various aspects of sperm cryopreservation, including chemical composition of extenders and their effects on the sperm plasma membrane, mitochondrial function, osmotic tolerance, hydraulic conductivity, and CPA permeability and toxicity suggest that each species' spermatozoa has unique cryobiological properties (Woods *et al.* 2004). Both quality of gametes and cryopreservation techniques influence fertilization success of post-thaw gametes.

A cryopreservation protocol aims to produce gametes with structural and functional integrity similar to that of unpreserved, healthy gametes. Damage of spermatozoan ultrastructure during cryopreservation will reduce fertilization success (Ciereszko *et al.* 2006). Sperm cells collected at the beginning or at the end of the spawning period may be of low quality (Billard and Cosson 1992). The reason for poor quality sperm at the onset of the spawning season is unclear. The low sperm quality at the end of the spawning season may be caused by ageing (Billard 1986). Even if sperm quality is excellent, sperm collection can introduce faeces and urine, which may modify sperm characteristics that are important to successful cryopreservation (Suquet *et al.* 2000). Investigations including seminal plasma composition, spermatogenesis and sperm storage with respect to spawning season are important for cryopreservation, For example, the composition of extender solutions used to inhibit motility of spermatozoa prior to cryopreservation would be designed to closely mimic those properties of the seminal fluid. Spermatogenesis and sperm storage would suggest the maturity state of

the gametes, thus identifying the appropriate timing for gamete acquisition prior to cryopreservation and, therefore, maximizing gamete quality. Seminal plasma creates optimal conditions for the storage of spermatozoa in the reproductive system by protecting their functional properties (motility and DNA integrity) and supporting spermatozoan metabolism. Most externally fertilizing teleost spermatozoa are within the genital tract and only become motile and metabolically active upon release into the external environment (Rurangwa *et al*. 2004). Results from investigations of gamete biochemistry and ultrastructure design are potentially important for the design of preservation procedures for gametes. Fish species which have been subjects of sperm cryopreservation are, generally, externally fertilizing teleosts with an anacrosomal aquasperm (Jamieson 1991). It was important to identify the type of sperm and its ultrastructure. to test the cryoprotective potential of *A. pectoralis* spermatozoa. This knowledge would contribute to cryopreservation procedures for teleosts with similar sperm type and ultrastructure and it may assist with the development of a cryopreservation technique for *A. pectoralis* spermatozoa.

The methods of acquiring and maintaining healthy broodstock determines the success of all aquaculture efforts. Trawling and long-lining are used to collect broodstock. However these methods may induce stress and barotrauma to potential broodstock. Longlines, however have been successfully used to capture Atlantic halibut *H. hippoglossus* brood fish, along the Norwegian continental shelf (Bolla *et al*. 1987). Similarly, successful cultivation and spawning have been achieved in the small-eyed flounder *P. microps*, acquired by trawling (Silva 2001). *A. pectoralis* were captured by a commercial trawling vessel operating at depths between 48-65 meters.

This study describes the ultrastrucure and spermatozoa type of *A. pectoralis* (Chapter 3). The cryopreservaton technique and preparation may be similar to that adopted for other species with an anacrosomal aquasperm. Histology and electron microscopy showed that mature spermatozoa were present for cryopreservation during August 2006. Due to sampling limitations seasonal collection was not possible. However, the study investigated sperm cryopreservation, thus samples were collected during a time when this was most likely successful. The short-term cryogenic potential of *A. pectoralis* spermatozoa to improve sperm availability for future artificial propagation was tested (Chapter 4). Results obtained by dual fluorescence flow cytometry suggest that cryopreservation of *A. pectoralis* spermatozoa may provide a tool for prolonged sperm storage and artificial propagation of this species.

Chapter 2 - General Methods and Materials

Collecting *Austroglossus pectoralis*

A. pectoralis were captured by a commercial in-shore sole trawler in August 2006 off Mossel Bay (34º08'S; 22º08'E), along the South coast of South Africa (Figure 2.1).

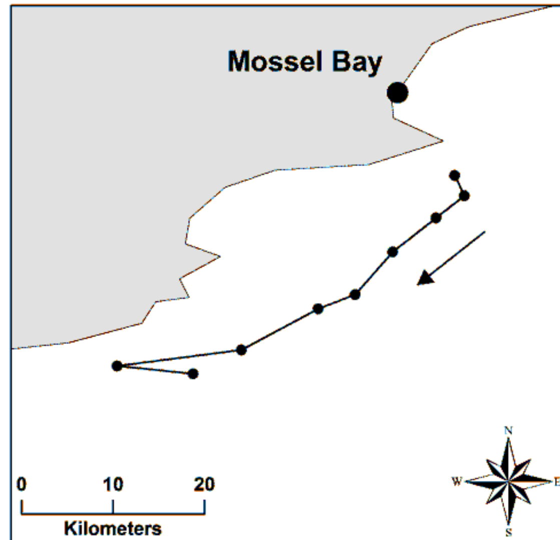


Figure 2.1: The GPS track line taken from the trawler during sampling. The start of the trawl was approximately one kilometre offshore in a south easterly direction off Mossel Bay. Thereafter, trawls proceeded in a south westerly direction as indicated by the arrow.

Collecting period and effort

Sampling was conducted on an inshore commercial trawler over 24 hours during August 2006. Five trawls were conducted: three during the day and two at night. The net was set and retrieved every three hours during the day and every five hours during the night, resulting in a total trawl time of 19 hours. Most of the *A. pectoralis* were caught during night trawls.

Sampling strategy

The depth of each trawl varied from 48 – 65 m. A trawl speed of 3 knots was maintained during the entire trawl. The trawl net had a maximum gape of approximately 10 m and a mesh size of 75 mm^2. Of the landed species, *A. pectoralis* comprised 10-15% of the catch (Figure 2.2). Other species included silver kob (*Argyrosomus inodorus*), shallow water hake (*Merluccius capensis*), skate and ray species (Rajiformes) and St. Joseph sharks (*Callorhinchus milii*).

Figure 2.2: Landings of *A. pectoralis* after a five-hour night trawl off Mossel Bay on the south coast of South Africa.

Of the *A. pectoralis*, approximately 10% were alive. Between 10 and 12 randomly selected live individuals from each trawl were placed into a 100-L container with aerated seawater. A total of 57 fish were sexed, of which 20 were male. A tape measure was used to measure total length for each of the 57 fish. An incision, using a sterile scalpel into the interperitonial cavity, was made and the testis was located. The testis was removed by cutting at the junction of the efferent ducts, using a sterile scalpel. The primary testis was sliced in half and both halves were placed into a test tube containing 4 ml Modified Mounib Medium (MMM) extender solution. One half of the testis was held with fine forceps and two tissue sections, one for histology and one for transmission electron microscopy, were cut, using sterile scissors. Cut sections were placed into their respective vials, sealed and stored. Both testis halves were tapped using a sterile glass rod to solicit sperm release for ultrastructure analysis and cryopreservation. All instruments used for testis removal and sperm acquisition were washed with 70% alcohol and dried prior to use.

Sperm samples from each specimen were cryopreserved in two-steps. Firstly, sperm were pre-cooled in a 4-L Dewar flask by placing samples 6 cm above the surface of liquid nitrogen for 10 minutes. Between batches of vials, the 4-L Dewar flask was refilled using a cryo-ladle to a pre-marked volume inside the flask, to ensure the distance from the surface to the sperm samples remained at 6 cm. To reduce movement of liquid nitrogen in the Dewar flask associated with the movement of the fishing vessel, the flask was held steady by hand.

Secondly, pre-cooled sperm samples were removed, and immediately dunked into a 30-L liquid nitrogen vat for storage. The vat was securely fastened to the vessel.

Port Elizabeth catches

A total of 70 *A. pectoralis* were obtained for gonad histology and sperm ultrastructure analysis from an inshore commercial trawling vessel off Port Elizabeth (33º57'S; 25º38'E) on the 31st August 2006. The method of capture was identical to that described above; however, live specimens from the last trawl were not kept alive. They were immediately placed on ice and stored for approximately five hours before testis samples could be obtained. Of the 70 fish processed, 33 were male. Total length was measured for each male before the fish were dissected for gonad histological and sperm ultrastructure samples.

Male *A. pectoralis* size frequency

Fish trawled off Mossel Bay ranged in length from 225 to 332 mm (n = 20) (Figure 2.3). The range of lengths obtained from Port Elizabeth was 293 to 450 mm (n = 33) (Figure 2.4).

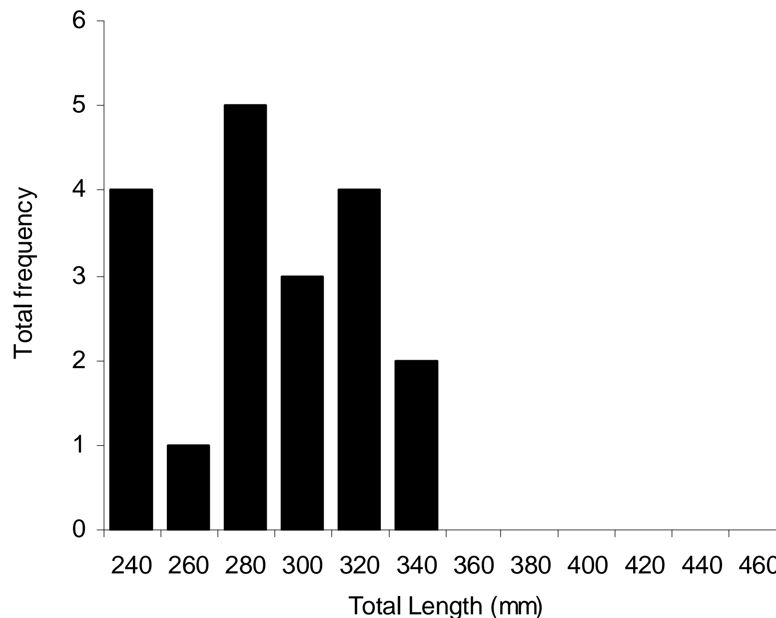


Figure 2.3: Length frequency distribution of *A. pectoralis*, captured on 28th August 2006 off Mossel Bay (34º08'S; 22º08'E) on the south coast of South Africa.

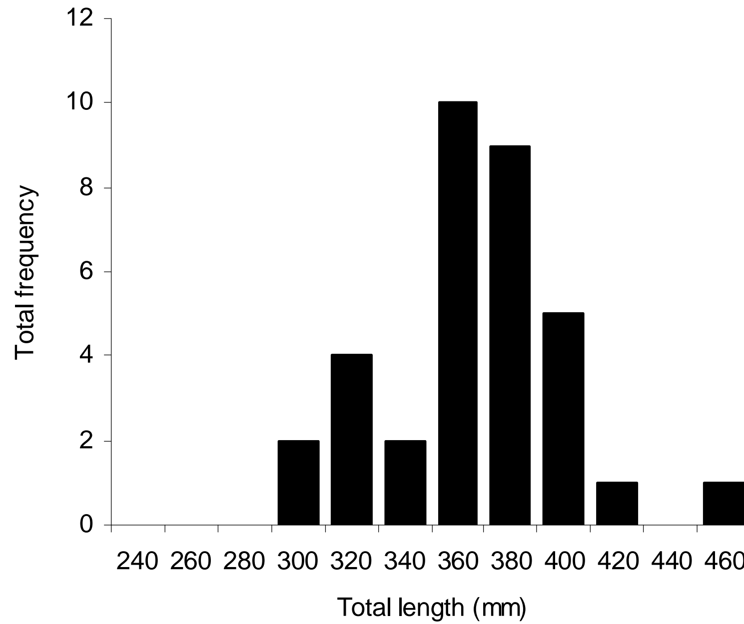


Figure 2.4: Length frequency distribution of *A. pectoralis* captured on the 31st August 2006 off Port Elizabeth (33º57'S; 25º38'E) on the south east coast of South Africa.

Chapter 3 – Sperm Ultrastructure in Teleosts

Introduction and a review of the literature

In most teleost fish, a mature testis is an elongate, bi-lobed white organ covered by thin connective tissue, the *Tunica albuginea* (Evans 1993, Weltzien *et al*. 2004). At the onset of the spawning season, approximately August and September, the testis colour of *A. pectoralis* changes from red (Figure 3.1A) to white and milky as males become ready to spawn (Figure 3.1B).

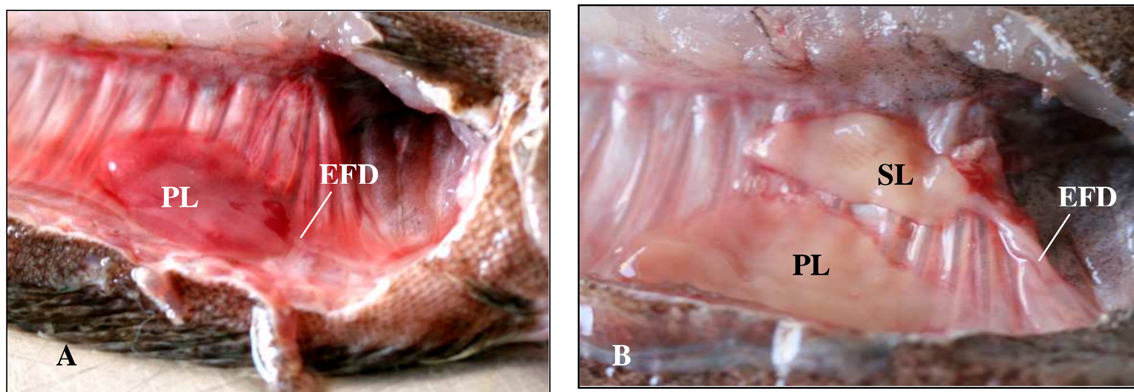


Figure 3.1: A The primary testis lobe of *A. pectoralis* that is not ready to spawn. The testis has a red vascular appearance and is relatively firm compared to a white milky testis.

Figure 3.1 B The white and milky bi-lobed testis of *A. pectoralis* at the onset of spawning season. SL = secondary lobe, PL = primary lobe and EFD = efferent duct.

The male gonad serves two main functions, i.e., to produce germ cells through spermatogenesis and to produce sex steroids via steroidogenesis to regulate reproduction both physiologically and behaviourally (Weltzien *et al.* 2002, 2004). A testis in teleost fish is divided into two compartments, the germinal and the interstitial, each with specific functions, according to the cell types of which they are composed (Grier and Neidig 2000).

The interstitial compartment contains the blood supply to the testis, contractile myoid cells and androgen-secreting Leydig cells (Grier and Neidig 2000). The myoid cells are characteristic of smooth muscle cells (Grier and Neidig 2000). The Leydig cells are important

for the regulation of spermatogenesis through their production of steroids, as well as the regulation of the development of secondary sexual characteristics (Evans 1993; Weltzien *et al.* 2004); however, secondary sexual characters have not been observed in flatfish (Weltzien *et al.* 2004).

The germinal compartment contains germ cells and somatic sertoli cells. These are organized in spermatocytes (Weltzien *et al.* 2004). The sertoli cells could be considered "nurse cells", as they are in close contact with developing germ cells. They provide support by modifying their chemical microenvironment during spermatogenesis (Evans 1993).

Testis classification

Teleost testes are classified into two types, based on the germinal compartment arrangement. These are the anastomosing tubular testis and the lobular testis (Grier and Neidig 2000). In the tubular testis, the germinal compartment forms a highly interconnected complex tubular system at the testis periphery. This, according to Grier and Neidig (2000), has only been observed in lower fishes such as bowfin *Amia calva* (Holostei), rainbow trout *Oncorhynchus mykiss* (Salmoniformes) and goldfish *Carassius auratus* (Cypriniformes). The lobular testis type is characteristic of higher fishes (Grier and Neidig 2000) where lobules terminate blindly at the testis periphery. Both testis types are divided into a restricted or unrestricted testis, based on the distribution of germ cells within the germinal compartment (Weltzien *et al.* 2002, 2004). In the unrestricted spermatogonial testis type, spermatogonia are distributed along the entire length of the tubule or lobule. In the restricted spermatogonial type, spermatogonia occur at the testis periphery (Weltzien *et al.* 2004).

The morphology of spermatozoa differs between species (Nostro *et al.* 2003, Gwo *et al.* 2004, Rurangwa *et al.* 2004, Shahin 2006). Despite the morphological simplicity of spermatozoa, structural differences yield a wide range of spermatozoa types, with phylogenetic implications (Jamieson 1991, in Medina *et al.* 2000; Gwo *et al.* 2004, 2006). The differences can be in part explained by the diversity of reproductive strategies and systematic position (Shahin 2006; Kime *et al.* 2001). Morphological differences may be expressed by flagella design (e.g. aflagellate, biflagellate), a wide range of nuclear structural variations (e.g., shape and size) and the presence, number and location of specific organelles (e.g. mitochondria and acrosome) (Rurangwa *et al.* 2004; Shahin 2006).

There are approximately 500 species of flatfish, flounders and sole in the order pleuronectiformes. They are a monophyletic group characterized by the absence of bilateral symmetry (Jamieson 1991). Although the group comprises potential aquaculture species, little work has been done on spermatozoon ultrastructure.

Spermatozoon ultrastructure has been described in eight pleuronectiform species (Medina *et al*. 2000), with detailed descriptions for *S. maximus* (Suquet *et. al.* 1993) and *P. flesus* (Jones and Butler 1988). There is interest in flatfish spermatozoa structure and quality, especially to assist in the development of cryopreservation protocols. The identification of mature spermatozoa, by observing a reduction in cytoplasmic mass around the nucleus and mid-piece and the formation of the flagella, would provide an indication to the correct timing of cryopreservation of *A. pectoralis* spermatozoa. Cryopreservation protocols for flounder *P. olivaceus* (Zhang *et al*. 2003), yellowtail flounder *P. ferrugineus* (Richardson *et al*. 1999), winter flounder *P. americanus* (Rideout *et al*. 2003) and ocean pout *M. americanus* (Yao *et al*. 2000) contribute to the knowledge about spermatozoa with respect to ultrastructural changes and fertilization capacity as a result of cryopreservation. There is a paucity of information on sperm ultrastructure within the pleuronectiformes and, according to Gwo *et al*. (2004), teleosts in general.

Spermatozoa of teleosts can be divided into two main categories: Aquasperm and introsperm (Figure 3.2). Aquasperm are released into the water column, where external fertilization takes place. Introsperm fertilize internally and this type is common amongst Chondrichthyes and Coelacanths (Jamieson 1991). The majority of teleost fish have spermatozoa of the primitive aquasperm type (Medina *et al*. 2000). Aquasperm is divided into two groups, ect-aquasperm and ent-aquasperm (Jamieson 1991). Both sperm types are released into the water column; however, ect-aquasperm fertilizes eggs externally while ent-aquasperm is drawn into the female, thus fertilizing internally (Jamieson 1991). Ect-aquasperm can be divided into type 1 and type 2 sperm (Figure 3.3). The characteristic difference between the two sperm types is the rotation of the flagellum axis relative to the nucleus. In type 2 spermatozoa, the flagellum remains parallel to the nucleus and, in type 1 spermatozoa, rotation causes the flagellum axis to be positioned perpendicular to the base of the nucleus (Figure 3.3 A and 3.3 B) (Jamieson 1991)

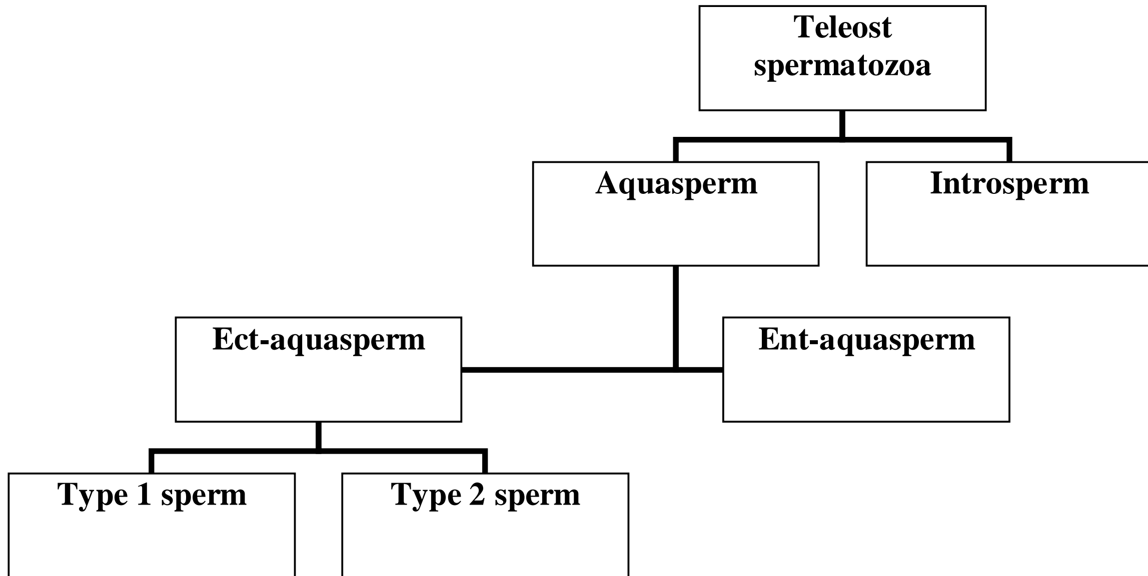


Figure 3.2: Spermatozoa classification, defined by mode of fertilization (Jamieson 1991).

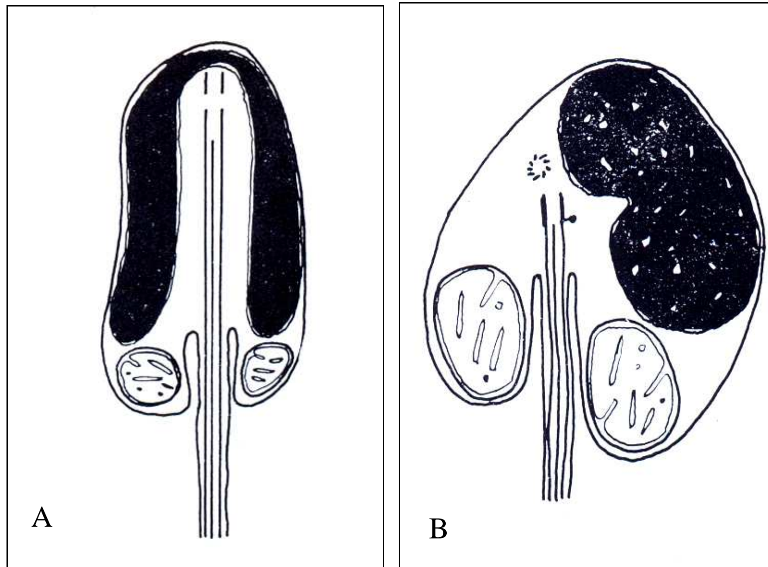


Figure 3.3: A Diagramatic representation of a type 1 sperm with flagellum perpendicular to the nucleus. B represents type 2 spermatozoa with the flagellum parallel to the nucleus (Diagrams modified from Mattei 1970 in Jamieson 1991)

Sperm morphology

A spermatozoon can be divided into three parts, which are: the head or nucleus, the mid-piece and the tail or flagellum.

Typically, externally fertilizing teleost aquasperm has a round to ovoid nucleus with a diameter of 2-3 µm. The nucleus length and width of *S. senegalensis* sperm is approximately 2.25 µm and 1.58 µm, respectively (Medina *et al*. 2000). Externally fertilizing aquasperm has no acrosome, examples of which are turbot *S. maximus* (Suquet *et al*. 1993), the puffer fish *Takifugu niphobles* (Morisawa 2001) and Senegalese sole *S. senegalensis* (Medina *et al*. 2000). The loss of the acrosome is thought to be the result of co-evolution: with the development of thick egg shells possessing a micropyle, an opening in the *zona pellucida* enables spermatozoa to access the oolemma (Suquet *et al.* 1993, Medina 2000). Diverse spermatozoa variations, with respect to nuclear morphology, occur in internally fertilizing fish species (Jamieson 1991). The nuclear length of coelacanth *Latimeria chalumnae* spermatozoa, including the acrosome, is approximately 26 µm The length, excluding the acrosome, is 25 µm, and the width is approximately 0.4 µm (Jamieson 1991). Australian lungfish *Neoceratodus forsteri* spermatozoa have a nucleus length of 70 µm and a maximum width of 2 µm, with a slender acrosome that does not enclose the tip of the nucleus (Jamieson 1991).

Depending on the species, a *nuclear fossa* is present, within which the proximal and distal centrioles are found (Grier and Neidig 2000). Pleuronectiforms characteristically have a deep *nuclear fossa* as reported in *P. flesus* (Jamieson 1991) and *S. senegalensis* (Medina *et al*. 2000). A relatively deep nuclear fossa was reported for four sparid species, *Acanthopagrus berda, A. australis, L. rhomboides and Archrosargus probatocepharus*; however, variations in depth of the *nuclear fossa* were noted amongst these four species (Gwo *et al*. 2005). Both pleuroneciforms and sparid species have a type 1 ect-aquasperm configuration (Figure 3.3 A). Sciaenid species have a type 2 spermatozoa configuration (Figure 3.3 B) represented by a double-arched lateral shallow *nuclear fossa* which does not house the centrioles as in pleuronectiforms and sparids (Gusmão-Pompiani *et al*. 2005).

The mid-piece consists of a cytoplasmic mass, in which a variable number of spherical mitochondria lie posterior to the nucleus, encircling the proximal portion of the flagellum. The mitochondria are separated from the flagellum by the cytoplasmic canal and they form a ring around the opening of the *nuclear fossa*. A small mid-piece is common in externally

fertilizing teleost species and a longer mid-piece characterizes spermatozoa of internally fertilizing species (Suquet *et al.* 1993).

Blue sprat spermatozoa *Spratelloides gracilis* (Clupeidae) have one oval-shaped mitochondrium, situated laterally in relation to the flagellum. Furthermore, there is no cytoplasmic canal separating the flagellum from the mid-piece (Gwo *et al*. 2006). In the flounder *P. flesus* eight spherical electron-dense cristate mitochondria make up the ring around the *nuclear fossa* (Jones and Butler 1988, in Jamieson 1991). In Senegalese sole *S. senegalensis* more than eight cristate electron-dense mitochondria have been identified (Medina *et al*. 2000). Turbot *S. maximus* spermatozoa have eight to ten spherical mitochondria forming the 'collar like ring' of the mid-piece (Suquet *et al.* 1993). In Sciaenidae the mid-piece and cytoplasmic canals are short with no more than 10 large spherical mitochondria (marine species) or elongate in the freshwater species, *P. squamosissimus* (Gusmăo-Pompiani *et al.* 2005). The condrichthyan *Hydrolagus collie* contains up to 70 mitochondria in the mid-piece (Jamieson 1991). This highlights the mitochondrial variability of the mid-piece amongst species. Mitochondria in the mid-piece of the spermatozoa are responsible for providing adenosine triphosphate (ATP).

The mid-piece housing the mitochondria is, however, not a key determinant of sperm swimming velocity (Malo *et al.* 2006). The hydrodynamic shape of the head and the forces generated by the relative length of the flagellum moderate swimming ability and velocity (Malo *et al*. 2006).

The flagellum of type 1 aquasperm contains an axoneme with nine peripheral doublet microtubules and two central single microtubules (9 + 2 configuration), typically seen in teleostean (and Neopterygian) spermatozoa (Jamieson and Leung 1991a). The axoneme of elopomorphs, which includes the European eel *Anguilla anguilla,* however, shows a 9 + 0 configuration (Bromage and Roberts 1995). In type 2 aquasperm, where the rotation of the flagella axis does not occur, the flagellum remains parallel to the base of the nucleus (Jamieson and Leung 1991). This axoneme configuration is different from type 1 spermatozoa in that the microtubule doublets 1, 2, 5 and 6 exhibit an intratubular differentiation resulting in the lumen appearing denser (Jamieson and Leung 1991).
The plasma membrane of the flagellum extends through 1, 2 or 3 or zero fin-like structures (Jamieson and Leung 1991). Puffer fish *T. niphobles* (Tetraodontiformes) sperm showed

swelling and shrinking of the plasma membrane in the sperm head region and the tail region independently, when sperm was subjected to osmolality tests (Morisawa 2001). This suggests that the function of the plasma membrane in the tail region may be physiologically different to that in the head region (Morisawa 2001). The author suggests that this aspect of sperm physiology should be further studied to determine whether it is common to all fish spermatozoa.

Biflagellate aquasperm utilize both the proximal and distal centrioles as basal bodies for the development of each flagellum (Jamieson and Leung 1991, Nostro *et al*. 2003). In *Synbrachus marmoratus* (Synbrachidae), the transformation of spermatids to mature spermatozoa involves: a reduction in cytoplasm which is lost as a residual body, the re-organization of the nucleus, and the formation of two flagella on each centriol prior to the mitochondrial ring formation of the mid-piece (Nostro *et al*. 2003). Aflagellate aquasperm is uncommon, but has been reported in osteoglossomorphs, i.e., the Mormyridae (Jamieson and Leung 1991).

Spermatogenesis is the process by which spermatogonial stem cells develop and differentiate into mature spermatozoa (Weltzien *et al*. 2004). The primary events of this modification process include morphological modifications of the nucleus, and the development of specialized organelles that must function during sperm transport and fertilization (Browder 1984).

Spermatogenesis in teleosts is similar to that reported for other vertebrates. The differences between taxa are small and relate to morphology of testis and spermatozoa, number of mitotic spermatogonial divisions prior to meiosis and the timing between successive cell development stages (Evans 1993). Spermatogenesis occurs in four steps: 1) Spermatogonial stem cells undergo mitotic proliferation producing differentiated spermatogonia; 2) spermatogonia undergo meiotic divisions to produce primary spermatocytes,with the nucleus starting to condense to form secondary spermatocytes; 3) the secondary spermatocytes undergo a second meiotic division to form haploid spermatids,and 4) further nuclear condensation occurs and the spermatids differentiate into flagellated spermatozoa, a process called spermiation (Weltzien *et al*. 2004). During spermiation, mature spermatozoa are released from the spermatocysts into the central lumen, which leads to the efferent ducts (Evans 1993; Weltzien *et al*. 2004). During spawning, sperm is released from the efferent ducts (Lahnsteiner 2003) where it is maintained viable prior to spawning into the external environment through the

urogenital pore, which may be modified as an intermittent organ in some fish species (Evans 1993; Weltzien *et al*. 2004). Sperm production in some teleosts is a single synchronous event; whereas in other species it is cyclic or continuous (Evans 1993).

Aims and contributions

The development of successful sperm cryopreservation techniques to facilitate artificial propagation of cultured species relies on an understanding of sperm structure and function. By analyzing the ultrastructure of the spermatozoa primarily for a reduction in cytoplasmic mass surrounding the nucleus and mid-piece, it could become possible to indicate the maturity state of the spermatozoa, thus suggesting their suitability for cryopreservation. Furthermore, the understanding of testis structure, spermatogenesis and sperm ultrastructure would provide information regarding the storage of mature spermatozoa, maturity state and seasonal sperm production.

Sperm morphology was further described by measuring nuclear lengths and widths, flagellum length, mid-piece length, and observing the presence of specific organelles, i.e., number of mitochondria and the absence of an acrosome. Testis arrangement and spermatogenesis were described from histology sections and the presence of mature spermatozoa in the lumen was noted. Spermatogenesis was further observed by identifying cells in different stages of development under transmission electron microscopy. The aim of describing the morphology of *A. pectoralis* in this study was first to contribute to the understanding of sperm morphology in the order Pleuronectiformes, and second to provide information regarding the suitability of *A. pectoralis* spermatozoa for cryopreservation.

Methods and Materials

Testis removal

To establish the sex of *A. pectoralis*, a small incision was made into the interperitoneal cavity to check for the presence of a testis. The connective tissue surrounding the primary testis lobe was cut, releasing the primary lobe from its position. The primary lobe was removed to allow cutting of the rib cage (Figure 3.4 A) to access the secondary lobe. The connective tissue surrounding the secondary lobe was cut and the testis was removed by cutting at the junction of the two efferent ducts (Figure 3.4 B). The testis was placed in 4 ml M M M extender solution from which sperm for cryopreservation was taken. The primary testis was sliced in half, of which one half was held with fine forceps. Two tissue sections, one for histology and the other for transmission electron microscopy, were cut using sterile dissection scissors. This process was repeated for the secondary lobe. Tissue sections for histology were placed in a vial containing a 10 % buffered formalin solution, while sections for transmission electron microscopy were stored in M M M extender solution.

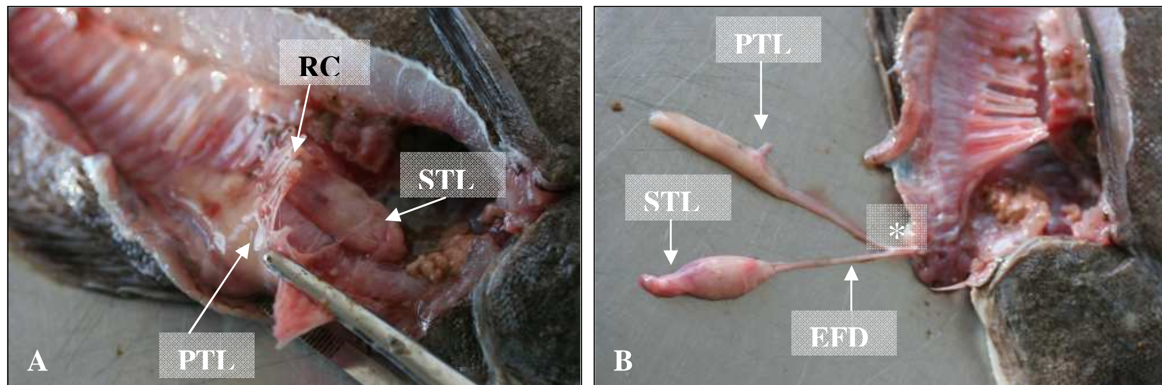


Figure 3.4 A: The primary testis lobe (PTL) located above the dorsal surface of the rib cage (RC) and secondary testis lobe (STL) at the ventral side of the rib cage**.**

Figure 3.4 B**:** the primary and secondary lobes of the testis. The testis was removed by cutting through the junction (*) of the efferent ducts (EFD).

Histology

Samples of both primary and secondary testis lobes were stored for histological and electron microscopy analysis. Histology samples were stored for 24 hours in a 10% buffered formalin solution and kept in 70% ethanol until prepared for histology.

Histology preparation

Histological preparation of *A. pectoralis* testes was conducted by the National Health Laboratory Services (NHLS) in Port Elizabeth, South Africa.

Tissue sections were treated in a 10% formalin/ ethanol (95%) bath for 5 minutes, whereafter they were placed into two ethanol baths, 70% and 95% for 10 minutes, respectively. Tissue sections were placed into a series of three absolute alcohol baths followed by a series of three xylene baths, each for 10 minutes. Tissue samples were then placed into two paraffin wax baths for 20 minutes each. Sections were removed from the second paraffin wax bath and allowed to cool for 60 minutes. Wax blocks were kept on ice until cut on a microtome at a thickness of 4 µm. Ribbons were floated in a water bath and a slide, treated with 30% ethanol, was used to take the ribbon-like samples out of the water bath. These were placed into an oven at 60℃ for 10 minutes.

Staining procedures

Tissue samples were placed into a 95% ethanol bath for one minute, rinsed in tap water and placed into a haemotoxylin bath for one minute. Samples were then removed, rinsed in tap water and placed into a 95% ethanol bath for one minute. Samples were then placed into two absolute alcohol baths, followed by two xylene baths each for five minutes, respectively. Samples were allowed to dry for 20 minutes. Dry slides were fixed with a coverslip using Entellan mounting solution.

Scanning Electron Microscopy (SEM)

SEM was conducted on unpreserved *A. pectoralis* sperm. A 2-µl sample was pipetted onto a gelatine-coated coverslip and allowed to air-dry. Coverslips were dipped into a series of ethanol washes (30%, 70% and 80%) for 15 seconds at each concentration and air dried between ethanol washes. After the 80% ethanol wash the coverslips were subjected to two absolute alcohol washes, each for 30 seconds. Samples were air-dried and splutter gold-coated.

Splutter gold coating

Cover slips were placed with double sided tape onto a gold stub, which was then placed into the centre of a splutter coater chamber until a vacuum of approximately 10^{-1} Torr was achieved. Argon was leaked into the system, which balanced the vacuum level at 5 x 10^{-2}

Torr. Once the vacuum was stable at 5 x 10^{-2} Torr, the field potential was adjusted to 2 kV. The argon leak valve was re-opened slightly, which allowed the spluttering current to rise to 15 mA. Spluttering lasted for 90 seconds before air was let into to the chamber to reduce the vacuum. Gold coated cover slips were examined using the SEM.

Transmission Electron Microscopy (TEM)

Both halves of each testis were placed into a test tube containing 4 ml MMM extender solution. A small sample of the testis was cut for TEM analysis and prepared as follows:

- Testis sections were fixed for 24 hours at 4 ℃ in a 2.5% buffered gluteraldehyde solution.
- The buffer solution was decanted and replaced with osmium tetroxide to completely cover the sample.
- After 60 – 90 minutes, the Osmium tetroxide was decanted and the vials were refilled with glutheraldehyde buffer solution and washed for 10 minutes.
- Buffer was decanted and vials were filled sequentially with 30%, 40%, 50%, 70%, 80%, 90% and 100% ethanol, each for 5 minutes. Finally, samples were rinsed twice in absolute ethanol.
- The absolute ethanol was decanted and vials were filled with propylene oxide and left to stand for 15 minutes.
- Tubes were decanted, refilled with propylene oxide and left to stand for 15 minutes.
- Propylene oxide was decanted and vials were refilled with a 75:25, 50:50 and 25:75 propylene: resin mixture, respectively. Each treatment was allowed to infiltrate for 60 minutes.
- After 60 minutes, vials containing 25:75 propylene:resin mix were decanted and refilled with pure resin. Samples were left overnight.
- Testis tissue sections were removed from the vials and placed into specimen capsules filled 2/3 with resin solution. The sections were placed at the base of the capsule and turned over to release air bubbles trapped under the tissue.
- Tissue sections were placed into an oven at 60 ℃ for 36 hours.

Trimming the resin block

- The resin block was firmly placed in an ultramicrotome specimen holder so that no more than 5 mm of the block protruded beyond the end of the holder.
- A glass knife was then placed in the knife holder, set at a right angle to the block. The block face was trimmed by advancing the knife approximately six to eight microtomes per stroke until approximately 0.2 mm^2 of tissue was exposed.

Cutting ultra thin specimen sections

- A trough was placed at the base of the glass knife. The trough was filled with water from a syringe until a meniscus was formed along 80% of the knife edge.
- The resin block was then cut at one to two micrometers and sections were checked for damage.
- A pair of fine forceps was used to scoop the cut sections from the water surface onto specific grids.
- Samples were placed on filter paper, to dry before staining.

Heavy metal staining for ultra thin sections

- One square layer of parafilm was placed onto the base of two Petri dishes. Lids were then placed on the Petri dish to prevent dust settlement.
- A droplet of one ml of uranyl acetate was pipetted onto the parafilm.
- Grids with sections were placed face-down onto the droplet and left for 30 minutes.
- Approximately 10 – 15 minutes before the uranyl acetate staining was complete, 10 sodium hydroxide pellets were placed just off the edge of the parafilm in the second Petri dish, to be used for lead citrate staining.
- Grids were removed after 30 minutes from the uranyl acetate and washed distilled water and excess water were removed by gently blotting the grids onto a piece of filter paper.
- The grids were placed onto a lead citrate droplet in the second Petri dish for 5 minutes, removed, washed with distilled water and placed on a marked piece of filter paper to dry.

Sections were examined using a Joel 1210 transmission electron microscope and images taken for ultrastructure measurements were captured at a 20 000 x magnification.

Sperm ultrastructure measurements

Sperm from 20 fish was measured, taking samples of 15 to 20 sperm cells per fish. Ultrastructural dimensions were measured using Analysis Software from images taken at 20 000x magnification, using a Joel 1210 transmission electron microscope. Sperm nuclear length, width, mid-piece length, cytoplasmic collar length and flagellum length were measured (Figure 3.5). Spermatozoa sections in which a deep *nuclear fossa* was not visible were not measured.

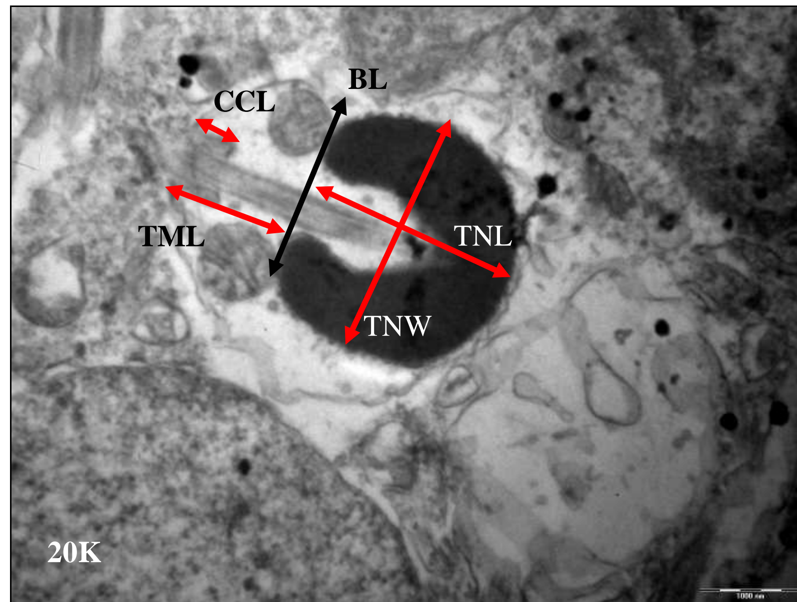


Figure 3.5: Ultrastructure measurements of *A. pectoralis* spermatozoa. TNL = total nuclear length, TNW = total nuclear width, BL = baseline for measurements, TML = total mid-piece length and CCL = cytoplasmic collar length. Measurements were taken on images captured at 20 000 x magnification under transmission electron microscopy.

Sperm flagellum measurements

Triplicate slides for each of the samples from the 20 fish were made by pipetting 5µl sperm onto each of the three slides. Slides were placed on a rack at 20ºC and allowed to dry. Dry slides were fixed for 30 seconds using RAPDIFF (Met-U-Lab) fixative. Excess fixative was poured off and slides were stained with RAPDIFF stain 2 and RAPDIF stain 3 for 30 seconds each. After staining, slides were gently rinsed in distilled water to remove excess stain and placed on the rack at 20ºC for 20 minutes. A cover slip was mounted onto the slide with DPX mountant and a drop of xylene. Slides were examined using light microscopy under a 1000 x magnification, using immersion oil.

Images of spermatozoa were captured using a JVC digital camera at a 1000 x oil magnification. Sperm flagellum length was measured using the measurement program Analysis software. Three images were taken per slide and only morphologically complete spermatozoa were measured. Spermatozoa that were broken damaged and or had their flagella tangled (Figure 3.6) were excluded from the analysis.

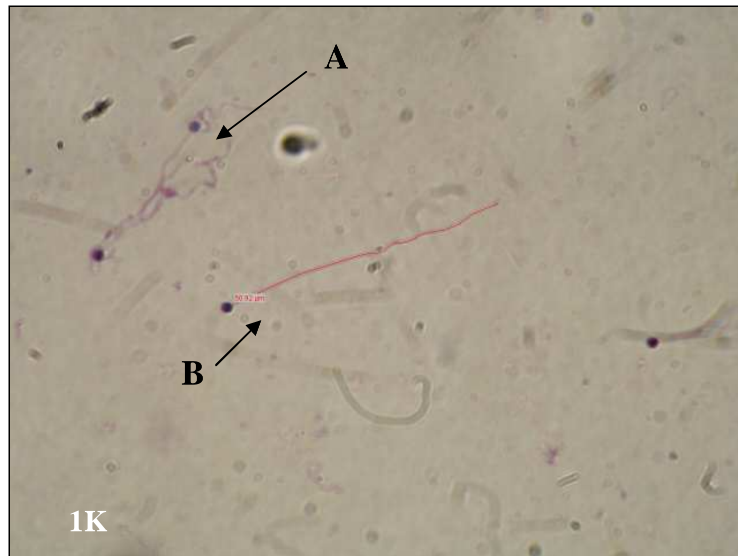


Figure 3.6: Stained spermatozoa observed through a light microscope at 1000 x oil magnification, showing tangled spermatozoa flagella for which no measurements were taken (A), and a fully intact spermatozoa (B) measured using the measurement program Analysis software.

Data Analysis

All measurements were recorded to the nearest micrometer. Measurements for each fish sample were represented by the mean, standard deviation and range**.**

Results

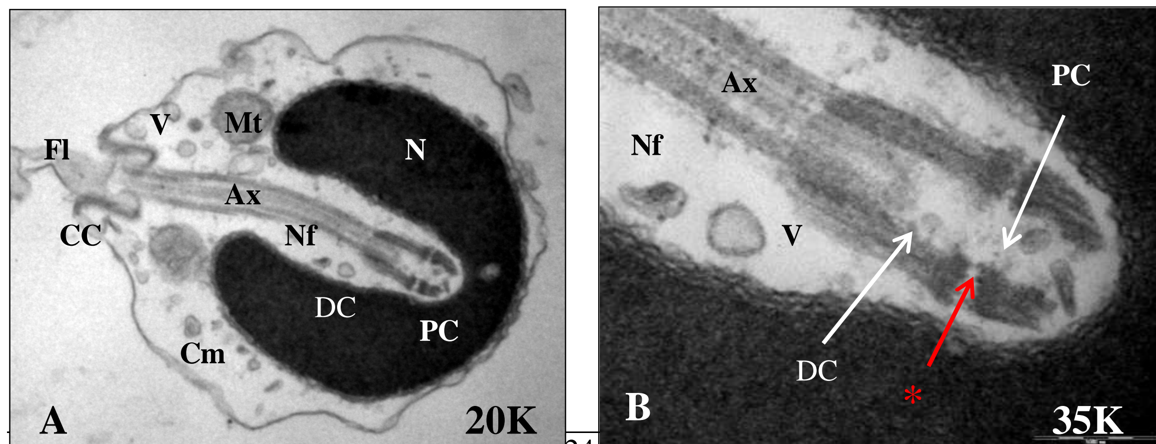
Spermatozoa

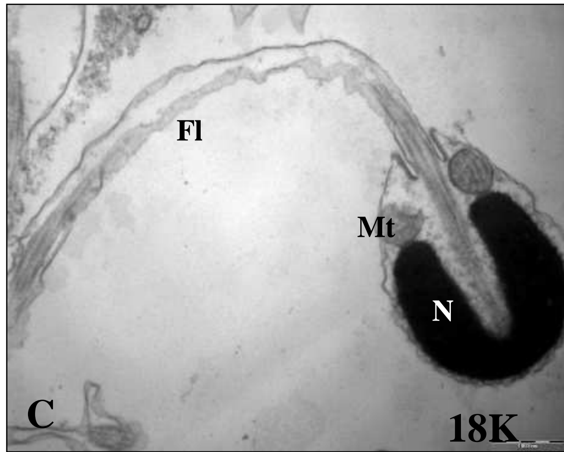
Mature spermatozoa of *A. pectoralis* consisted of an acrosome-free head, a mid-piece and a tail. The nucleus appeared as an electron-dense ovoid shape with an average length ± standard deviation of 1685.3 ± 162.4 nm (n = 18) (Table 3.1) and an average width of 1742.0 ± 165.9 nm (n = 18) (Table 3.2). The average range of nuclear length and width was 171.8 and 170.8

nm, respectively. The nucleus was enclosed by a nuclear membrane which ended at the cytoplasmic collar. Here, an axoneme with a 9 + 2 microtubule configuration penetrated into the nucleus through the deep *nuclear fossa* (Figure 3.7 A). The cytoplasmic collar had an average length of 309.3 ± 74.9 nm (n = 18) with a dense membrane-like structure on the surface adjacent to the extending flagellum. The bulk of the cytoplasmic mass was situated at the distal end of the nucleus. Vesicles (Figures 3.7 A and B) were found throughout the cytoplasmic mass and deep within the *nuclear fossa*, often surrounding the distal and proximal centrioles (Figure 3.7 B). The distal centriol (Figure 3.7 B and E) formed the basal body from which an axoneme extended. The distal centriol was aligned in a co-axial arrangement with the proximal centriol, in the direction of the longitudinal axis of the spermatozoon. Both the distal and proximal centrioles showed an electron-dense matrix at the sides adjacent to each other (Figure 3.7 B).

The total mid-piece length averaged 539.3 ± 101.8 nm (n = 18), with an average range in length of 304.4 nm (Table 3.3). A cross-section of the mid-piece showed a ring of seven spherical electron-dense mitochondria (Figure 3.7 F). The mitochondrial ring was situated within the cytoplasmic mass of the mid-piece just posterior to the nucleus (Figures 3.7 A and C). The mitochondria had longitudinal cristae which were irregular in shape. The mitochondrial ring did not protrude past the width of the nucleus (Figure 3.7 A and C).

Average flagellum length was 47.4 ± 4.8 µm (n = 13), with an average range of the length values of 19.7 µm (Table 3.4). The flagellum consisted of two lateral fins; however, three lateral fins were observed in some sections while some spermatozoa did not have any (Figures 3.7 G, H, I and J). At the base of the cytoplasmic canal the flagellum appeared to be a continuous extension of the plasma membrane of the mid-piece (Figure 3.10)

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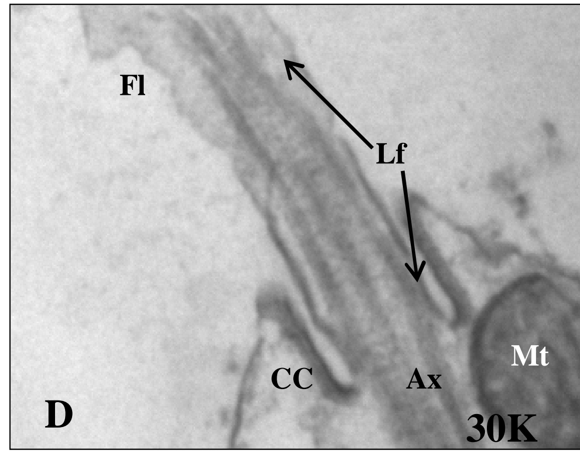
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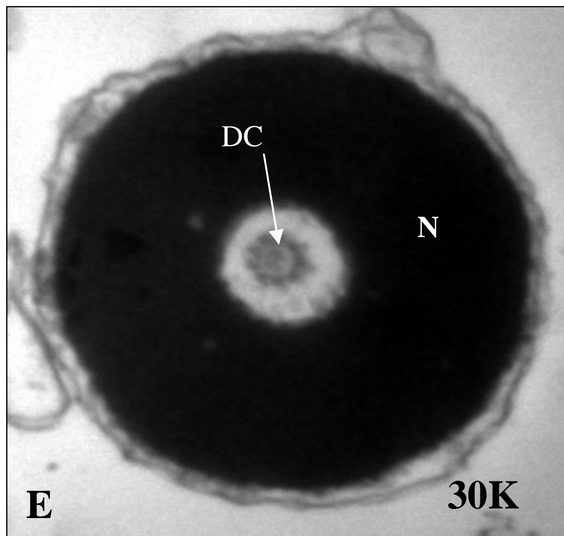
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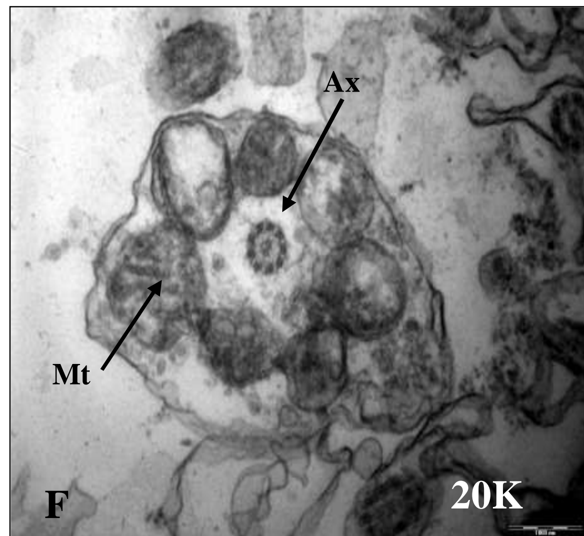
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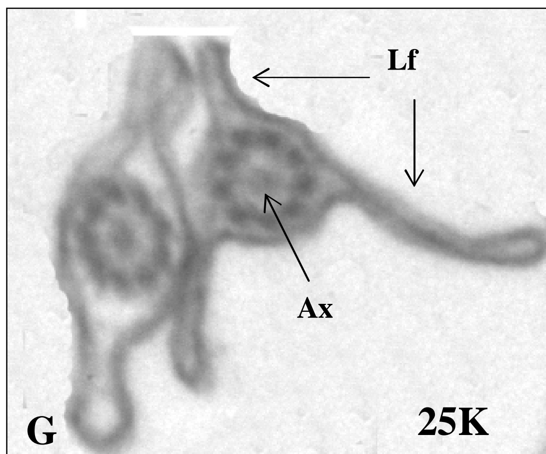
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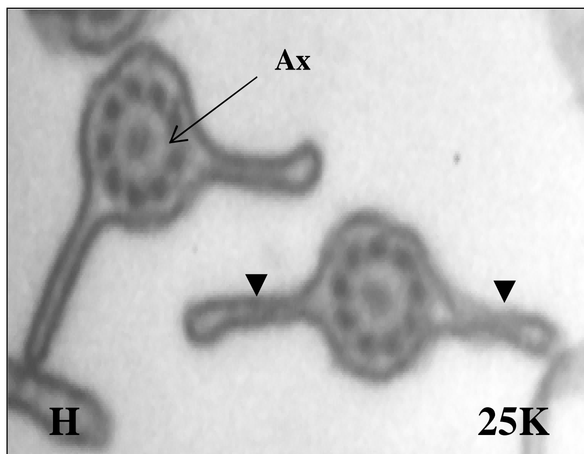
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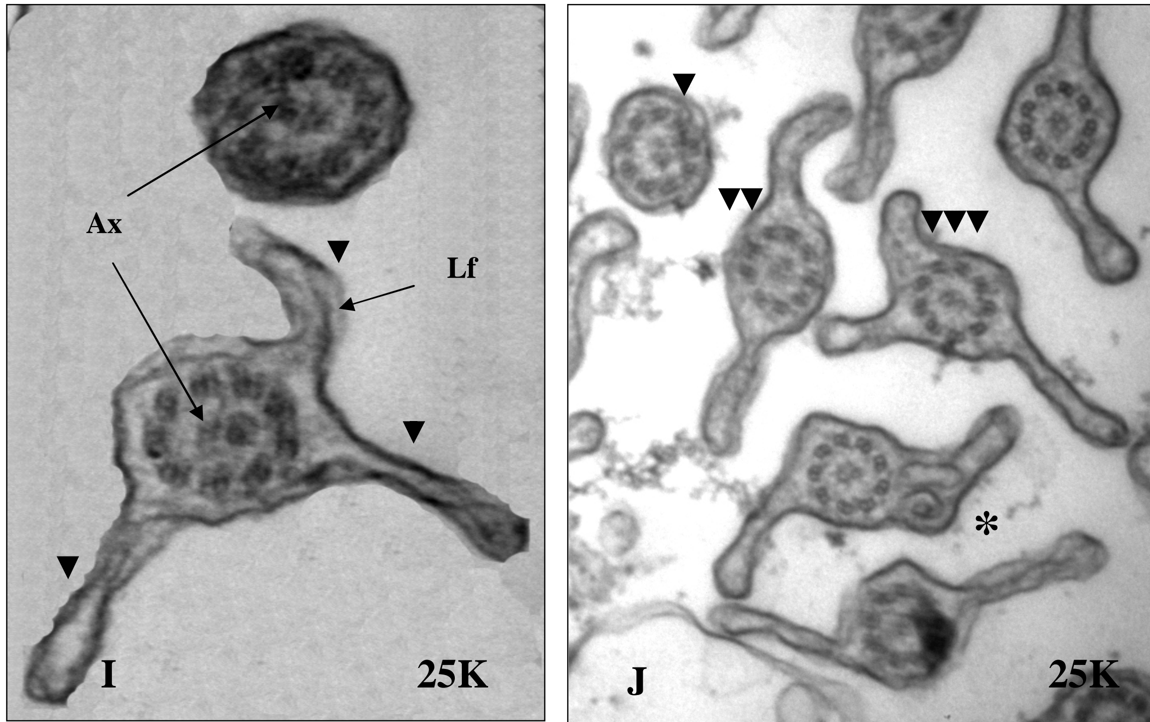

Figures 3.7 A-J Illustrate spermatozoa of *A. pectoralis* under transmission electron microscopy

Figure 3.7: A – D) Longitudinal sections through the nucleus (N) of the spermatozoa, showing the deep *nuclear fossa* (Nf) housing the distal and proximal centrioles (DC and PC). The electron-dense matrix on adjacent sides of the proximal and distal centrioles is denoted by (*). Randomly distributed vesicles (V) are present in the cytoplasmic mass (Cm) which surrounds the mitochondria (Mt). The cytoplasmic canal (CC) with a dense membrane structure on the surface adjacent to the extending flagellum (Fl) allows the axoneme (ax) to extend to the distal centriol deep within the nuclear fossa (Nf). The flagellum contains lateral fins (Lf) which start projecting from the spermatozoa as the flagellum (Fl) extends through the cytoplasmic collar (CC).

Figure 3.7: E) A cross-section through the nucleus (N) at the level of the distal centriol (DC). F) A cross-section through the mitochondrial ring, showing seven electron-dense and irregularly shaped mitochondria (Mt). The axoneme (Ax) with a 9 + 2 microtubular arrangement is present in the centre of the ring.

Figure 3.7 G – J**:** Cross-section of spermatozoan flagella (Fl), showing flagella with zero, two and three lateral fins (Lf) (number of fins denoted by arrow heads). A potential deformation of the lateral fin is denoted by (*).

Table 3.1: The mean, standard deviation (SD) and range of nuclear lengths (nm) of *A. pectoralis* spermatozoa. There were 18 animals with different numbers of measurements for each. Values from measurements of each animal were averaged.

Fish number	n	Mean ± SD	Min	Max
1	5	1819.7 ± 131.5	1683.2	2009.1
2	8	1729.2 ± 90.0	1524.3	1824.9
3	8	2000.3 ± 356.6	1664.5	2640.9
4	6	2248.9 ± 677.9	1600.4	3402.5
5	8	1804.9 ± 88.2	1641.1	1892.1
6	14	1801.5 ± 88.4	1704.2	2010.5
7	9	1431.7 ± 68.0	1349.5	1530.3
8	10	1711.1 ± 171.7	1479.3	1941.2
9	9	1438.9 ± 61.4	1370.9	1559.1
10	9	1497.9 ± 171.0	1304.4	1911.3
11	8	1534.8 ± 207.2	1342.5	1980.0
12	13	1657.9 ± 129.5	1438.0	1805.1
13	9	1481.3 ± 99.1	1310.8	1602.4
14	7	1639.7 ± 130.5	1472.6	1849.1
15	7	1661.7 ± 172.5	1427.0	1884.9
16	7	1438.9 ± 135.8	1298.1	1681.9
17	6	1556.5 ± 118.5	1403.3	1705.3
18	5	1880.6 ± 108.3	1762.5	2039.0

Table 3.2: The mean, standard deviation (SD) and range of nuclear width (nm) of *A. pectoralis* spermatozoa. There were 18 animals with different numbers of measurements for each. Values from measurements of each animal were averaged.

Fish number	n	Mean ± SD	Min	Max
1	5	1936.5 ± 121.7	1799.6	2070.7
2	8	1805.7 ± 116.2	1548.3	1913.4
3	8	2090.3 ± 307.5	1692.5	2525.4
4	6	2452.2 ± 648.7	1873.9	3573.7
5	8	1877.8 ± 112.5	1708.8	2055.7
6	14	1867.9 ± 103.0	1670.4	2059.5
7	8	1465.3 ± 142.4	1268.6	1640.5
8	10	1738.5 ± 160.0	1495.3	1972.8
9	9	1483.9 ± 41.9	1394.5	1525.8
10	9	1575.1 ± 148.1	1485.4	1958.1
11	8	1606.6 ± 220.1	1427.9	2100.4
12	13	1648.8 ± 185.4	1223.0	1865.7
13	9	1487.4 ± 70.2	1366.3	1567.7
14	7	1671.3 ± 113.7	1550.0	1840.6
15	7	1674.2 ± 96.4	1514.6	1792.0
16	7	1452.9 ± 155.7	1260.2	1695.8
17	6	1619.9 ± 108.6	1470.3	1725.1
18	5	1903.3 ± 134.7	1753.3	2113.3

Table 3.3: The mean, standard deviation (SD) and range of mid-piece length (nm) of *A. pectoralis* spermatozoa. There were 18 animals with different numbers of measurements for each. Values from measurements of each animal were averaged.

Fish number	n	Mean ± SD	Min	Max
1	4	596.2 ± 124.0	454.6	736.4
2	7	575.5 ± 61.4	494.4	692.7
3	7	703.7 ± 89.0	559.3	825.4
4	6	783.6 ± 167.4	492.9	958.8
5	7	425.5 ± 98.7	289.9	526.9
6	14	553.9 ± 158.3	354.0	897.8
7	9	460.5 ± 127.0	314.9	645.1
8	10	548.5 ± 122.6	301.9	723.4
9	9	505.0 ± 102.0	354.0	663.4
10	9	501.7 ± 138.5	199.1	692.3
11	8	492.8 ± 76.4	368.9	583.2
12	13	576.7 ± 113.2	369.4	814.9
13	9	453.0 ± 106.7	286.2	631.1
14	7	566.7 ± 65.5	488.0	645.5
15	7	545.4 ± 43.9	502.5	622.6
16	7	439.3 ± 64.0	362.7	522.1
17	6	470.4 ± 102.5	307.8	607.7
18	5	509.4 ± 72.2	404.3	588.2

Table 3.4: The mean, standard deviation (SD) and range of flagella length (nm) of *A. pectoralis* spermatozoa. There were 18 animals with different numbers of measurements for each. Values from measurements of each animal were averaged.

Fish number	n	Mean ± SD	Min	Max
1	30	48.2 ± 5.5	34.4	61.6
2	27	47.9 ± 5.9	35.4	68.1
3	12	44.0 ± 5.7	32.7	51.5
4	10	46.4 ± 4.8	38.3	52.4
5	24	48.6 ± 3.2	40.7	56.0
6	12	50.8 ± 2.4	44.6	53.7
7	7	46.0 ± 4.7	40.7	53.2
8	32	46.1 ± 4.0	37.4	52.3
9	23	45.3 ± 6.9	31.3	59.1
10	13	47.0 ± 4.9	39.9	58.6
11	34	50.5 ± 4.7	42.0	58.9
12	20	48.0 ± 5.3	37.7	58.3
13	38	48.1 ± 6.0	31.7	59.2

Testis

The testis of *A. pectoralis* is an elongated, paired organ. It conforms to an unrestricted spermatogonial testis type as spermatogonia are distributed along the entire length of the lobule (Figure 3.8 C), and are not restricted to the testis periphery. The testis is divided into the interstitial and the germinal compartment, with the latter arranged in lobules (Figure 3.8 C and D) and terminating blindly at the periphery of the testis. Branching and elongation of the lobules result in a complex formation within the germinal compartment (Figure 3.8 D). The germinal compartment contains germ cells and somatic sertoli cells, organized in spermatocysts (Figure 3.8 C). The interstitial compartment comprises of connective tissue containing fibroblast cells blood vessels and Leydig cells (Figure 3.8 C).

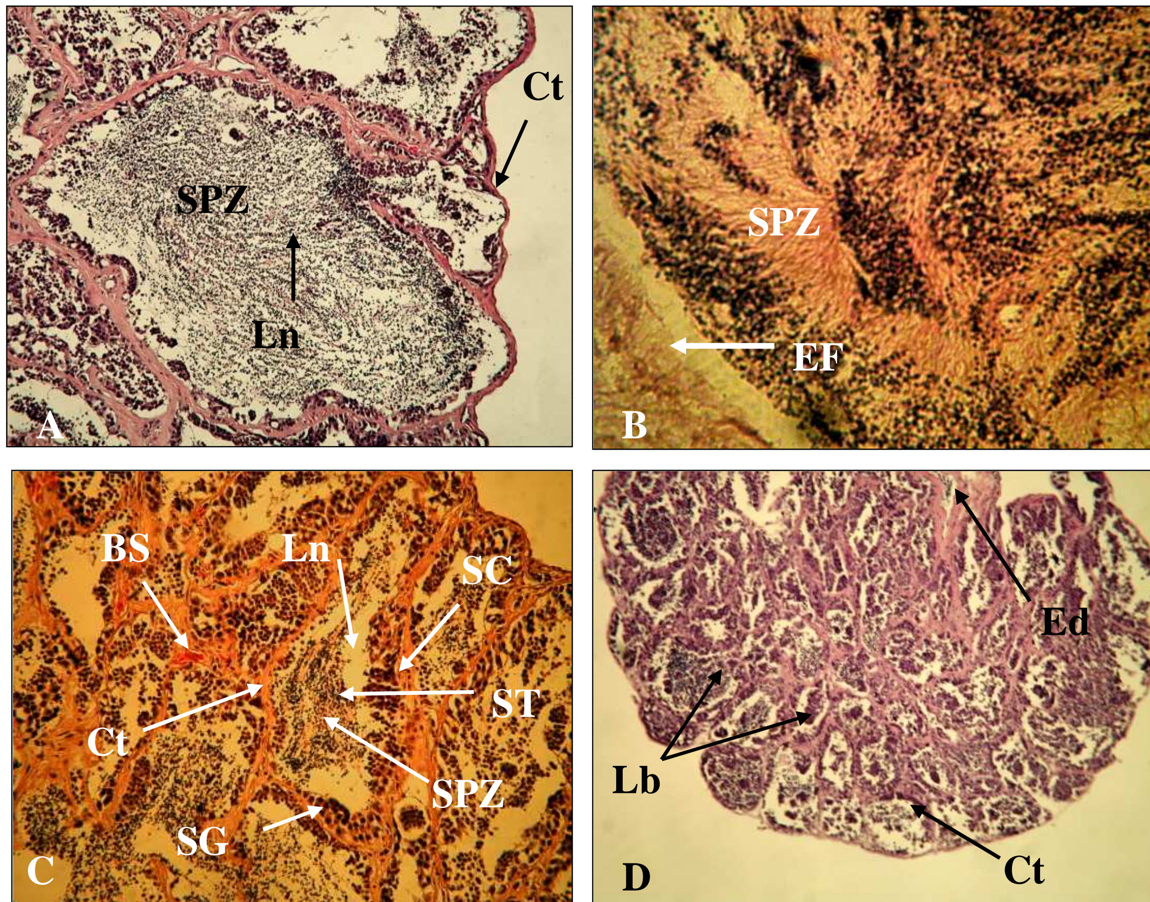

Figure 3.8: Histological sections of *A. pectoralis* testis using light microscopy. A) Mature spermatozoa (SPZ) at 400 x magnification in the lumen (Ln) of a lobule encased by connective tissue (Ct). B) Mature Sopermatozoa (SPZ) at 1000 x magnification grouped together in the lumen of a lobule prior to spawning. C) Lobules showing various cell developmental stages during spermatogenesis at 400 x magnification. The germinal compartment shows spermatogonia (SG). The differentiation of the spermatogonia occurs in the spermatocyst (SC). Haploid spermatids (ST) develop into mature spermatozoa (SPZ) which are released into the lumen (Ln) of the lobule. The interstitial compartment shows the connective tissue (Ct) and the blood supply (BS) for the testis. D) Longitudinal section through the primary testis at 100 x magnification, showing the distribution of lobules (Lb) surrounded by connective tissue (Ct) and the efferent duct (Ed).

Spermatogenesis, the process of male spermatogonial germ cell development, marks the beginning of maturity. *A. pectoralis* males (n = 54) ranging in size from 225 mm to 450 mm and weight from 80 g to 600 g were captured during the sampling period in August 2006.

All fish were mature with spermatozoa (Figures 3.8 A and B). During spermatogenesis spermatogonia undergo mitotic proliferation (Figure 3.9 A). Cellular volume reduces by the reduction of cytoplasmic mass to form primary spermatocytes (Figure 3.9 B). The first meiotic division in conjunction with chromatin condensation, mitochondrial migration and a reduction in cytoplasmic mass occurs to form secondary spermatocytes (Figure 3.9 C). A second meiotic division produces haploid spermatids (Figure 3.9 D and F). In spermatids a flagellum is present, and the mitochondria have migrated to the base of the nucleus, forming a mitochondrial ring in the cytoplasmic mass, the mid-piece. Further chromatin condensation occurs, before a spermatid becomes a spermatozoon (Figure 3.9 E and F). Spermatozoa are released into the lumen of the lobules from where they migrate to the efferent ducts where they are stored until spawning (Figures 3.8 A and B).

Figures 3.9 A – E: The cellular changes associated with spermatogenesis. (Transmission electron microscopy at 20 000 x magnification) F: A scanning electron microscopy image of mature spermatozoa (SPZ) and immature spermatids (SPT) at 10000 x magnification.

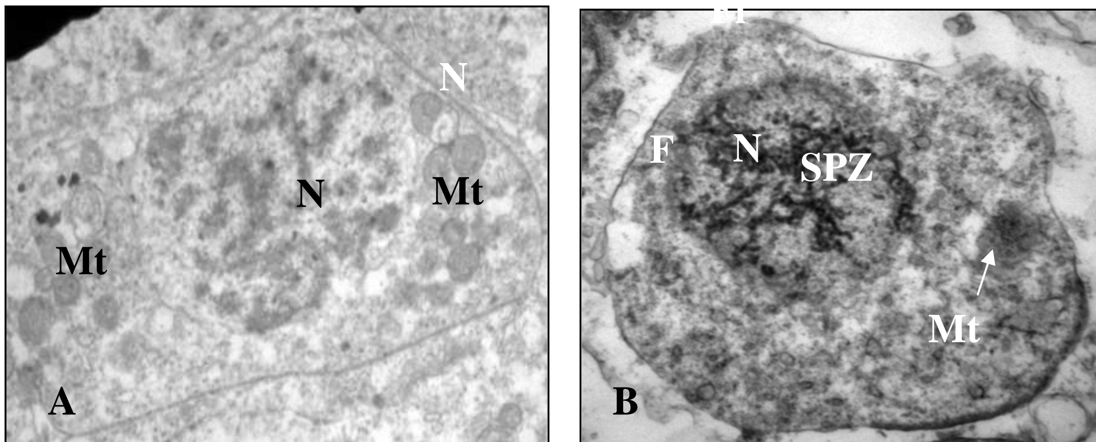

Figure 3.9 A: A large spermatogonia cell with numerous mitochondria (Mt) and a non-condensed nucleus (N) due to active mitotic proliferation.

Figure 3.9 B: Primary spermatocyte with a granular nucleus (N) and a low number of mitochondria (Mt).

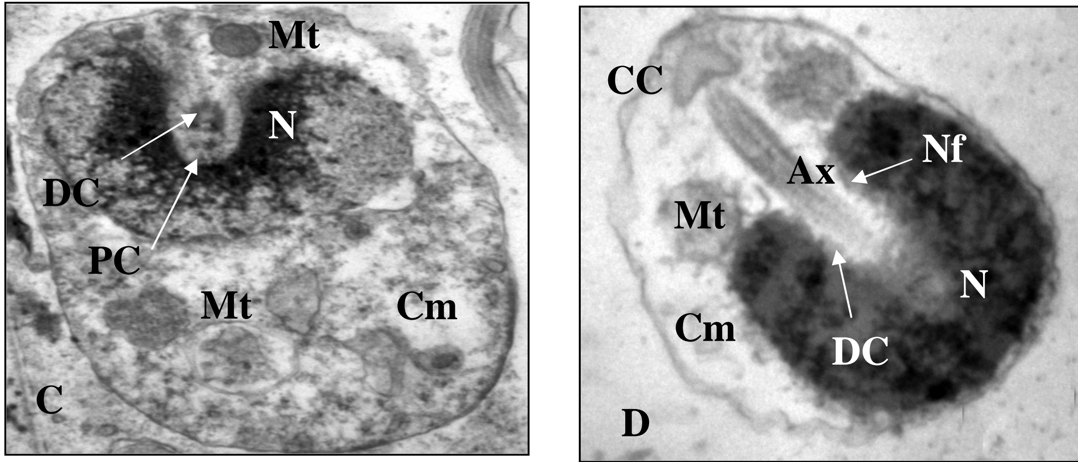

Figure 3.9 C: Secondary spermatocytes showing mitochondria (Mt) migrating to the base of the nucleus. There is a reduction in cytoplasmic mass (Cm) and condensation of the nucleus (N) occurs.

Figure 3.9 D: Meiotic divisions produce a haploid spermatid. In the spermatid the mitochondria (Mt) forms a ring, which surrounds the axoneme (Ax). The axoneme extends into the *nuclear fossa* (Nf) to the distal centriol (DC). A flagellum (Fl) is present at this stage. Further condensation of the nucleus (N) and reduction in cytoplasmic mass (Cm) occurs as spermatids mature into spermatozoa.

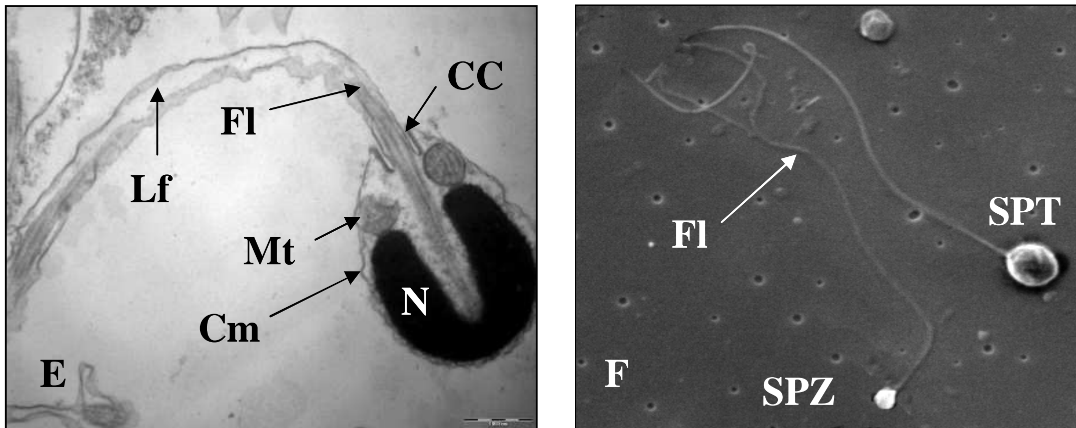

Figure 3.9 E: Mature spermatozoa showing a condensed electron-dense nucleus (N), reduced cytoplasmic mass (Cm) and a functional flagellum (Fl) with lateral fins (Lf) extending from the cytoplasmic collar (CC).

Figure 3.9 F: Scanning electron microscope image of a large immature spermatid (SPT) and a smaller mature spermatozoon (SMZ) with flagella (Fl) at 10 000 x magnification.

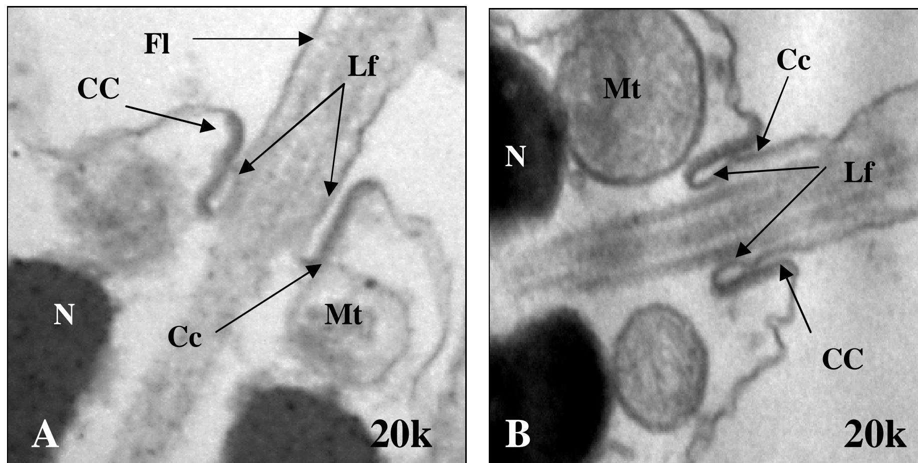

Figure 3.10 A: Spermatozoon of *A. pectoralis* under transmission electron microscopy at 20000 x magnification, showing the lateral fin projections (Lf) identified on the flagellum (Fl) within the cytoplasmic canal (Cc). The lateral fins show a continuous extension of the plasma membrane of the mid-piece

Figure 3.10.B. Mitochondria = (Mt), cytoplasmic collar = (CC) and nucleus = (N).

Discussion

Testis description

The testis appeared as a bi-lobed (a primary and a secondary lobe) elongated, milky white organ surrounded by a connective tissue capsule, the *t. albuginea*. The primary lobe of *A. pectoralis* was situated on the dorsal surface of the rib cage. The secondary lobe was situated on the ventral side. The efferent ducts of the primary and secondary lobes joined at the urogenital pore.

The vertebrate testis comprises two compartments the germinal and the interstitial, each with its own function (Grier and Neidig 2000). Furthermore, the testis can be classified into two morphological designs with respect to the organization of spermatogonia within the germinal compartment (Weltzien *et al*. 2004). The 'restricted' anastomosing tubular testis type is where the germinal compartment forms a highly complex system of interconnected tubules. This type is only reported for lower fish species such as the Northern pike *Esox lucius*, rainbow trout *O. mykiss*, Florida gar *Lepisosteus platyrhinchus* and goldfish *Carassius auratus* (Grier and Neidig 2000, Weltzien *et al*. 2004). In the 'unrestricted' lobular testis type the germinal compartment is arranged into blindly ending lobules at the periphery of the testis. This

arrangement is characteristic of higher fish species for example perciformes (Grier and Neidig 2000, Weltzien *et al*. 2004). Similar to Atlantic halibut *H. hippoglossus* (Weltzien *et al*. 2004) and flounder *P. flesus* (Lye *et al*. 1998) *A. pectoralis* conforms to the ‘unrestricted’ lobular testis type, based on the identification of spermatocytes with spermatogonia in the central parts of the lobules (Weltzien *et al*. 2004).

Spermatogenesis

Spermatogenesis can be divided into four processes: Firstly, spermatogonial cells undergo mitotic proliferation, resulting in differentiated spermatogonia. Secondly, the differentiated spermatogonia undergo the first meiotic division and become primary spermatocytes. A reduction in cytoplasmic mass combined with condensation of the chromatin network results in secondary spermatocytes. Thirdly, the secondary spermatocytes undergo a second meiotic division which produces haploid spermatids and, fourthly, the spermatids differentiate into mature flagellated spermatozoa (Weltzien *et al.* 2004). Each of the above processes was observed in *A. pectoralis*.

The time required for spermatogenesis was not determined as this would have required repeated sampling over time. However, 54 *A. pectoralis* had been sampled in August 2006, and estimated two months prior to the onset of spawning, as described by Hecht (1976). Each testis contained mature spermatozoa, observed through histology. The testes comprised two types: a large white, milky and swollen section, containing mature spermatozoa and a smaller, firm, reddish, non-swollen testis. In the latter section, only a small number of mature spermatozoa could be observed. Thompson (2004) reported no pronounced difference in testis size during the spawning season in the white-margined sole *D. marginatus* as spermatogenesis occurred all year round. This is in contrast to the Atlantic halibut *H. hippoglossus*, where distinct seasonal variations in absolute and relative testicular size and developmental stage were found (Weltzien *et al.* 2002). Here, spermatogenesis in *H. hippoglossus* was prenuptial, whereby reproductively quiescent fish with fully regressed testis enter into a testicular growth phase prior to spawning. Further investigations should acquire spermatozoa prior to, during and post spawning to determine the onset of spermatogenesis and assess sperm quality as spawning season progresses. This information can help understand the reproductive physiology of male *A. pectoralis*.

Spermatozoa

The spermatozooa of *A. pectoralis* measured 47.4 ± 4.96 µm total length, which was similar to that reported for turbot *S. maximus* at 45 µm total length (Suquet *et al.* 1993). The morphology of *A. pectoralis* spermatozoa conforms to that of a primitive type I aquasperm, typically found in fish species using external fertilization (Jamieson 1991).

The nucleus was devoid of an acrosome. This is justified by the presence of a micropyle in the eggs, which facilitates the penetration of spermatozoa into the egg (Suquet *et al.* 1993). The nucleus was ovoid to round in shape and had an electron-dense matrix. The nucleus measured 1.68 ± 0.16 µm in length and 1.74 ± 0.16 µm in width. This is similar to that described by Jamieson (1991), where the general morphology of an aquasperm nucleus was round to ovoid in shape with a diameter of 2-3 µm. Internally fertilizing species, such as *L. chalumnae* have an elongate nucleus, with a length of 25 µm (Jamieson 1991).

The nucleus and the mid-piece were surrounded by a plasma membrane, which extended to the cytoplasmic collar. A reduction in cytoplasm occurred around the nucleus during cell maturation, thus providing an indication of the maturity of the sperm cell. The maturity of spermatozoa is important in determining an appropriate time for cryopreservation, as immature spermatozoa may have reduced tolerance to freezing (Suquet *et al.* 2000). Using sperm maturity as an indicator for timing the cryopreservation of *A. pectoralis* spermatozoa may reduce the decline in motility, storage and fertilization capacity, as well as alteration of the plasma membrane that was reported for turbot, *S. maximu*s spermatozoa as the milting season progressed (Suquet *et al*. 2000, Dreanno *et al*. 1997). Furthermore the highest rate of motility for winter flounder, *P. americanu*s spermatozoa was at the beginning of the milting season (Shanngguan and Crim 1995).

The nucleus had a deep *nuclear fossa*, which has been reported for pleuronectiform species. The proximal and distal centrioles were housed deep within the *nuclear fossa*, which is considered a plesiomorphic state amongst neopterygians (Jamieson 1991). The proximal and distal centrioles were in co-axial alignment, with the proximal and distal centriols located in the same direction as the longitudinal axis of the spermatozoa. The same coaxial arrangement of centrioles was observed in turbot *S. maximus* spermatozoa (Medina *et al*. 2000), however the arrangement of the centrioles in *S. maximus* was reported to be at right angles and not co-

axially aligned (Suquet *et al.* 1993). The difference, however, between *S. maximus* sperm when compared to Soleidae sperm was the anterior depression of the nucleus in *S. maximus* sperm (Medina *et al.* 2000; Suquet *et al.* 1993). The anterior depression of the nucleus was also reported in *Paralichthys olivaceus*, but not in any other pleuronectiform species (Hara and Okiyama 1998).

Mid-piece

The mid-piece length of *A. pectoralis* averaged 0.53 ± 0.10 µm with a range of 0.30 µm. A short mid-piece with respect to internally fertilizing teleosts was observed in *S. maximus* (Suquet *et al.* 1993) and in the Nile pebblefish *Alestes dentex* (Shahin 2006) the latter having a mid-piece length of 0.5 µm. A reduced mid-piece is consistent amongst teleosts that use external fertilization (Suquet *et al.* 1993; Mattei 1991). In species that reproduce internally, the spermatozoa have characteristically elongated mid-piece (Mattei 1991). The mid-piece reaches its maximum length at 45 µm in the osteoglossoid *Pantodon bucholzi* (Jamieson 1991). Other species with a relatively long mid-piece include the hagfish *Eptatretus stoutii* (12 µm) and the coelacanth *L. chalumnae* (5 µm) (Jamieson 1991).

A cross-section of the mid-piece had seven mitochondria arranged in a ring-structure at the opening of the *nuclear fossa* and the proximal region of the flagellum. The mitochondria of *A. pectoralis* were irregular in shape and size, had cristae and an electron-dense matrix. Mitochondria of turbot *S. maximus* and flounder *P. flesus* showed a collar-like appearance and comprised of eight to ten irregularly shaped electron-dense mitochondria, respectively (Suquet *et al.* 1993; Jamieson 1991). The mitochondrial variation observed in *S. maximus* could be linked to the maturation stage of the spermatozoa (Suquet *et al.* 1993). The number of mitochondria in *S. senegalensis* was variable, with more than eight mitochondrial profiles (Medina *et al.* 2000); however, the variable mitochondrial profile did not appear to be linked to the maturation state of the spermatozoa.

A mid-piece length of 3.5 µm and a mitochondrial count of 70 were reported for the chondrichthyan *H. colliei* (Jamieson 1991), whilst a single large mitochondrion with a diameter of 0.92 µm was found in *S. gracilis* (Clupeiformes) (Gwo *et al.* 2006).

The relationship between mid-piece size and number of mitochondria may influence swimming speed. Mitochondrial variation and number with respect to mid-piece size is poorly

documented. However, a study conducted on Iberian red deer showed that spermatozoa with longer mid-pieces swim more slowly, a finding which does not support the hypothesis that the size of the mid-piece determines the amount of energy translated into swimming speed (Malo *et al.* 2006). *A. pectoralis* is an externally fertilizing species, with spermatozoa of the ect-aquasperm type. A small mid-piece length of 539.3 nm, housing seven mitochondria in a ring formation and a long flagella 47.4 µm, suggests that *A. pectoralis* spermatozoa need to move quickly through the water column to fertilize a free floating egg, or that they need to overcome turbulences created by the close proximity of the male and female during spawning. Fast swimming speeds may help reduce the distance between the dispersing gametes within the water column. The distance between gametes during spawning may, however, be better understood by evaluating the duration of motility of the spermatozoa. Given that *A. pectoralis* spermatozoa are fast swimmers, if they are able to sustain their activity over prolonged periods of time, this may suggest that the gametes are far apart upon release, or that the environment in which they are released is particularly turbulent. Further investigations of swimming speed, with respect to mitochondrial variation and mid-piece length and motility duration, need to be conducted before information regarding the predictive ability of sperm morphology can be used to accurately determine spawning strategies of fish species.

The mid-piece terminates at the cytoplasmic collar, a folding of the cytoplasmic membrane forming a shallow collar-like structure which extends around the base of the flagellum from which it is separated by a periaxonemal space (cytoplasmic canal) (Jamieson 1991). The cytoplasmic canal can separate the flagellum from the mid-piece (Gwo *et al.* 2006). The internal membrane of the cytoplasmic collar had a thickened dense appearance. A study to investigate the hypothesis that the thickened dense appearance of the internal membrane of the cytoplasmic collar may provide structural support for the movement of the flagellum during swimming should be conducted. This information could provide useful information regarding the movement of the flagellae with respect to the swimming dynamic of the spermatozoa.

Tail

The axoneme forms the cytoskeletal structure within the flagellum, providing support and structure to the flagellum primarily during movement. The characteristic feature of the axoneme is the nine pairs of "doublet" microtubules, forming a ring around a single central pair of microtubules, to create a '9 + 2' arrangement of microtubules.

This configuration is reported for many teleosts (Mattei 1988), however Nile pebblefish *A. dentex* and eel *Anguilla anguilla* both show a '9 + 0' axoneme configuration (Shahin 2006; Billard and Ginsburg 1973, in Suquet *et al.* 1993).

A cross section of the nucleus and mid-piece of *A. pectoralis* showed the axoneme with a '9 +2' microtubule configuration extending from the distal centriol deep within the *nuclear fossa* to the cytoplasmic collar of the mid-piece.

A single flagellum was encased by a plasma membrane, which extended as one, two or three longitudinal flat projections, called lateral fins (Jamieson 1991). The presence of lateral fins appears to be common amongst pleuronectiformes (Medina *et al*. 2000). Both turbot *S. maximus* and Senegal sole *S. senegalensis* spermatozoa showed two lateral fins; however, variations including zero and one lateral fin were observed (Suquet *et al.* 1993; Medina *et al.* 2000). Cross-sections through the flagella of *A. pectoralis* showed mostly two but also zero and three lateral fins. In S. *senegalensis* lateral fins were not present at the proximal end of the flagella, the region at which the flagellum passes through the cytoplasmic canal (Medina e *al.* 2000). In *A. pectoralis* the lateral fins were an extension of the plasma membrane of the mid-piece and, therefore, occurred within the cytoplasmic canal (Figures 3.10 A and B). At the distal end of the flagella in *S. senegalensis,* no lateral fins were observed and the flagella had a cylindrical shape (Medina *et al*. 2000). Cross-sections of *A. pectoralis* flagella with no lateral fins were cylindrical. It is hypothesised that there are no lateral fins at the terminal end of the flagella, as shown in *S. senegalensis*.

A description of type 1 aquasperm by Jamieson (1991), showed that the plasma membrane of the flagellum may extend as one, two or sometimes three longitudinal, flattened, fin-like projections. In *A. pectoralis,* three lateral fin projections were observed in 14 of the 18 fish sampled, where as zero and two lateral fin projections were recorded in each of the 18 fish sampled. Future investigations should quantify the relative occurrence of spermatozoa with three lateral fins. This information may suggest that the presence of three lateral fins could potentially influence the functioning of the spermatozoa by enhancing or reducing the sperm's efficiency to swim and fertilize. Further investigations should be conducted to test the hypothesis that the three lateral fin projections observed in *A. pectoralis* spermatozoa may influence buoyancy and/or swimming speed and fertilization rate. They may also be the outcome of mutation events that have no advantage to spermatozoa function.

Chapter 4: Cryopreservation Protocol

Introduction

During cryopreservation, cells or tissues are preserved by cooling them to sub-zero temperatures, usually -196ºC, which is the boiling point of liquid nitrogen. Freezing and thawing of biological material involves a series of complex and dynamic physiochemical processes of heat and water transport between cells and their surrounding medium (Leung 1991). A cryopreservation protocol can be divided into four categories, gamete or tissue collection, freezing, storage, and thawing. Empirically derived protocols for one species may not apply to other species. Woods *et al.* (2004) highlighted a complication in cryobiology, by stating that "the unavoidable variation in defining a successful cryopreservation protocol lies within the complications of biology". Optimization of a cryogenic protocol depends on the cell type of a species and its susceptibility to storage at sub-zero temperatures (Woods *et al.* 2004).

Cryopreservation

Cryopreservation of gametes is routinely applied in animal husbandry. Artificial insemination and embryo transfer in the livestock industry have been made possible through the cryopreservation of sperm and embryos (Chao and Liao 2001; Woods *et al.* 2004; Muchlisin *et al.* 2004). The benefits of cryopreservation to terrestrial plant and animal breeding programs caused an interest in its use for the propagation of aquatic organisms (Chao and Liao 2001). The first successful trials of cryopreservation of herring sperm *Clupea harengus* were reported by Blaxter (1953). Cryopreservation of sperm has been applied in about 200 finfish species (Zhang *et al.* 2003), for example, tropical bagrid catfish *Mystus nemurus* (Muchlisin *et al*. 2004), red drum *Sciaenops ocellatus* (Wayman *et al.* 1998), turbot *S. maximus* (Chen *et al*. 2004), Tilapia *Oreochromis* spp. (Rana and Mcandrew 1989), sea perch *Lateolabrax japonicus* (Ji *et al*. 2004), yellowtail flounder *P. ferrugineus* (Richardson *et al.* 1999), Japanese eel *Anguilla japonica* (Tanaka *et al.* 2002), rainbow trout *O. mykiss* (Cabrita *et al*. 2001), flounder *Paralichthys olivaceus* (Zhang *et al*. 2003) and winter flounder *Pseudopleuronectes americanus* (Rideout *et al.* 2003). There are a few published studies on shellfish species including Pacific oyster *Crassostera gigas* and *Crassostera tulipa.* (Dong *et al*. 2005; Yankson and Moyse 1991) and abalone *Haliotis diversicolor supertexta* (Gwo *et al.* 2002).

Fewer studies have been published about cryopreservation of shellfish and finfish embryos (Chao and Liao 2001). Cryopreservation research focuses, primarily, on economically important species with respect to food production, and in the context of species conservation.

Gamete cryopreservation of aquatic organisms is a novel field with little progress on a commercial scale (Chao and Liao 2001; Ji *et al*. 2004). Benefits to aquaculture include a reliable supply of gametes for hatchery production (Chao and Liao 2001; Fabbrocini *et al*. 2000; Rideout *et al.* 2003; Muchlisin *et al.* 2004; Carolsfeld *et al*. 2003; Richardson *et al.* 1999), easy and economic transport of gametes (Chao and Liao 2001; Richardson *et al*. 1999), improved selective breeding and production of hybrids (Chao and Liao 2001; Fabbrocini *et al*. 2000), an increased number of offspring from selected males (Rideout *et al*. 2003; Richardson *et al*. 1999) and improved utilization of each male within the broodstock. Benefits to fisheries' conservation programmes include the ability to maintain gene banks of endangered species genes (Carolsfeld *et al*. 2003; Ji *et al*. 2004; Chao and Liao 2001; Otha *et al*. 2001; Richardson *et al*. 1999).

Cryoprotective agents

Cryo-injuries, associated with cooling and thawing, could be reduced through the addition of non-permeating and permeating organic cryoprotective compounds (Table 4.1) to the dilutants (Bromage and Roberts 1995). Cryoprotective agents (CPA), such as dimethyl sulphoxide and glycerol, can bind water, lower the homogenous nucleation temperature, raise the glass transformation temperature, reduce ice crystal formation and bind electrolytes to prevent them from concentrating in the residual unfrozen solution, thereby decreasing the freezing point of intracellular fluid during freezing (Gwo 2000, Leung 1991). Non-permeating cryoprotectants include sugars, e.g., sucrose and glucose, polymers, for example., dextran, hydroxyethyl starch and proteins, e.g. egg yolk, bovine serum albumin. As these compounds do not enter the cells, their cryoprotective ability can complement a permeating CPA by lowering the freezing point and raising the glass transformation temperature of the extra-cellular solution (Leung 1991). The choice of cryoprotective agent is based upon permeability, water solubility and toxicity towards cells (Chao and Liao 2001). The toxicity of a cryoprotectant depends on its type and concentration, the equilibration time prior to freezing and temperature during the loading of samples (Chao and Liao 2001). Some compounds destroy gametes

during pre-treatment and post-thawing by denaturing cellular proteins, thus reducing cell viability (Bromage and Roberts 1995).
It is, therefore, important to determine cryoprotective efficiency and the toxicity tolerance of the cell types that will be cryopreserved (Chao and Liao 2001).

Since the introduction of glycerol by Pogle *et al*. (1949) and dimethyl sulphoxide (DMSO) by Lovelock and Bishop (1959), many cell and tissue types have been cryopreserved (Woods *et al*. 2004). Several cryoprotective agents are available (Table 4.1); however DMSO, glycerol and methanol are most widely used in fish gamete preservation (McAndrew *et al*. 1993). In the cryopreservation of marine fish sperm good results, have been obtained by using DMSO at a concentration between 5 and 20% (Wayman *et al*. 1997; Bromage and Roberts 1995; Gwo 2000). This has been reported in turbot *S. maximus* (Dreanno *et al*. 1997; Chen *et al*. 2004), seabream *Sparus aurata* (Fabbrocini *et al*. 2000), shortnose sturgeon *Acipenser brevirostrum* (Horváth *et al*. 2005), yellowfin seabream *A. latus* (Gwo 1994) and yellowtail flounder *P. ferrugineus* (Richardson *et al*. 1999). Propylene glycol and ethylene glycol have been chosen for their low toxicity and their ability to penetrate cells (Gwo 1994). Propylene glycol proved to be effective for yellowtail flounder *P. ferrugineus* and Atlantic halibut *H. hippoglossus* (Billard *et al*. 1993, Richardson *et al*. 1999); however, ethylene glycol appeared to be toxic to turbot *S. maximus* sperm (Dreanno *et al*. 1997). Methanol showed cryoprotective potential for sperm of barramundi *Lates calcarifer* (Leung 1987), Atlantic croaker *M. undulatus* (Gwo *et al*. 1991), yellowtail seabream *A. latus* (Gwo 1994), summer whiting *Sillago ciliate* (Young *et al.* 1992) and turbot *S. maximus* (Dreanno *et al*. 1997). The optimum concentration may, however, vary between cryoprotectants, fish species, equilibration time and criteria used for post-thaw evaluation (Bromage and Roberts 1995). For *Oreochromis niloticus,* sperm methanol was the best cryoprotectant at a concentration of 10%, whereas 5% methanol was suggested for *O. mossambicus* sperm with regard to post-thaw motility (Bromage and Roberts 1995; Rana and McAndrew 1989).

Table 4.1: Type of penetrability of cryoprotective agents. As described by Denniston *et al*. (2000).

Penetrating cryoprotective agents	Non-penetrating cryoprotective agents
Dimethyl sulfoxide (DMSO)	Polyvinyl pyrolidone
Glycerol	Hydroxyethyl starch
Ethylene glycol	Dextrans
Methanol	Albumin
Dimethyl acetamide	Polyethylene glycol

Extender solutions

Undiluted gametes are not suitable for freezing. They must be diluted with an extender solution, i.e., a physiological solution comprising mainly of salts and various organic compounds (Leung and Jamieson 1991). Extender solutions range in complexity from solutions which mimic the chemical composition of seminal plasma to a glucose solution (Cloud 2000). In freshwater and marine species, the composition of the extender solution influenced post-thaw fertility of spermatozoa; however there was no extender solution that maximized post-thaw fertility of all species (Cloud 2000). The extender solution acts by osmotically inhibiting sperm activation prior to cryopreservation. Extender compositions reported in two studies on turbot are presented in Table 4.2, of which MMM supplemented with 10 % Bovine Serum Albumin (BSA) showed best results (Dreanno *et al*. 1997). Post-thaw motility of sperm frozen in TS-2 extender was higher than for sperm frozen in Modified Place Ringers Solution (MPRS) at a motility score of 73.7% and 50%, respectively (Chen *et al*. 2004). However, Dreanno *et al*. (1997) reported no significant difference between Modified Mounib Medium (MMM) and Modified Ringers Medium (MRM), although a lower variability of sperm motility was recorded using MMM. There is no universally applicable extender, and every species' spermatozoa have different cryobiological properties and sensitivity to cryopreservation (Woods *et al*. 2004).

Table 4.2: Composition of extenders used for turbot *S. maximus* spermatozoa (Dreanno *et al*. 1997, Chen *et al*. 2004). MMM = Modified Mounib Medium; MRM = Modified Ringer Medium; MPRS= Modified Place Ringers Medium.

	MMM	MRM	MPRS
Osmolality (mOSm/kg)	310	200	202
Bovine Serum Albumin (mg/ml)	10	10	
Tris-Cl (mM)			1.3
Reduced glutathione (mM)	6.5		1.8
Sucrose (mM)	125		
$CaCl_2$ (mM)		1.8	
KCL (mM)		26.8	5.23
$KHCO_3$ (mM)	100		
$NaHCO_3$ (mM)		1.59	3.0
D-Glucose (mM			55.55
NaCl (mM)		74	60.35
pH	7.8	8.0	6.6

Osmotic and volume effects on cells

Chemical reactions associated with cryopreservation are the result of osmotic properties of the cell (Denniston *et al*. 2000). The cell comprises water, organelles, dissolved salts, sugars, proteins and lipids, all surrounded by a semi-permeable membrane which provides a barrier to larger molecules. The movement of water across cellular membranes helps balance concentrations of compounds extra- and intra-cellularly (Denniston *et al*. 2000). This solute concentration is measured in osmolality with a range between 280-310 mosM/kg in animal cells (Denniston *et al*. 2000).

During cryopreservation freezing reduces cellular water content. Both extra-cellular and intra-cellular solutions become more concentrated (Leung 1991) which could reduce post-thaw cell viability. Cellular injuries resulting from high solute concentrations include membrane injuries, due to the denaturing effects of salts on membrane lipoproteins, or due to direct osmotic effects of concentrated solutions on the cellular membrane (Leung 1991). Shrinkage of the cell during freezing is caused by hyperosmosis due to extra-cellular ice formation

(Leung 1991). Furthermore, cryo-injuries can occur if the cellular membrane at the point of convexity becomes compressed at the sides and stretched at the point of concavity (Leung 1991). During rapid thawing, solutes can not leave the cells fast enough, thus allowing an influx of water into the cell (Leung 1991). Swelling of cells stretches cellular membranes, which may cause considerable cellular damage, including leakage of macromolecules, destruction and denaturing of proteins, structural deformation of cell organelles and abnormalities in sperm chromatin structure (Li *et al*. 2006). The design of a cryopreservation protocol requires an understanding of both static and dynamic osmometric behavior of the cells to be cryopreserved. Such information can be used to understand volumetric responses (Chao and Liao 2001) of cells to cryopreservation and to develop a freezing protocol which minimizes cry-oinjuries resulting from osmometric and volumetric changes.

Freezing rates

The freezing of teleost milt generally requires a two-step-procedure. The milt is first cooled in liquid nitrogen vapor either on a floating tray or raft, or suspended in the neck of the Dewar flask, and then plunged into liquid nitrogen at -196ºC for storage (Suquet *et al*. 2000). The freezing protocol varies among species (Chao and Liao 2001; Suquet *et al*. 2000) and the rate at which cells are cooled influences post-thaw survival (Denniston *et al*. 2000). If cells are cooled too rapidly, adequate dehydration does not occur and intracellular ice formation will cause damage to cell membranes and organelles. If the cooling rate is too slow cells will be exposed to a high concentration of solutes for too long. This may result in post-thaw injury of cells based upon osmotic and volumetric changes.

No significant differences in fertilization rate were found among freezing rates ranging from 10ºC/minute to 150ºC/minute for Atlantic croaker sperm (Gwo *et al*. 1991). The optimum freezing rate ranged from 20° to 154ºC/minute for yellowfin seabream sperm (Gwo 1994). Freezing rates between 5° and 50ºC/minute for *O. niloticus* sperm had no significant effect on post-thaw fertilization success (Rana and McAndrew 1989). A freezing rate of 40ºC/minute from 5ºC to 120ºC/minute was successfully adopted for striped bass sperm *Morone saxatilis* (He and Woods 2004). A simple successful freezing protocol was adopted by Rideout *et al*. (2003) for winter flounder *P. americanus*, where straws were floated 5.5 cm above liquid nitrogen for 12 minutes before being immersed in liquid nitrogen.

The freezing protocol for sea perch sperm *L. japonicus* (Ji *et al*. 2004) included equilibrating cryovials for 10 minutes in liquid nitrogen vapour at 2,6cm and 13 cm above the liquid nitrogen surface, followed by 5 minutes on the surface of liquid nitrogen before immersion. Equilibration at 6 cm above the liquid nitrogen surface for 10 minutes resulted in a linear temperature decrease from 16° to -15ºC at rate of 31ºC/minute. The freezing rate from -12° to -180ºC was 18.6ºC/minute. Ji *et al*. (2004) showed that equilibration at 6 cm above the surface of liquid nitrogen for 10 minutes resulted in a 73.3% motility score, which was significantly higher than equilibrating at 2 cm (41.7%) and 13 cm (48.3%). Two freezing rates for sea bream *Sparus aurata* were compared by Fabbrocini *et al*. (2000). These were 15ºC/minute and 10ºC/minute each from 0° to -150º C. Motility scores after cryopreservation were higher at the slow freezing rate. A successful freezing rate of 45ºC /minute for red drum *Sciaenops ocellatus* sperm was adopted until samples reached -80ºC when they were transferred into a liquid nitrogen Dewar flask for storage. Turbot *S. maximus* sperm should be cooled at 99ºC/minute; at 46ºC/minute and 148ºC/minute post-thaw motility assessed 10 seconds post-activation decreased by 8% and 30%, respectively (Dreanno *et al*. 1997).

Thawing rate

Even under optimal freezing conditions a small amount of thermodynamically unstable intracellular ice crystals may undergo crystallization and cause cell damage (Woods *et al*. 2004). A rapid thawing rate can minimize crystallization by reducing the time during which the dehydrated cells may absorb the amount of water lost during freezing (Leung 1991; Suquet *et al*. 2000).

A wide range of thawing temperatures and temperature rate changes have been investigated for cryopreserved marine teleost spermatozoa. A change in thawing temperature from 1ºC for 7 seconds to 30ºC for 30 seconds showed no reduction in post-thaw motility of ocean pout *Macrozoarces americanus* spermatozoa (Yao *et al*. 2000). Flounder *P. olivaceus* spermatozoa was thawed in two steps. Firstly, vials were removed from the liquid nitrogen and suspended in the nitrogen vapor for 2 minutes. In the second step vials were removed from the nitrogen vapor and thawed in a water bath at 28ºC. Spermatozoa had functional motility and fertilization capacity (Zhang *et al*. 2003). Post-thaw motility was achieved in seabream *S. aurata* by thawing straws in a 30ºC water bath at a rate of 15ºC/minute. Thawing temperatures examined for turbot *S. maximus* included 20°, 30° and 40ºC and the best post-

thaw motility was obtained at 30ºC (Dreanno *et al*. 1997). A successful thawing temperature of 37ºC with regard to post-thaw sperm motility was adopted in an investigation on turbot *S. maximus* spermatozoa by Chen *et al*. (2004). Thawing temperatures for cryopreserved spermatozoa of marine fish species are shown in Table 4.3 and are lower than those reported for freshwater fish, 30° to 80ºC (Suquet *et al*. 2000; Rana 1995).

Table 4.3: Thawing temperatures successfully used on cryopreserved spermatozoa of marine teleost species.

Species	**Scientific name**	**Thawing temperature (ºC)**	**Reference**
Atlantic halibut	*Hippoglossus hippoglossus*	10 or 40	Bolla *et al*. (1987)
Yellowfin seabream	*Acanthopagrus latus*	20	Gwo (1994)
Turbot	*Scophthalmus maximus*	20,30,37, and 40	Dreanno *et al*. (1997) Chen *et al*. (2004)
Seabream	*Sparus aurata*	30	Fabbrocini *et al*. (2000)
Flounder	*Paralichthys olivaceus*	28	Zhang *et al*. (2003)
Winter flounder	*Pleuronectes americanus*	30	Rideout *et al*. (2003)
Yellowtail flounder	*Pleuronectes ferrineus*	30	Richardson *et al*. (1999)
Ocean pout	*Macrozoarces americanus*	1 or 30	Yao *et al*. (2000)

Post-thaw analysis

Assessment of thawed spermatozoa is helpful in predicting the success of a cryopreservation protocol. According to Leung and Jamieson (1991) and Rurangwa *et al*. (2004), spermatozoa motility is a good measure of estimating cryopreservation success. However, Rurangwa *et al.* (2004) suggest that only quantification of fertilization is a reliable indicator of cryopreservation success. Motility of thawed spermatozoa is not a reliable measure of fertilization potential (Gwo 2000). In Atlantic croaker *Micropogonias undulates,* no post-thaw sperm motility was observed. However, when diluted with 1% sodium chloride extender solution containing 30% DMSO, fertilization was recorded (Gwo *et al*. 1991).

Motility is difficult to estimate and visual assessment may be inaccurate (Gwo 2000). Direct observation, using a microscope or via videotape, provides motility estimates on a point scale, for example, 5 = excellent motility to 1 = poor motility (Kime *et al*. 2001). Due to the large number of spermatozoa per unit volume, short duration of post-thaw motility, rapid sperm movement, human error and lack of standardization in defining motility, accuracy of the measurements depends on the skill of the observer (Gwo 2000). Methods to rapidly, reliably and effectively estimate sperm quality and assess damage following cryopreservation are required for the evaluation of cryopreservation success of fish spermatozoa.

Advanced methods to estimate sperm motility include the use of Computer Assisted Sperm Analysis (CASA). This technology can rapidly and objectively quantify sperm motility and estimate fertilizing capacity (Rurangwa *et al*. 2004). CASA systems are expensive and could not be considered for this study.

Viability of spermatozoa is a key determinant of sperm quality (Flajšhans *et al*. 2004). A method for determining post-thaw sperm viability is dual DNA staining using flow cytometry. This staining procedure combines two DNA fluorescing dyes, SYBR 14 and Propidium iodide. SYBR 14 stains the DNA in living cells where the plasma membrane integrity has not been breached. Propidium iodide stains the DNA in damaged cells which have lost their plasma membrane integrity and are considered non-viable (Flajšhans *et al*. 2004). Flow cytometry allows rapid characterization of spermatozoa in terms of cellular function and plasma membrane integrity (Baulny *et al.* 1997). This makes it a valuable method for objective and quantitative post-thaw sperm viability analysis.

The aim of this study was to use dual fluorescing flow cytometry incorporating SYBR 14 and propidium iodide to test the effect of two cryoprotective agents DMSO and Glycerol on the plasma membrane integrity of *A. pectoralis* spermatozoa during a 64-day cryopreservation trial. Furthermore, based on wide acceptance of DMSO as a cryoprotective agent and its ability to rapidly permeate into cells, it was hypothesized that DMSO would provide better protection to *A. pectoralis* spermatozoa than glycerol, due to the short time available for sperm to equilibrate prior to freezing.

Methods and Materials

Fish capture

A. pectoralis were captured by trawling off Mossel Bay (34º08'S; 22º08'E) along the South coast of South Africa. The details of their collection at sea are described in Chapter 2.

Sperm acquisition

Live *A. pectoralis* were immediately collected after each trawl and placed in a 100-L container with sea water. Sex was determined by making a small incision into the interperitoneal cavity to check for the presence of a testis. Prior to the removal of the testis length and weight of each fish were recorded to the nearest mm and g, respectively. The testis was removed and its colour and texture were noted. The primary testis was sliced in half and both halves were placed into a test tube containing 4 ml MMM extender solution (Figure 4.1). Before tapping the testis to solicit sperm release, tissue samples were preserved for Transmission Electron Microscopy (TEM) and histology. Samples for TEM were stored in a 2.5% glutaraldehyde phosphate buffer. Histology samples were stored for 24 hours in a 10% buffered formalin solution, and then transferred to a 70% ethanol solution. The testis was tapped with a sterilized glass rod for approximately 10 seconds to induce sperm release. A 270-µl sperm sample was pipetted into a cryovial containing 30 µl of a 2.5% buffered glutaraldehyde solution for Scanning Electron Microscopy (SEM) analysis. A 270-µl sperm sample was pipetted into experimental vials for cryopreservation (Figure 4.1). Sex identification and preparation of sperm samples took between four to six minutes. Four males were processed per trawl.

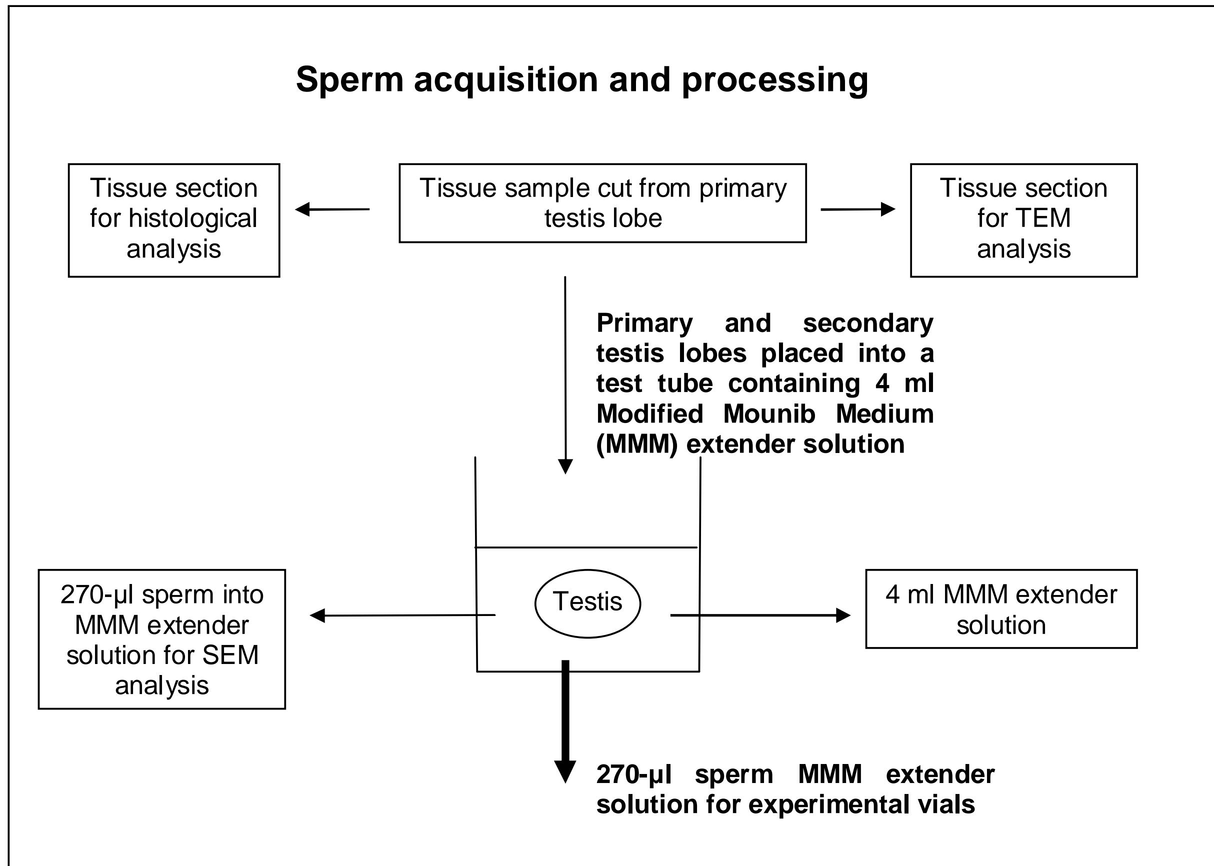

Figure 4.1: Preparation of testis tissue samples for Transmission Electron Microscopy, histology and the storing prior to cryopreservation.

Extender and cryoprotectant constituents and preparation

A 500-ml stock solution of MMM extender was prepared one day prior to use. The composition of MMM is given in Table 4.4. Care was taken not to stir the solution. The extender was stored on ice in a sealed Styrofoam container.

Two 50ml cryoprotective agent stock solutions were prepared using Modified Mounib Medium extender. The first contained 10% dimethyl sulphoxide (DMSO) and the second 10% glycerol. The stock solutions were prepared and refrigerated two days prior to sampling and kept on ice during pipetting and sampling of sperm.

Table 4.4: Composition of Modified Mounib Medium (MMM) extender solution.

Components	Modified Mounib Medium	Concentration
$KHCO_3$	100 mM	10.012 g/L
Sucrose	125 mM	42.79 g/l
Reduced glutathione	6.5 mM	1.997 g/L
Bovine serum albumin (BSA)	10 mg/ml	5 g/L
pH	7.8	7.8
Osmotic Pressure	310 mOsmol/kg	310 mOsmol/kg

Experimental design

Sperm from 19 male *A. pectoralis* were available for the cryopreservation experiment. Sperm from each male were subjected to three treatments run in triplicate (Figure 4.2). Treatment 1 consisted of three cryovials each containing 30 µl of a 10% DMSO stock solution. Treatment 2 comprised three cryovials containing 30 µl of a 10% glycerol stock solution. The control treatment consisted of three cryovials, each containing 30 µl MMM extender solution but no cryoprotective agent. A total of 270 µl of sperm/MMM extender solution was pipetted into each of the treatments (Figure 4.2). The total volume in each cryovial amounted to 300 µl.

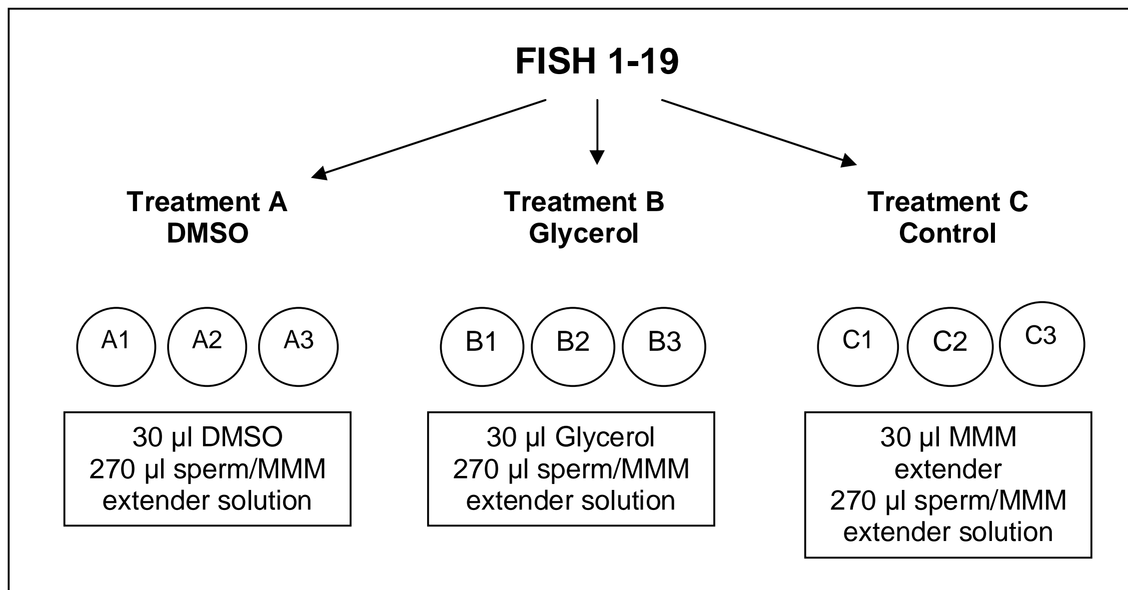

Figure 4.2: Experimental design of the three cryopreservation treatments, DMSO, glycerol and the control. There were 19 fish (replicates) in each treatment. MMM represents Modified Mounib Medium extender solution.

Freezing rate and storage

Sperm samples from each specimen and in each treatment were cryopreserved in two-steps. Sperm was taken from the testis and placed into a test tube containing 4 ml pre-cooled MMM extender solution and pipetted into the cryovials. Cryovials for each fish were placed in a circular vial holder designed for this study (Figure 4.3). A 10mm thick, wooden dowel was pushed through the centre of the vial holder so that the vials were placed 6 cm above the surface of the liquid nitrogen for 10 minutes during pre-freezing. After 10 minutes, the samples were removed from the Dewar flask and immediately dunked into a 30*l* cryopreservation vat of liquid nitrogen for 64 days.

Once a week a 10mm thick dowel rod was briefly inserted into the container, removed and checked to determine the level of liquid nitrogen in the container. Liquid nitrogen was added when the vat was approximately 30% full.

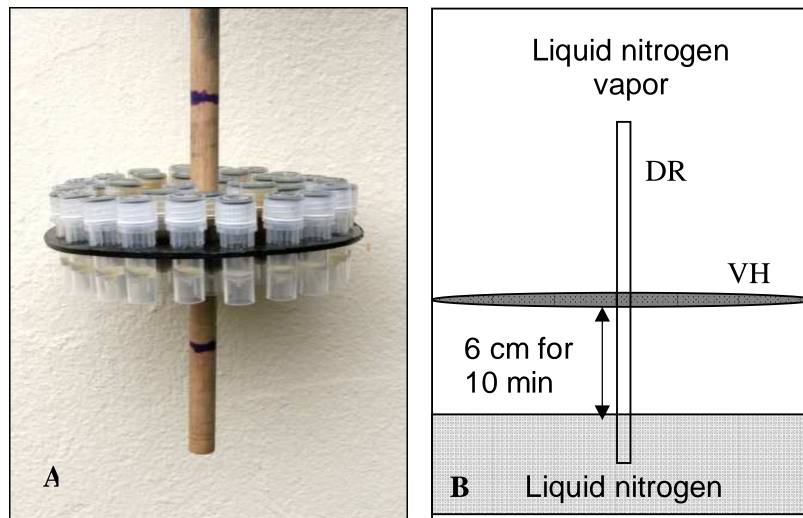

Figure 4.3.A-B: Experimental vials in the circular holder (A) were placed in a pre-freeze Dewar flask 6 cm above the surface of the liquid nitrogen (B) for 10 minutes. DR = dowel rod; VH = vial holder.

Thawing of cryopreserved sperm

Liquid nitrogen from the sample storage vat was poured into a polystyrene box and the vials were scooped out of the liquid nitrogen with a meshed metal strainer. The vials were submerged in a 30ºC water bath for one minute, whereafter they were kept on ice and transported within one hour to the University of Cape Town, Department of Immunology and Medical Research on Infectious Diseases, for flow cytometry.

Preparation of the fluorescing LIVE/DEAD Sperm Viability Kit

The LIVE/DEAD Sperm Viability Kit (Molecular Probes L-7011) was used to analyze samples by flow cytometry. SYBR 14, a membrane-permanent nucleic acid stain, and Propidium iodide, a conventional stain which indicates dead or dying cells were used as stains. The stains were prepared as follows:

- A 25-ml stock solution of HEPES-buffered saline solution (10 mM HEPES, 150 Mm NaCl, 10% BSA, pH 7.4) was prepared and kept at 4ºC.
- A 1:50 dilution of SYBR 14 stock solution (Component A) in the HEPES-buffered saline solution was prepared immediately before use and new solutions were used for each treatment.
- 1.5 µl of the diluted SYBR 14 stock solution were added to the 300-µl sperm sample resulting in a SYBR 14 concentration of 100 nM.
- To differentially stain live/dead cells, 1.5 µl of propidium iodide were added into each 300-µl sperm sample.
- Samples containing both dyes were incubated in a water bath at 25° C for 5-10 minutes where after 500 µl PBS were added to each sample.
- Samples were kept dark prior to flow cytometry analysis.

Flow cytometry

For the flow cytometry-based assay individual samples were placed into a Becton Dickinson FACS Vantage flow cytometer.

The sperm cells were identified and gated on a forward scatter (FSC) versus a side scatter (SSC) dot plot, as well as a fluorescent channel 1 (FL1 – FICT) versus fluorescent Channel 3 (FL3 – PERCP). All fluorescent parameters were measured using a logarithmic amplification

scale and the FSC and SSC data were on a linear scale. A threshold of 52 FSC channels was set to remove sample debris. Fresh *A. pectoralis* sperm was used for calibration. A total of 50 000 cells were counted per sample.

Cells stained positive for SYBR 14 (FITC) but negative for Propidium iodide (PERCP) were rated as potentially live cells, based on membrane integrity. Cells stained positive for Propidium iodide (PERCP) and negative for SYBR 14 (FITC) were considered non-viable. By using the dot plot of FL1 versus FL3, the percentage of potentially viable cells could be estimated.

Data analysis

There were three treatments with 19 replicates each. These were DMSO, Glycerol and a control treatment. Although three measurements were taken for each of 19 fish, they were averaged for the analysis to avoid pseudoreplication. Thus, a fish was taken as the experimental unit. Since sperm from each fish was represented in all treatments, within-subject-variation could be estimated using Repeated Measures Analysis of Variance. Tukey's multiple range test was used to compare averages. Significant differences between treatment means were accepted at a 5% error level ($p<0.05$).

Results

Positive reaction for SYBR 14

There was a significant difference between treatments in the positive reaction to SYBR 14 (repeated measures analysis of variance, F (2,36), p = 0.0032). Best results (61.38 %) were obtained in the glycerol treatment, while the DMSO treatment and the control did not differ from each other (Tukey's multiple range test; p = 0.915). Average values for DMSO, Glycerol and the control were 47.4, 61.4 and 45.5, respectively (Figure 4.4).

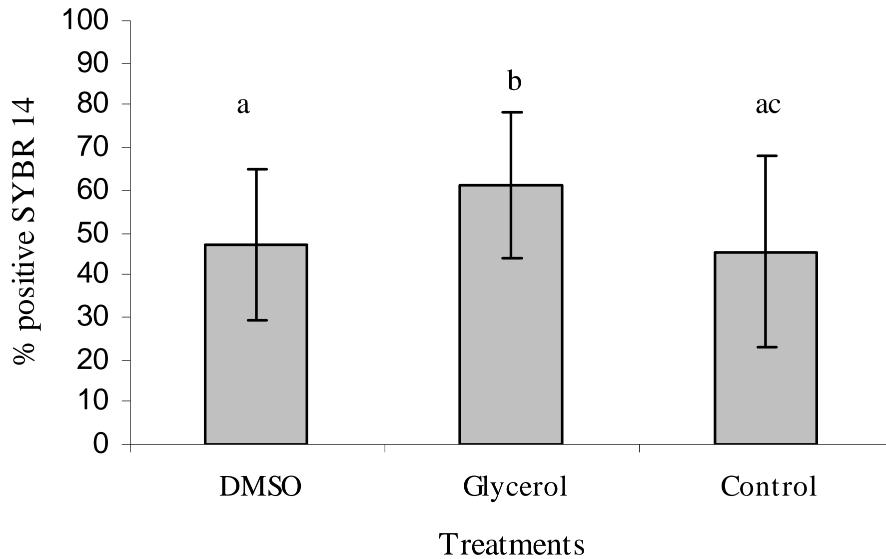

Figure 4.4: The mean and standard deviation of potentially viable cells based on plasma membrane integrity (positive reaction to SYBR 14) for each treatment. Different letters represent a significant difference ($p < 0.05$), between pairs of comparisons. Vertical bars are standard deviations of the mean.

Positive reaction to Propidium iodide

There was a significant difference between treatments for a positive reaction to propidium iodide (repeated measures analysis of variance, F (2, 36), p = 0.0007). Cells which reacted positive to Propidium iodide were considered non-viable as Propidium iodide stains DNA in degenerated cells which have lost their membrane integrity. The highest value was obtained in the DMSO treatment (35.2 %), while the means of the glycerol treatment and the control

treatments did not differ significantly from each other (Tukey's multiple range test; $p = 0.236$). Average values for the DMSO, glycerol and the control treatment were 35.2, 23.0 and 27.8, respectively (figure 4.5).

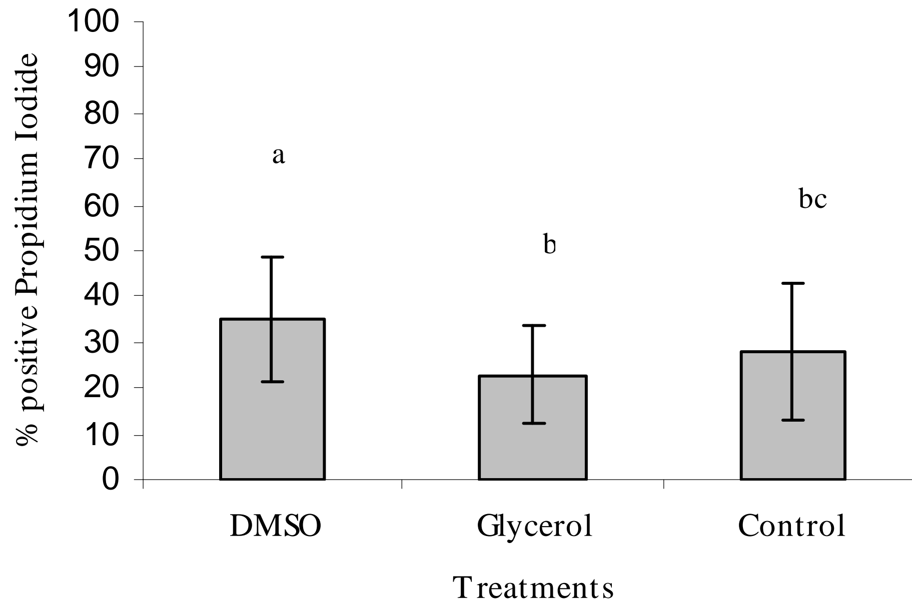

Figure 4.5: The mean and standard deviation of the percentage of cells reacting positive to staining with propidium iodide. Different letters represent significant differences ($p < 0.05$) between pairs of comparisons (Tukey's multiple range test) and vertical bars are standard deviations of the mean.

Negative reaction to propidium iodide and SYBR 14

There was a significant difference between treatments for a negative reaction to both propidium iodide and SYBR 14 (repeated measures analysis of variance, $F_{(2, 36)}$, $p = 0.0046$). These cells were sufficiently damaged during cryopreservation that they were unable to incorporate either of the fluorescing stains. The highest value was obtained in the control (26.2%), while glycerol and DMSO treatments did not differ significantly from each other ($p = 0.80$). Average values for DMSO, Glycerol and the control were 17.1, 14.9 and 26.2, respectively.

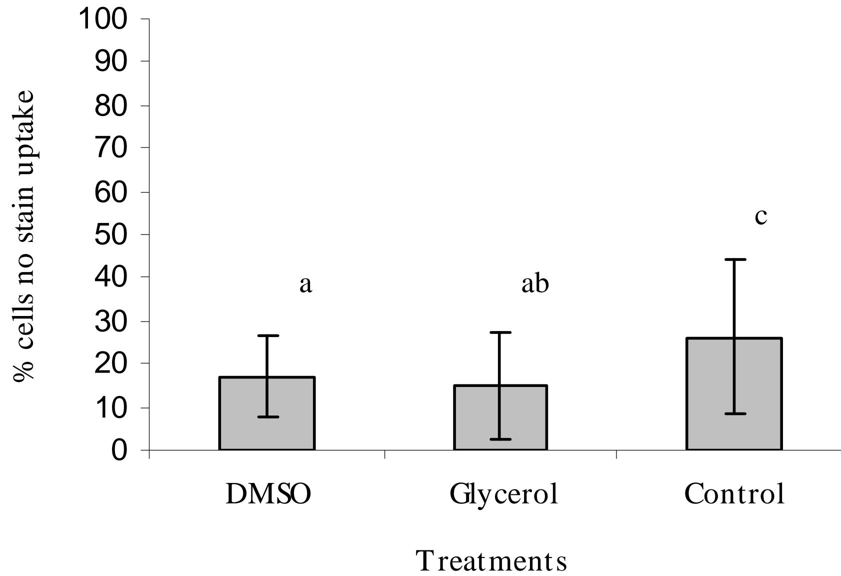

Figure 4.6: Percentage of cells that did not stain positive to either SYBR 14 or Propidium iodide. Different letters denote significant differences ($p < 0.05$) between treatments. Vertical bars are standard deviations of the mean.

Discussion

A cryopreservation protocol can increase sperm availability and facilitate farming of sole in South Africa. Flow cytometry data suggested that *A. pectoralis* spermatozoa can be frozen and thawed while maintaining 63.2% potential cellular viability based on nuclear membrane integrity. Although viability of spermatozoa must ultimately be defined by their capacity to find and fertilize eggs, staining with a Live/Dead Sperm Viability Kit can help predict sperm viability as it addresses an important aspect of cryopreservation, i.e. the integrity of nuclear membranes of thawed spermatozoa. These results provide a foundation for studies to establish a correlation between viability, estimated using a Live/Dead Sperm Viability Kit, and fertilization success.

The process of freezing and thawing results in cryo-injuries which can be reduced by the addition of non-permeating and permeating organic cryoprotective compounds (Bromage and Roberts 1995). In an evaluation on the cryopreservation of sperm in marine fish, it was suggested DMSO obtained better results than propylene glycol, glycerol, methanol and

ethylene glycol (Suquet *et al.* 2000). Methanol provided poor cryoprotection for marine fish spermatozoa (Suquet *et al.* 2000). This has also been observed for turbot (*Scophthalmus maximus*) spermatozoa (Dreanno *et al.* 1997) and Atlantic croaker (*Micropogonias undulatus*) (Gwo *et al.* 1991). Glycerol provided a higher motility and fertilization rate of flounder sperm than DMSO and methanol and it was, thus, the preferred cryoprotectant for flounder sperm (Zhang *et al.* 2003). Therefore, the cryoprotectants adopted for this study were DMSO based on rapid permeability and low toxicity to cells and glycerol, due to its low toxicity and relative permeability, both at a concentration of 10 %.

During the 64-day cryopreservation period, 10% glycerol provided significantly better cryoprotective support to *A. pectoralis* spermatozoa than both 10% DMSO and the control treatment, respectively. In the glycerol treatment 63.2% of sperm cells were potentially viable, based on the positive reaction to SYBR 14. In the DMSO treatment 47.4% of post-thaw spermatozoa were potentially viable, similar to the 45.5% in the control. Although DMSO was considered an effective cryoprotective agent for sperm in marine teleosts at concentrations ranging from 5-20% (Rideout *et al.* 2003, Jamieson 1991), toxicity studies suggested that DMSO is more toxic than glycerol to cells and isolated cellular components (Arakawa *et al.* 1990; Chen *et al.* 2004). Motility of rainbow trout *O. mykiss* sperm was reduced at DMSO concentrations above 20% (Jamieson 1991).

The toxic effects of DMSO were reported for striped bass *Morone saxatilis* (He and Woods 2004) sperm. Mitochondrial function decreased with increasing DMSO concentration from 2.5%, to 5% and 10%, respectively. In this study, the preparation time of sperm samples was four to six minutes at ambient temperature prior to freezing. It was not quantified whether DMSO may have damaged the plasma membrane of sperm cells during this time. Arakawa *et al.* (1990) pointed out that DMSO was more toxic to isolated proteins at elevated temperatures, i.e., room temperature and higher. Such toxic effects of DMSO may have been responsible for the lower viability of DMSO-treated sperm relative to the glycerol treatment and the control treatment, as was suggested by the positive reaction to propidium iodide. In the DMSO treatment, 35.2% of sperm cells were non-viable, compared with 23.0 and 27.8% for glycerol and the control treatment, respectively.

Although DMSO and glycerol provide cryoprotective ability to spermatozoa of many species, no cryoprotectant is suitable for all species. This is emphasized by Gwo (1994), who mentions that variations in the composition of seminal plasma, sperm membranes and their structure point to species-specific requirements for extenders and cryoprotectants. For example, while a 10% solution of glycerol could be recommended based on the present study, post-thaw analysis on turbot *S. maximus* spermatozoa showed that glycerol at a 5%, 10% and 15% concentration caused irreversible damage, such as the modification of membrane fluidity, reduction of membrane electrical capacity and the polymerization and depolymerization of microtubules (Dreanno *et al*. 1997). Glycerol is a slow-permeating cryoprotectant (Chen *et al.* 2004; Dreanno *et al*. 1997) and this may explain why 10% glycerol was a less effective cryoprotective agent for winter flounder sperm *P. americanus* than DMSO and propylene glycol (Rideout *et al.* 2003). Motility of bluefin tuna *Thunnus thynnus* spermatozoa increased with an increase in equilibration time from 10 to 30 minutes when glycerol was used as a cryoprotective agent (Doi *et al.* 1982, in Rideout *et al.* 2003); however, yellowfin seabream *Acanthopagrus latus* sperm, diluted in 10% glycerol, did not become motile within less than 60 minutes (Gwo 1994). In this study, it took four to six minutes to prepare the samples. It is hypothesised that this was sufficient for glycerol to penetrate into the sperm cells of *A. pectoralis.* However, it was not tested whether the preparation time of four to six minutes was optimal. It is, therefore, suggested that future investigations in defining a cryopreservation protocol focus on a range of equilibration times, using different cryoprotectants and their concentrations.

With regard to the negative reaction to both SYBR 14 and propidium iodide, the average value of the control treatment was significantly different from the DMSO and glycerol treatments, respectively. Spermatozoa reacting negatively to both SYBR 14 and Propidium iodide were considered destroyed. In the control 26.2 % of the spermatozoa were destroyed, compared to 17.1% and 14.9% in the DMSO and glycerol treatment, respectively. As no cryoprotectant was added to the control, structural damage to the plasma membranes could have been caused by a combination of two factors, i.e. internal ice formation during freezing, resulting in cell rupture and excessive dehydration of the cells due to an elevated concentration of solutes, resulting in an osmotic imbalance. Furthermore, depending on the freezing rate, the concentration of extracellular electrolytes may increase during freezing, thereby influencing interactions between ions that stabilize the plasma membrane (Li *et al*. 2006).

In the control treatment, 45.5% of *A. pectoralis* spermatozoa showed a positive reaction to SYBR 14, which was similar to that observed for DMSO 47.4%. Cryoprotective agents require dilution with an extender solution. The MMM solution contained sucrose. Anchordoguy *et al*. (1987) showed that sugars such as trehalose and sucrose, were effective non-permeating cryoprotectants. The authors suggested that sucrose interacts directly with membrane phospholipids, thereby providing protection during freezing. Although the control showed a positive reaction to SYBR 14, permeating cryoprotectants, such as glycerol, are necessary to reduce the cellular destruction during cryopreservation of *A. pectoralis* spermatozoa. This was supported by the significant differences in sperm viability between glycerol and the control treatment.

In summary, based on a review of published information, this study aimed,firstly, to define appropriate cryoprotectants, extenders, cooling and thawing rates for the cryopreservation of *A. pectoralis* spermatozoa. Dual fluorescing flow cytometry provided an objective method to describe post-thaw spermatozoa viability based on membrane integrity. The study did not aim at defining optimal conditions for the cryopreservation of *A. pectoralis* spermatozoa, as experimental conditions were kept constant and only one concentration was tested for each CPA. The results lead to a rejection of the null hypothesis, i.e. that DMSO and glycerol provided equal cryopoprotective support for *A. pectoralis* spermatozoa because glycerol provided the best protection.

In this study, cryo-injuries associated with freezing and thawing were not examined. Such information could provide useful insights into the fertilizing capacity of the spermatozoa, by highlighting structural damage which may prevent successful fertilization. For example, flow cytometry, using Rhodamine 123 and SYBR 14, would assess post-thaw spermatozoa quality with respect to mitochondria and plasma membrane integrity. This could help in understanding if there is a link between membrane integrity and fertilization capacity, therefore providing an assessment of cryopreservation success. Furthermore, an evaluation of cryo-injuries of pre-freeze and post-thaw spermatozoa could highlight the potentially damaging effects of cryoprotective agents.

Chapter 5: General Discussion

This study was designed to investigate the ultrastructure of *A. pectoralis* sperm, to compare the two cryoprotectants glycerol and DMSO, and to evaluate the use of dual fluorescence flow cytometry in fish sperm cryopreservation using *A. pectoralis* as a study animal.

Spermatozoon ultrastructure was described with reference to sperm type in order to compare spermatozoa from this species to that of other pleuronectiformes. Histology of *A. pectoralis* testis provided an insight into spermatogenesis, sperm maturity, and morphology of the flagellum. The maturity of spermatozoa is important in determining the appropriate timing for cryopreservation. Knowledge of spermatozoan ultrastructure can be useful for studies of cryo-injuries during freezing and thawing. Thus, the study provides the first reference description for sperm from this species. Researchers can use this for comparisons and an evaluation of the safety of cryopreservation methods.

Post-thaw sperm viability was analyzed using dual fluorescence flow cytometry on sperm that had been cryopreserved for 64 days. This technique determines sperm viability with respect to plasma membrane integrity and the usefulness of this method for the evaluation of sole sperm cryopreservation will be discussed.

This chapter aims to discuss the relevance of the findings for the development of a cryopreservation protocol for *A. pectoralis* spermatozoa.

Sperm morphology

Diversity among teleost spermatozoa has been reviewed by Mattei (1991) and Jamieson (1991). This diversity is expressed through structural variations, for example nuclear shape and the presence of lateral fins at the flagellum, or biochemical differences in milt composition. According to Gwo (1994), there is a species-specific requirement for extenders and cryoprotectants for cryopreservation. This may be due to variations in the composition of seminal plasma, species-specific differences in sperm membrane morphology, or cell ultrastructure.

Pleuronectiform spermatozoa, in general, are type 1 ect-aquasperm with an ovoid 2-3 μm long nucleus devoid of an acrosome with a short mid-piece, a collar-like arrangement of mitochondria and a flagellum with lateral fins (Jamieson 1991). Despite few published investigations, important differences in sperm ultrastructure have been reported between families within the pleuronectiformes (Medina *et al*. 2000). The authors discussed spermatozoon morphology of Senegal sole *S. senegalensis* and its implications for phylogenetics. Two sperm morphotypes have been described in the order Pleuronectiformes based on the orientation of the centrioles (Medina *et al.* 2000). Spermatozoa of the Citharidae, Pleuronectidae and Paralichthydae differed from that of the Soleidae by the retention of their plesiomorphic perpendicular arrangement of the centrioles (Medina *et al.* 2000). In the Senegal sole *S. senegalensis* centrioles were arranged in a co-axial alignment along the longitudinal axis of the spermatozoa (Medina *et al*. 2000). This is similar to the arrangement described for *A. pectoralis* in this study. The co-axial arrangement of the centrioles in Soleidae has been suggested to be an apomorphic state and it may contribute to the phylogenetic separation of Soleidae from Pleuronectidae (Medina *et al.* 2000; Mattei 1991).

A. pectoralis lateral fin morphology was compared to that described for *S. senegalensis*. In *A. pectoralis,* the lateral fins were an extension of the plasma membrane of the mid-piece and, therefore, occurred within the cytoplasmic canal. *S. senegalensis* did not have lateral fins at the proximal end of the flagellum at the cytoplasmic canal region (Medina *et al*. 2000). The number of lateral fins, although commonly found in the Pleuronectiformes, varies between species. Both turbot *S. maximus* and Senegal sole *S. senegalensis* spermatozoa had two lateral fins; however, some sperm had no lateral fins while others had only one (Suquet *et al*. 1993; Medina *et al*. 2000). Where lateral fins were observed with respect to teleost spermatozoa, no indication which suggests that they may be temporary structures was found in the literature to the best of the authors knowledge. Cross-sections through the flagella of *A. pectoralis* showed two lateral fins, while some spermatozoa had none or three lateral fins respectively. This occurred in sperm samples from all 18 fish, but flagella with three lateral fins were observed in 14 fish. There are no papers suggesting a reason for the existence of three lateral fins. Studies should be done to determine the frequency at which flagella with three lateral fins occur. It is hypothesised that three lateral fins may affect the buoyancy or swimming speed of spermatozoa. Results from such studies may help predict under which conditions this species spawns. It should also be tested whether three lateral fins have a function, or if they are mutations.

In pleuronectiformes, the mitochondria form a ring-like structure surrounding the *nuclear fossa* at the caudal end of the nucleus. Both turbot *S. maximus* (Suquet *et al*. 1993) and the flounder *P. flesus* (Jamieson 1991) had eight to ten mitochondria. *A. pectoralis* possessed seven electron-dense mitochondria. This is different from published information on other pleuronectiform species. Studies are needed to provide data on mitochondrial counts within the mid-piece of mature spermatozoa of Peuronectiformes. Such information may have phylogenetic implications.

Spermatozoa of Pleuronectiformes are grouped as ect-aquasperm, based on their ultrastructure. This suggests that fertalisation is external (Jamieson 1991). Differences in sperm ultrastructure between *A. pectoralis* and *S. senegalensis,* within the family Soleidae, suggest that spermatozoa can not be grouped into a single archetype if spermatozoa of the Soleidae do not conform to a uniform sperm configuration.

Chapter 3 described ultrastructure and morphology of *A. pectoralis* spermatozoa by reporting structural variations in mitochondria number, lateral fin morphology and variations within the Pleuronectiformes, specifically the Soleidae. Histology showed that during August 2006 there were mature sperm in the lumen of the testis. Cryopreservation can result in damage to cellular structures, such as the plasma membrane, nucleus, flagellum and mitochondria predominantly, by causing swelling of the cells, resulting in their rupture (Li *et al*. 2006). Such damage can lead to structural deformation of cell organelles, abnormalities in sperm chromatin structure and result in impaired viability and reduced fertility (Li *et al*. 2006). The description of the ultrastructure of *A. pectoralis* spermatozoa may provide a reference that can be used when investigating cryo-injury in other studies on this or other species. Although flow cytometry was used to determine the potential viability of thawed spermatozoa by assessing the cryo-injury of plasma membranes, this type of cryo-injury was not investigated. To optimize a cryopreservation protocol, ultrastructual damage should be studied using information from electron microscopy and flow cytometry. This could provide information about sensitivity of spermatozoa to cryoprotective agents, extender solutions, freezing and thawing rates, and it could help outline steps to improve the cryopreservation success of *A. pectoralis* spermatozoa. Such research may become relevant in the artificial spawning of rare species with a very small milt volume and a skewed sex ratio such as some soleids.

Cryopreservation

The consistently high prices on the European markets for flatfish have stimulated research and development of their culture. Despite successful culture of two soleid species, the Senegal sole *S. senegalensis* (Anguis and Cañavate 2005), and the common sole *S. solea* (Howell 1997), low milt volumes and skewed sex ratios have been reported. This suggests that the low availability of males may limit the success of soleid culture. In addition, in greenback flounder *Rhombosolea tapirina* and common sole *S. solea* it has been difficult to obtain milt prior to spawning (Rijnsdorp and Witthames 2005). Similarly, milt release could not be induced in two soleid species: the white margined sole *D. marginatus* (Thompson 2004) and *A. pectoralis* (this study). Thompson (2004) reported a skewed sex ratio of 1:5:1 (male:female) in *D. marginatus*. Skewed sex ratio favouring females has also been reported for *Cynoglossus zanzibarensis*, 1:2.4, (Booth and Walmsley-Hart 2000) similarly to the study by Hecht (1976) on *A. pectoralis*, 1:2.4. This highlights the need to develop techniques for sperm acquisition, storage and utilization. A cryopreservation protocol developed for *A. pectoralis* can increase sperm availability for their culture and become a tool for broodstock management, conservation of genetic diversity and improve continuous supply of synchronized gametes for hatchery production or research and development. This study contributed to the establishment of a cryopreservation protocol by comparing the cryoprotective potential of dimethyl sulphoxide (DMSO) and glycerol for *A. pectoralis* spermatozoa during a 64-day cryopreservation period.

Dual fluorescence flow cytometry, combined with staining using SYBR 14 and propidium iodide was used to examine the plasma membrane integrity of cryopreserved spermatozoa of *A. pectoralis*. Glycerol at a concentration of 10% showed better cryoprotection towards *A. pectoralis* spermatozoa than DMSO, based on a positive reaction to both SYBR 14 (intact plasma membrane) and propidium iodide (non-intact plasma membrane). However, it was hypothesised that 10% DMSO would provide the highest percentage of potentially viable spermatozoa. For example, DMSO was expected to penetrate into the cells faster than glycerol and, thereby, providing better cryoprotection. It was also thought that the toxic effect of DMSO towards the cells would be reduced at an equilibration time of four to six minutes. Although glycerol is a slow-permeating cryoprotectant (Chen *et al.* 2004), four to six minutes of equilibration time may have been sufficiently long for glycerol to provide better cryoprotection.

Sperm samples were prepared at ambient temperature aboard a trawler. Arakawa *et al.* (1990) suggested that the toxic effects of DMSO on isolated proteins increased with temperature. The authors did not mention which temperature range was considered elevated. It is, therefore, possible that the temperature at which the samples were prepared may have been sufficiently high for DMSO to cause cell damage. Thus, future investigations should determine the effect of temperature and equilibration time on DMSO toxicity.

A total of 45.5% of the spermatozoa in the control treatment reacted positive to SYBR 14. However, no permeating cryoprotective agent had been added to the control. Sucrose in the MMM extender solution and trehalose can interact with membrane phospholipids (Anchordoguy *et al.* 1987) and provide protection during freezing. Glycerol and DMSO were, however, significantly different from the control in the negative reaction to SYBR 14 and propidium iodide. Thus, permeating cryoprotectants are needed for the cryopreservation of *A. pectoralis* spermatozoa, as cryoprotectants, such as DMSO and glycerol, reduce the freezing damage to cells by affecting the size and shape of ice crystals that form during freezing (Denniston *et al*. 2000).

Summary and conclusions

The aim of this study was to determine the cryogenic potential of *A. pectoralis* spermatozoa. A skewed sex ratio, favouring females, was observed for *A. pectoralis* by Hecht (1976), which is common amongst soleids, thus the need to develop cryopreservation methods and an understanding of sperm morphology. By analyzing the ultrastructure of spermatozoa, this study made a contribution to sperm morphology in the order Pleuronectiformes and provided information on spermatogenesis, sperm maturity and storage for cryopreservation.

The cryoprotective agents DMSO and Glycerol both reduced cell destruction of *A. pectoralis* spermatozoa after a 64-day trial. This was the first study to cryopreserve *A. pectoralis* spermatozoa. Using dual fluorescence flow cytometry to estimate the viability of thawed spermatozoa based on plasma membrane integrity, it is suggested that when preparing spermatozoa samples for cryopreservation on-board a vessel, glycerol will be a better cryoprotectant than DMSO. Future studies should test the effects of freezing and thawing rate and equilibration time on the viability of *A. pectoralis* spermatozoa. A correlation between membrane integrity of post-thaw spermatozoa and fertilization success should be established.

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