# GAMETOGENESIS, GONADAL RECRUDESCENCE, RESTRAINT AND SPAWNING PATTERNS IN NILE PERCH, *Lates niloticus* (LINNAEUS, 1758)

**A thesis submitted in fulfilment of the requirements of the degree of**

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**By**

**DAVID KAHWA**

**April 2013**

# DECLARATION

## DECLARATION

I, **DAVID KAHWA,** declare that this thesis is my original work and has never been submitted for a degree in any other university. Where other sources of information have been used, acknowledgement has been made.

Signature: ..... Date: 23/04/2013

# CERTIFICATE OF APPROVAL

We hereby approve this thesis that has been submitted for examination with our authority as university supervisors.

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# ABSTRACT

The Nile perch, *Lates niloticus* (Linnaeus, 1758), is a predacious freshwater fish widely distributed throughout the Afro-tropic eco-zone. The species was introduced to Lake Victoria in the early 1950s and by 1980 it had dominated the fisheries of Lake Victoria. This was followed by a dramatic decrease in the Nile perch fisheries production due to uncontrolled exploitation. The purpose of this thesis is to provide fundamental knowledge that can be applied in aquaculture and fisheries management through the study of the reproductive biology of *L. niloticus.*

The research was aimed at the studying of the diverse aspects of the reproductive biology of *L. niloticus* in the Lake Victoria, Ugandan populations. This included reproductive patterns in relation to proximate environmental conditions, size at sexual maturity, gonad and gamete structure, gametogenesis and induced ovulation. The size at 50% sexual maturity for female Nile perch was 59.4 cm, which is lower than the earlier reported size of greater than 90 cm total length. Male *L. niloticus* matured at 57.8 cm total length in Lake Victoria. Microscopy revealed that *L. niloticus* from Lake Victoria had one spawning period that started in November and ended in March. Type I atresia occurred at high frequency from March to June, and type III atresia was present from July to September and between November and December.

Spermatogenesis in *L. niloticus* is cystic and sperm development is the result of asynchronous activation of the germ cells. Type II spermatozoa are simple, uni-flagellate aquasperm with no acrosome. Oogenesis in *L. niloticus* differed from that of other fishes in that no cortical alveoli were present in any stage of oogenesis. Numerous oil globules were present in the primary yolk

vesicle stage. This formed one centrally positioned, large oil globule in the tertiary yolk vesicle oocytes during final oocyte maturation.

Clove oil was an effective sedative and an anaesthetic for the handling of *L. niloticus*. Induction time was more rapid at clove oil concentrations of 50 - 100 µl $L^{-1}$ than in fish exposed to clove oil concentrations less than 50 µl $L^{-1}$. Fish exposed to high concentrations exhibited significantly short induction times of less than 240 seconds. On average, fish recovered within 673 ± 58 seconds for all the concentrations used. Prolonged exposure of *L. niloticus* to low clove oil concentrations of 2.5 - 10 µl $L^{-1}$ did not change the blood plasma cortisol, glucose, and the lactate and chloride ion concentration, relative to the control treatment. Captive breeding was attempted by conducting induced spawning experiments. Only final oocyte maturation was achieved using a decapeptide Gonadotropin Releasing Hormone (Dargin, sGnRH-MET), combined with a water-soluble dopamine receptor antagonist metoclopramide.

This thesis suggests a research approach that provides a basis for aquaculture of the new species by first studying reproductive biology patterns and then linking the information to gonad and gamete structure so that spawning times can be estimated. It further provides insights into aspects of the reproductive biology of the species and the effects of hormonal intervention on oocytes by showing at which stage of oocyte development hormones should be applied in *L. niloticus*. Clove oil can be used to sedate and anaesthetise *L. niloticus* broodfish to reduce the stress related to the handling of large specimens.

# Dedication

To my ever loving wife, Hellen Abwooli, our daughters, Brenda, Bridget, Bethany and Bernadette, our sons Benjamin and Julius, my mother Jane Rose and to my ever-caring and loving father, the late Mzee Maliko Byegarazo, who inspired me in education by always stressing to me that ***"Your Future Lies in Education".***

And finally, to all those who endeavour to make this a world worth living in.

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## ABBREVIATIONS

CIA = Central Intelligence Agency

COVAB = College of Veterinary Medicine, Animal Resources and Bio-security

DFR = Department of Fisheries Resources

FAO = Food and Agriculture Organisation

GnRHa = Gonadotropin releasing hormone analogue

MAAIF = Ministry of Agriculture, Animal Industry and Fisheries

MET = Metoclopramide

NARO = National Agricultural Research Organisation

NEMA = National Environmental Management Authority

NRF = National Research Fund of South Africa

PYVO = Primary Yolk Vesicle Oocyte

POF = Post Ovulatory Follicle

SYVO = Secondary Yolk Vesicle Oocyte

TYVO = Tertiary Yolk Vesicle Oocyte

UBOS = Uganda Bureau of Statistics

UNCST = Uganda National Council for Science and Technology

WAAR = Wildlife Aquatic Animal Resources

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Above all, to The Almighty GOD I give the GLORY. Without him I would not have been able to come so far.

# CHAPTER ONE

## Introduction

The Nile perch, *Lates niloticus* (Linnaeus, 1758), is a predacious freshwater fish in the family *Latidae* of the order *Perciformes*. It is widely distributed throughout the Afro-tropic eco-zone and it occurs in all major river basins including the Nile, Chad, Volta, Senegal and Niger, as well as in the south of the Congo River basin, in Lake Albert, Lake Chad, Lake Volta and Lake Turkana. It also occurs in the brackish waters of Lake Mariout of Egypt (Hopson, 1972).

The genus *Lates* is widely distributed in Africa. It occurs in most Rift Valley Lakes, including Lake Tanganyika *Lates microlepis* (Boulenger, 1898) *L. angustifrons* (Boulenger, 1906) and *L. mariae* (Steindachner, 1909). In Lake Albert, the endemic species *L. macrophthalmus* (Worthington, 1929) is sympatric with *L. niloticus*. Meanwhile in Lake Rudolph, the genus is represented by *L. n. rudolfianus* (Worthington, 1932) and *L. n. longispinus* (Worthington, 1932) (Hopson, 1972). The genus *Lates* has one marine species, the barramundi, *Lates calcarifer* (Bloch, 1790). It is widely distributed throughout the Indo-Pacific region, inhabiting coastal waters. *Lates. calcarifer* is the only species that has been cultured in Australia, Israel and certain Asian countries.

Fossil records show that *L. niloticus* inhabited the Arabian plate in sites including, As-Sarrar, Ad-Dabtiyah (Saudi Arabia), Ghaba of the Sultanate of Oman and the Negev of Israel during the Lower Oligocene and Miocene period (Otero and Gayet, 2001). The species is reported to have existed among the freshwater fish fauna of Koro-Toro (Chad), during the Pliocene approximately 3.58 million years ago (Otero et al., 2010) (Fig. 1.1).

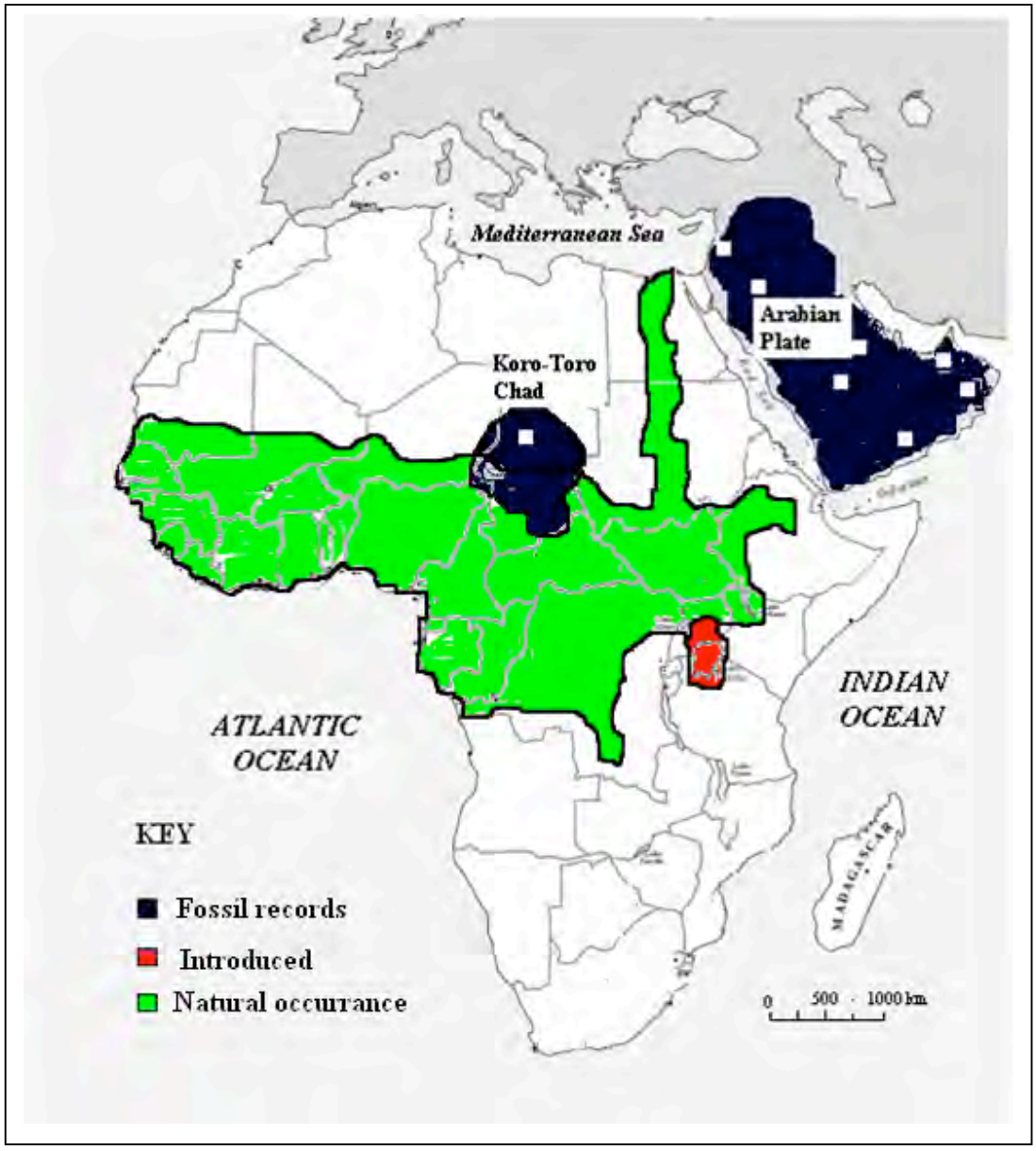


**Figure 1.1:** Nile perch prehistoric and current population distribution (*Sources: Hopson, 1972; Otero and Gayet, 2001; Otero et al., 2010*)

In Uganda *L. niloticus* distribution was limited to Lake Albert and the Albert-Nile ecosystems by the presence of the Semuliki rapids to the south and Murchison Falls to the east. In the 1950s and the early 1960s some specimens from Lake Albert and Lake Turkana were introduced to Lake Kyoga, Lake Victoria and Lake Nabugabo with the aim of boosting the fisheries resources of

those water bodies, which were by then dominated by tilapiines and haplochromines, the latter accounting for 80% of the total biomass in Lake Victoria (Ogutu-Ohwayo, 2004).

*Water resources in Uganda*

Uganda is a landlocked country located at the equator between 4° north and 1° south and stretching from 29.5° to 35.5° east. It has a total area of 241 040 $km^2$. The major lakes in Uganda include Lake Victoria, Lake Albert, Lake Edward, Lake George, Lake Kyoga, Lake Kwania and Lake Bisina. There are also over 160 small water bodies, covering an area of 1707 $km^2$. Uganda has numerous wetlands that are widespread throughout the country. Approximately 24 000 $km^2$ are covered by wetlands (swamps), of which about one-third are permanently flooded. All these water resources account for 20% of the total surface area of the country and they are an important fisheries resource (NEMA, 2008).

*The fisheries resource*

The ecosystems within and around these water bodies are amongst Uganda's richest sources of biodiversity. The current fisheries resource comprises of capture fisheries (artisanal) and aquaculture. Uganda harvests ornamental fishes, in particular haplochromines, especially from Lake Victoria, Lake Kyoga and other smaller lakes (NEMA, 2008). The main fish species in the order of commercial importance include Nile perch, *L. niloticus*, Nile tilapia, *Oreochromis niloticus*, "Mukene", *Rastrineobola agentea*, catfish, *Clarias gariepinus*, *Bagrus docmac*, and *Protopterus spp.* Others are *Alestes baremose* and *Hydrocynus* and *Barbus* species (MAAIF, 2004; NEMA, 2008).

By 1964 a breeding population of *L. niloticus* had been established in the Northern part of Lake Victoria (Gee, 1964). The Nile perch began appearing in the fisheries of Lake Victoria in the 1980s, which coincided with a further decline of populations of indigenous species and biodiversity (Barel et al., 1985). Witte et al. (1991) estimated that 150 to 200 of the endemic fish species, most of which had not been described, could be considered extinct. In addition, about 200 littoral and demersal species that exhibited a wide range of trophic specialization had not been collected for some time (Goldschmidt et al., 1993). The number of haplochromine species, pelagic zooplanktivores and insectivores in particular, has declined. All this has been blamed on the Nile perch and has been termed as the greatest vertebrate extinction reported in recent times (Kaufman and Ochumba, 1993). While conservation biologists decry the loss of extraordinary fish diversity in Lake Victoria, the social and economic benefits of the current fisheries should be discussed. The total annual fisheries yield increased from approximately 100 000 t to 300 000 t and to a further 500 000 t, of which 80% to 90% comprised of Nile perch in the 1980s (Kitchell et al., 1997).

*Current status of the Nile perch Fisheries in East Africa*

Lake Victoria is the most productive lake in East Africa whose fishery is supported by three major fish stocks, the Nile perch (*Lates niloticus*), Dagaa, Omena or Mukene (*Rastrineobola argentea*) and Nile Tilapia (*Oreochromis niloticus).* Total fish catch from the lake has increased over the years due to increase in fishing effort. The estimated total annual landing has been increasing from about 620,000 t in 2000 while in 2005 the total catch was 804,000 t and in 2006 it was estimated at 1,061,107.6 t. On the contrary a reverse trend in the contribution of *L. niloticus* to the fisheries has been observed. *Lates niloticus* contribution to the total annual fish

landing decreased from 42% in 2000,through 29% in 2005 to 24% in 2006 (http://www.lvfo.org, 2013).

*Declining fish production*

The fisheries sector in Uganda was estimated to provide employment to more than 136 000 artisan fishermen, while over 700 000 people were indirectly employed through secondary and tertiary activities such as processing, trading, boat building, net-making and the providing of diverse support services (MAAIF, 2004; Matsuishi et al., 2006; Njiru et al., 2008; Hammerle et al., 2010). Regionally, the Nile perch fishery alone earns Uganda, Kenya, and Tanzania US$ 600 million per annum and provides employment to over three million people (Matsuishi et al., 2006; Njiru et al., 2008). Captured fish provides the most inexpensive source of protein with an average per capita consumption per year of 10 kg, accounting for more than 50% of the animal protein intake of an average Ugandan diet (MAAIF, 2004; NEMA, 2008). This is below the FAO global fish, per capita, consumption which is estimated at 17 kg live weight (FAO, 2010).

The Nile perch fishery contributes an estimated 2.6% to Uganda's Gross Domestic Product (Odongkara et al., 2009). In 2006 fish exports generated US$141 million for Uganda, but this figure has dropped to less than US$80 million in 2008 (FAO, 2010; Hammerle et al., 2010). The dramatic decline has been attributed to over-exploitation of the fish stocks, hastened by an increasing demand for fish on local, regional and international markets, as well as nutrient loading from farmlands, urbanisation of the catchment areas and illegal and unregulated fishing methods (NEMA, 2008; Njiru et al., 2010).

*Future demand for fish*

Globally, most fisheries are now considered either fully exploited or exploited beyond maximum sustainable yields (FAO, 2010) and yet the demand for food fish has continued to rise as the global human population reached the 7 billion mark (CIA, 2011). Ugandan population size was projected to reach 32 million by 2015 but this estimate was already reached in 2009, six years earlier than the forecast period (UBOS, 2011). The population was estimated at 34.6 million in July 2011 (CIA, 2011). Therefore, more than the projected 380 000 t of food fish will be required if Uganda is to meet her current domestic annual-per-capita-fish-consumption levels as well as projected export volumes. This means that the production of fish will have to increase by more than 160 000 t above the 2001 catch of 220 726 t by 2015 (MAAIF, 2004). Uganda's total fish production has stagnated to below 220 000 t per year since 1995, probably due to declining fish stocks (Nyombi and Bolwig, 2004). This calls for an increase in the fisheries production to avert the expected social and economic crisis as a consequence of decreased capture fisheries production and increased demand for the fisheries resources (MAAIF, 2004; NEMA, 2008). One way of increasing fish supply is through aquaculture (NEMA, 2008).

*Choice of Nile perch as a species for aquaculture*

The Nile perch, *L. niloticus* grows fast and generally attains a weight of 2 to 3 kg body weight in the first year (Hughes, 1992), while adults of the species have been reported to grow to a body weight of up to 200kg at a total length of 1.9 m (Ribbink, 1987). *Lates niloticus* has white muscle tissue which is rich in omega-3 poly-unsaturated fatty acids (Mbatia et al., 2010). Based on the high quality of the flesh, exporters have established marketing channels in Europe and North America despite the high freight costs. As a result, processing and handling facilities were

established in Uganda, with an installed capacity of 400 t of fresh and frozen fillets per annum. Unfortunately several ventures closed down and the few remaining factories are operating below 50% of their expected capacities, due to the dwindling wild stock of Nile perch. There is therefore a need to increase Nile perch production so as to provide fish for these factories and meet the domestic, regional and global demand for Nile perch products.

There are two potential methods of increasing Nile perch production. One is an effective and sustainable capture fisheries management and the other is to culture the species. However, capture fisheries, if not properly managed, as it removes large individuals from wild populations, may impose selection pressure that reduces the frequency of large individuals (Campton and Gall, 1988; Allendorf and Hard, 2009). Aquaculture breeding programmes have the specific goal of increasing the frequency of desirable broodstock, hence contributing to the sustained production of the species. It does not impose selection that reduces the frequency of those desirable phenotypes in the species (Allendorf and Hard, 2009). A sustainable way to increase the Nile perch fishery production may be through aquaculture technology development.

Development of a farming protocol for a new candidate fish species for aquaculture requires an understanding of its reproductive biology as well as the environmental factors that influence the reproduction. Information about reproductive biology is required for the development of farming technologies and the design of fisheries management measures such as decisions on closed areas or seasons. These policies are based on biological data. They are best based on information of the species' reproductive biology such as size at sexual maturity, duration of spawning season and periodicity of spawning (Wootton, 1984). For aquaculture development of a species, there is a

need for production of gametes and progeny to ensure year-round production of fish (Bromage, 1995). Several aspects of biology and ecology of Nile perch, such as its ecology and biology in Lake Chad (Hopson, 1972), predation and its interactions with the fisheries in Lakes Victoria and Kyoga (Ogutu-Ohwayo, 1990; Kitchell et al., 1997), reproductive potential of the Nile perch, (Ogutu-Ohwayo, 1988), Nile perch growth patterns (Hughes, 1992), and sex differentiation (Basiita et al., 2011) have been published. There is, however, a paucity of information on gametogenesis, reproductive seasonality and environmental effects on reproduction. Research on the Nile perch has concentrated on the stock abundance and distribution, exploitation patterns and general biology, like growth and feeding habits with less research being focused on their reproductive biology (Kaelin and Cowx, 2002). Information is needed on the species' reproductive biology such as gonadal development and recrudescence, spawning patterns, the physiological responses to handling stress associated with induced spawning, environmental factors influencing reproduction, and larval development under aquaculture conditions. This thesis examines gonadal development, gonadal recrudescence, spawning patterns, physiological and behavioral responses to clove oil used as an anaesthetic, the influence of rainfall, temperature and wind speed on reproduction and the use of a hormone preparation (GnRHa + MET) to induce final oocyte maturation in Nile perch. These factors are key determinants of economic viability for the commercial production of a species. The need for research in these areas is motivated in the following paragraphs.

*Reproductive seasonality*

Rainfall, water temperature and photoperiod modify reproductive cycles in teleost fishes (Sumpter, 1990). They show considerable, but reliably-timed annual fluctuations in temperate

regions, whereas in the tropics, dry seasons alternating with wet seasons, lead to seasonal differences in water quality and food availability. In addition, proximate cues related to flooding and consequent availability of suitable spawning sites or substrate, as well as food availability are important factors that control reproduction in fish species (Sumpter, 1990).

*Proximate factors controlling spawning*

Several factors control the onset of breeding in fishes. Rainfall influences hydrological factors such as water quality and quantity. Changes in these variables provide cues that may be used by fish to detect the onset of floods that provide conditions for spawning (Hopson, 1972; Munro, 1990). Photoperiod is considered a predictive cue and may play a role in at least some species in areas close to the equator as low as $12^{o}$N (Munro, 1990). Light intensity may be used by some fish species as a predictive factor (Migaud et al., 2010). However, in equatorial regions, factors like cloud cover, water depth and turbidity may affect light intensity in the water and their seasonal changes may be used as cues (Munro, 1990).

Ambient temperature in the tropics is usually highest at the end of the dry season and low during the rainy season. Such cycles may have a predictive role in higher latitudes but are likely to be less important at low latitudes, especially in small and shallow water bodies like Lake Nabugabo in Uganda where Nile Perch was introduced. Changes in water temperature had a capacity to affect endocrine function by either advancing or retarding gametogenesis or maturation (Pankhurst and King, 2010).

Food supply for broodstock and larvae is important in determining the time of reproduction. Surplus food is stored as fat in the mesenteric fat tissue in both broodstock and larvae. The mesenteric fat index provides useful information on energy storage for future metabolic demands relative to other energy stores (Brown and Murphy, 2004). Lloret et al. (2008) showed that female European hake pre-spawners with higher lipid reserves, had high lipid content in their ovaries and they suggested that maternal condition may affect the inherent reproductive capacity in the species.

Atmospheric pressure, wind speed and rainfall are presumed to act as predictive cues for the commencement of breeding in some fish species. This study aimed to quantify to what extent rainfall, temperature and wind speed moderate reproductive activities (GSI, gametogenesis and spawning) in the Nile perch.

Information on seasonal variation of gonadal somatic, mesenteric fat and hepatosomatic indices is important in estimating spawning times or spawning events of a fish species (Lloret et al., 2008; Santos et al., 2010). Such information contributes to our understanding of timing of biological energy reserve allocation to various biological needs of the organism, such as reproduction (Sutharshiny and Sivashanthini, 2011). There is a paucity of knowledge on seasonality of reproduction and the relationship between these indices and environmental factors in the reproductive biology of *L. niloticus*.

*Gonad and sperm structure*

Gonadal histology provides information based on oocyte developmental stages (West, 1990). In *L. niloticus* macroscopic staging of the gonads, oocyte size (diameter), staging based on

appearance of oocytes, size at maturity and gonadal indices have been studied (Okedi, 1970; Hopson, 1972; Ogutu-Ohwayo, 1988 and 2004). Histomorphology of *L. niloticus* gonads have been used to investigate sex differentiation and gonad development (Basiita et al., 2011). However, no studies on the testis and sperm ultrastructure have been conducted in this species. This study aimed at generating information on sperm and oocyte structure and development in *L. niloticus*. This information can be used to evaluate milt quality (Billard, 1978) for artificial spawning and hatchery management. Sperm ultra-structure is useful in the classification of fishes into different reproductive strategies (Mattei, 1991).

*Stress and anaesthesia in fish*

Stress in fish is a non-specific response that can be categorized into three stages i.e. a primary, secondary and tertiary response (Stoskopf, 1993). Stress can manifest in fish as a result of handling and transport or physical injury, resulting in the suppression of the immune response and even mortality. The large size of *L. niloticus* limits broodstock management during capture, handling and transportation both from the wild and on a farm. There is therefore a need to immobilize the fish before handling. This is especially relevant to the handling of *L. niloticus*, a large species that has not been kept under captive conditions.

Studies have been carried out on the efficacy of various anaesthetics in many species; *Carassius auratus* (Weyl et al., 1996), *Oncorhynchus mykiss* (Pirhonen and Schreck, 2003), *Salmo salar* (Iversen et al., 2003), *Colossoma macropomum* (Roubach et al., 2005) and *Haplochromis obliquidens* (Kaiser et al., 2006). The dose responses have been reported to be species-specific and substance-specific (Stoskopf, 1993). No research work has been done on the stress-effects and the efficacy of any anaesthetic agent used to sedate *L. niloticus*. This chapter established

clove oil working doses as an anaesthetic and the influence of clove oil on the physiology of the Nile perch broodfish.

**Objectives of the study**

Life-history characteristics such as, gonadal development, size-at-fifty-percent-maturity, spawning patterns, recrudescence patterns and factors influencing these parameters, gonadal and gamete ultrastructure, chemical restraint and hormonal influence on oocyte and follicular structure were studied. The reproductive biology of some indigenous fish species in Lake Victoria, such as *Labeo victorianus* (Rutaisire, 2003) and *Bagrus docmak* (Aruho, 2009) has been studied. Hopson (1972) studied the breeding biology and growth of *L. niloticus* in Lake Chad, and Hughes (1992) reported on the growth and size at maturity in *L. niloticus* from the Nyanza gulf, Lake Victoria. Ogutu-Ohwayo (1988) studied size at first sexual maturity and fecundity of *L. niloticus* from Lake Kyoga. There is no information on the reproductive biology and environmental factors moderating gametogenesis in *L. niloticus*.

This thesis has seven chapters. Chapter one gives an overview of the topic. The study areas and general methods used are outlined in Chapter two. Chapter three investigates the reproductive biology and factors influencing Lake Victoria *L. niloticus* populations. Testicular, ovarian and gamete structure, together with a histomorphological description of recrudescence is studied in Chapter four. The potential use of clove oil as an anaesthetic has been evaluated with the aim of establishing anaesthetic and sedative working doses that can be applied in the handling of broodstock. To achieve this, physiological responses to exposure to clove oil have been evaluated in Chapter five. The data obtained in Chapters three, four and five were used in timing

the collection and management of live fish and to induce oocyte maturation and ovulation in the species. In Chapter six, induced oocyte maturation and ovulation and early embryo development is described. Chapter seven contains a discussion of the findings with the aim of providing a perspective of aquaculture development and fisheries management of *L. niloticus*.

# CHAPTER TWO

## Study area and general methods and materials

Nile perch, *Lates niloticus*, is a large fish that inhabits most niches in Lake Victoria, from deep waters to waters as shallow as 3.5 m in depth. This species prefers rocky sites in low light intensity areas of the lake. Juveniles are easily caught in beach seine nets from around seven o'clock in the evening, throughout the night and using light-fishing gear as generally employed in the African sardine fishery (own observations). Adult Nile perch feed both during day and night as they have night vision; their eyes reflecting light in the night (Fig. 2.1).

In addition, Nile perch has a swim bladder with no pneumatic duct, suggesting that it regulates the pressure inside the swim bladder by physiological means, using a gas gland. The gases in the swim bladder are released slowly into the bloodstream. This results in a delay to balance the swim bladder internal pressure build-up due to decreasing external pressure, resulting from rapid elevation of the fish from the deep water column to the surface. This presented a challenge in acquiring broodstock, especially those caught at a water depth of greater than ten metres. Nile perch caught in these water depths usually had an inflated swim bladder. In some cases the swim bladder is ruptured, or the stomach was forced out through the mouth and the eyes protrude (Fig. 2.2). They often present with a gas-filled vascular system and are lethargic. Fish with these symptoms rarely survive beyond 18 hours from the time of capture. Usually a swim bladder puncture improves survival, provided that the fish had not spent too long on the hook.

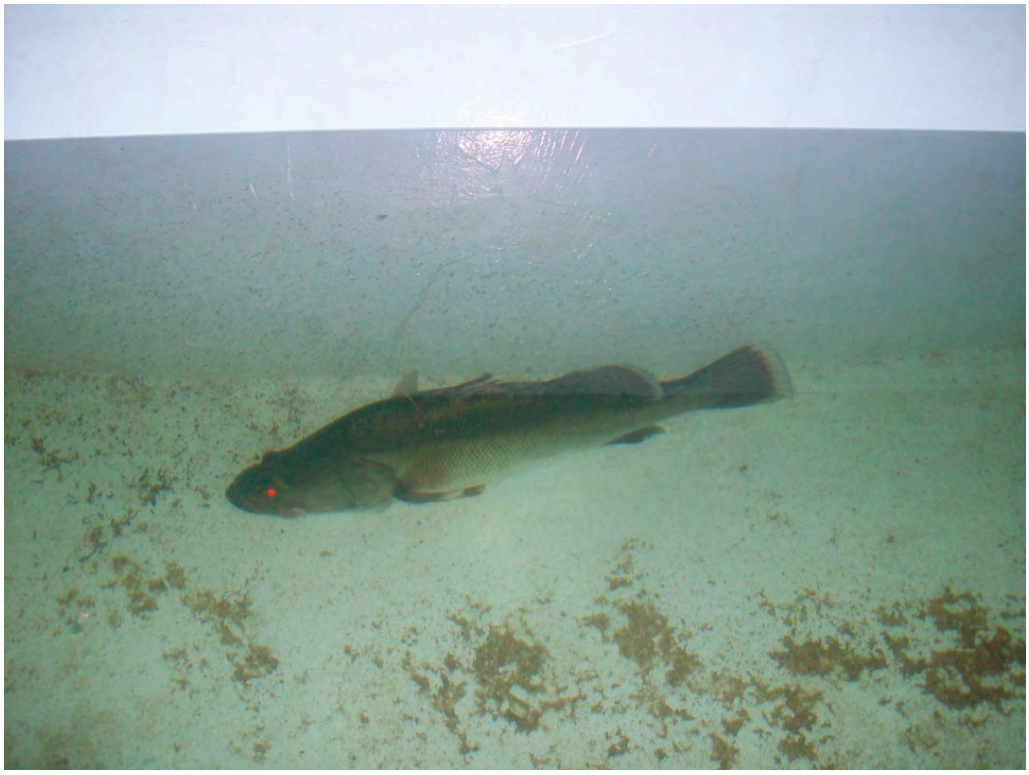

**Figure 2.1:** Female Nile perch in a concrete tank with glaring eyes for night vision

**Figure 2.2:** Nile perch specimens with averted stomachs and protruding eyes due to baro-trauma

These issues influenced the selection of the sites chosen for the sampling of specimens. Sites such as sheltered, shallow and rocky areas of the lake were chosen in order to ensure an adequately large sample size and because that is where the broodstock fish required for the maturation and anaesthesia trials, were most likely to be found.

*Study areas*

The study was conducted in shallow areas around Entebbe, the Wakiso district, Wayia Bay, ($0^o$ 04' 47.96 N, $32^o$ 26' 23.28"E), Zanga Bay, sheltered rocky waters, Kamutenga rocks in Mpigi district ($0^o$ 03' 15.71"S, $32^o$ 14'28.28"E), and Mawala Islands in Wakiso District ($0^o$ 01'42.81" S, $32^o$ 24' 22.94" E) (Fig. 2.3).

The specimens used for this research were from three bays, Wayia, Entebbe and Zinga as well as the surrounding islands. The choice of the site was also influenced by the proximity to the Aquaculture Research and Development Centre in Kajjansi, a government-run research centre, eight kilometres from the Lake Victoria shore, and Interfish Fish Farm, a privately owned fish farm on the shores of Lake Victoria. Interfish Fish Farm uses water from Lake Victoria and is thus ideally suited to condition the Nile perch broodstock. The conditioning of the broodstock is done in large, circular tanks with a volume of 30 $m^3$.

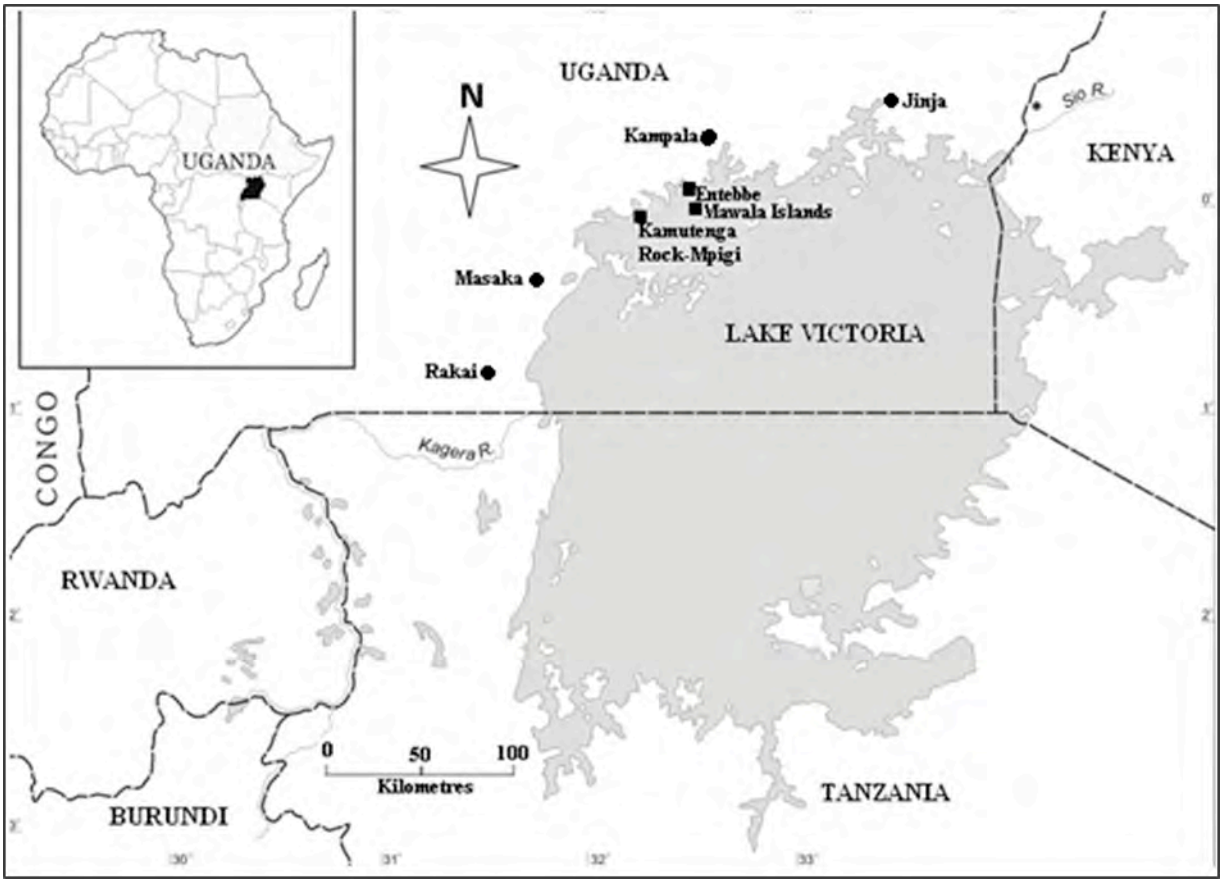


**Figure 2.3:** Map of Lake Victoria, Uganda, showing the areas selected for sampling. Inserted is a map of Africa showing where Uganda is located. Solid squares = sampling sites, solid circles = cities/municipalities/towns

**Field collection methods**

Fish used (in Chapters three and four) were caught with long-lines with hooks ranging from 6 to 12 inches and baited with catfish juveniles. Samples were collected every month, for 24 months, from May 2007 to April 2009. Samples were labelled and morphometric measurements were done. The gonads were scored according to Hopson (1972) (Tables 3.1 and 3.2 in Chapter three of this thesis) and preserved in Bouin's solution for histology. In the laboratory, fixed specimens were trimmed to a size of 2 $mm^3$ and processed for light microscope histological examinations, following standard histological procedures (Genten et al., 2009).

Gonad samples, for ultra-structural studies, were taken from fish caught from Entebbe Bay, using beach seine nets. Blocks of gonad samples (2.5 mm) from freshly killed fish were fixed in 2.5% glutaraldehyde, in a phosphate buffer, at pH 7.2 for 24 hours. These samples were then couriered to the Rhodes University Electron Microscopy Unit. Here the samples were processed further for scanning and transmission electron microscopy. They were viewed using a JEOL 1210 electron microscope and JEOL 840A scanning electron microscope, as reported in Chapter four of this thesis.

Specimens for the anaesthesia experiment (a description of the methods are outlined in Chapter five of this thesis), were obtained from Entebbe and Wayia Bays by using long-line hooks and beach seine nets. Live fish were transported in a 1000 litre tank, supplied with medical oxygen. The oxygen concentration was maintained above 6 mg/L. The specimens were then delivered to Kajjansi and released in a pond (4000 $m^2$), where they remained for three months to give the fish time to recover from the stress of their capture. Juvenile tilapia that had been stocked in those ponds two months earlier, served as live food for the Nile perch.

Specimens for the induced ovulation experiment (Chapter six) were collected from Entebbe, Wayia and Zinga Bays, using long-line hooks. These bays are shallow, with a depth ranging from 2.5 metres to 5.5 metres and the area is sheltered from strong water currents. The fish were transported under clove oil sedation to Kajjansi Aquaculture Research Centre and Interfish Fish Farm where they were kept for three hours before the experiment could commence. Before the hormonal injection was administered, fish were evaluated for suitability to spawn, by a biopsy of eggs. A 2.5 mm silicon cannula was used and the eggs were examined under a dissecting microscope at a 40 x magnification. Details of the methods are outlined in Chapter six of this thesis.

# CHAPTER THREE

## Aspects of the reproductive biology of Nile perch, *Lates niloticus,* from Lake Victoria

### Introduction

Many studies on reproductive seasonality in tropical freshwater teleost fishes have been based on data from mature fish, while some authors studied breeding behaviour, fish with spent gonads or the presence of their offspring (Munro, 1990). Photoperiod, temperature and seasonal rainfall, for example, can modify the reproductive cycle of teleosts (Sumpter, 1990; Migaud et al. 2010; Pankhurst and King, 2010). Fish show considerable, but precisely-timed annual fluctuations in temperate regions, while in the tropics, dry seasons alternate with wet seasons, leading to seasonal differences in water quality and food availability (Migaud et al. 2010). Proximate cues related to flooding and consequent availability of suitable spawning sites, substrate and food abundance have been reported as the most important factors to moderate reproduction in some tropical fish species (Sumpter, 1990).

The environmental factors that influence teleost reproduction are categorised as either ultimate or proximate (Munro, 1990). Ultimate factors relate to offspring survival and growth, while proximate factors are related to gonadal cycle synchronisation with the environment, so as to maximize an individual's reproductive fitness. This strategy allows for spawning when environmental conditions are optimal for survival and growth of the progeny (Munro, 1990). The relationship between reproduction and the environment is mediated through the neuro-endocrine system (Dufour et al., 2010; Migaud et al., 2010). The neuro-endocrine system perceives environmental cues and acts as a transducer of signals that influence the structure of the gonads and their function (Peter and Yu, 1997; Dufour et al., 2010; Migaud et al., 2010). The

interactions of such environmental and intrinsic factors have, however, not been studied in *L. niloticus*.

Size or age at first maturity is defined as the age or size at birth of offspring in animals and the age at first seed ripening in plants and not the age or size at which some of the physiological or morphological conditions of maturation are being met (Stearns, 1992). An individual must reach a threshold size or age before it can invest in reproduction or initiate gametogenesis in response to environmental cues (Munro, 1990). Mean size, or age at sexual maturity varies among populations within species and this has been attributed to genetic divergence in response to environmental variability (Stearns, 1983).

In *L. niloticus,* size at maturity varied over the last 50 years, since its introduction into Lakes Victoria and Kyoga. Variations in the ranges of size at maturity have been reported in lakes where the species naturally occurs (Okedi, 1970; Hopson, 1972; Ogutu-Ohwayo, 2004). Female Nile perch matured at a larger size than males, based on data collected from 1970 to 2003 (Okedi, 1970; Ogutu-Ohwayo, 2004). Although data collected from Lake Victoria fish-processing factories suggest a smaller difference in size at maturity between females and males than previously estimated (LVFO, 2007), fish were probably not from random samples. Methods may also have been biased.

Several studies on Nile perch have addressed ecology, stock assessment, harvesting, feeding and growth (Ogutu-Ohwayo 1988, 1990 and 2004; Hughes, 1992; LVFO, 2007). Few studies on the reproduction of Nile perch have been done. Basiita et al. (2011) gave an overview of sex

differentiation and provided fecundity estimates. Age at sexual maturity was calculated by Hopson (1972), Ogutu-Ohwayo (1988) and LFVO (2007). There is paucity of data on the reproductive cycle, gametogenesis, and environmental factors influencing reproduction in *L. niloticus.*

Understanding the reproductive biology of a fish species is the basis for the establishment and development of farming methods, as well as the management of wild stocks. Catch quotas are based on information of the species' reproductive biology such as size at sexual maturity and the duration and periodicity of spawning (Wootton, 1984). In aquaculture, there is a demand for well-planned production of gametes and offspring to ensure fish production throughout the year (Bromage, 1995). This requires manipulation of the reproductive system of the fish under aquaculture conditions, based on an understanding of natural spawning patterns as well as those factors influencing timing and the rate of gametogenesis. This chapter investigates reproductive seasonality, sexual maturity and causal factors influencing timing of reproduction in the Nile perch, *L. niloticus,* in Lake Victoria, Uganda.

**Materials and methods**

Nile perch specimens (n = 1332) were collected using commercial gill nets of 10 - 21.6 cm mesh size and long-line hooks. The fish were sampled from May 2007 to April 2009. Fish were sexed, based on the morphology of the genitalia, and weighed to the nearest 0.1 g. The total and standard lengths were measured to the nearest 0.1 cm. All fish were dissected and gonads were classified according to Hopson (1972), with some modifications. Hopson (1972) classified male Nile perch gonads into six stages; Stage 1: immature; Stage 2: early developing; Stage 3: late

developing; Stage 4: mature/resting; Stage 5: mature ripe and Stage 6: ripe/running. Hopson (1972) did not stage Nile perch males that had spent their gametes (Table 3.1).

**Table 3.1:** Female and male gonad maturity stage classifications of *L. niloticus*, as modified from Hopson (1972). Stage VI spent males were not included in Hopson's classification

| **Gonad stages** | **Females** | **Gonad stages** | **Males** |
|---|---|---|---|
| **Stage I:** Immature | Ovary paired, thin, transparent running longitudinally dorsal to body wall. Sex indistinguishable. | **Stage I:** Immature | Testes were a pair of thin transparent filaments running longitudinally, along the dorsal wall of the body cavity. Sex was indistinguishable. |
| **Stage II:** Resting | Ovaries greyish-white, transparent, smooth cylindrical and lightly vascularised. They did not span over the entire length of the peritoneal cavity. | **Stage II:** Developing | Testes semi-transparent greyish-white, flat with longitudinal fat attached by the mesentery to its vertical surface. The testes were small in transverse section. Testes were well vascularised, with no milt. |
| **Stage III:** Early maturing | The ovaries were reddish-pink, semi-transparent, pear shaped in transverse section and well vascularised. | **Stage III:** Mature/ Resting | Testes opaque, white-pinkish, soft and triangular in section, well vascularised and flattened along the edge. The fat was smaller than the testes but still |

| | | | |
|---|---|---|---|
| | | | prominent. Milt exuded from the lumen of cut testes. |
| **Stage IV:** Late maturing | Ovaries were pinkish with clearly visible small, opaque, pear-shaped yolky oocytes. Small blood vessels were present. | **Stage IV:** Mature/ Ripe | Testes opaque, ivory-white or pinkish, triangular in cross section, edges rounded with the fat strand reduced, lying in the longitudinal groove on the ventral surface of the testes. Large volumes of milt. |
| **Stage V:** Ripe | Ovaries were opaque with large, yellow, yolky oocytes discernible through a superficial membrane. Large blood vessels were prominent on the surface of the ovaries. | **Stage V:** Ripe/ running | Testes appearance similar to those in stage VI. A large volume of milt was released through the cloaca on applying gentle pressure on the abdomen. Testes turgid. |
| **Stage VI:** Running | Oocytes were yellow-brownish in colour. Slight pressure resulted in eggs exuding from the vent. | **Stage VI:** Spent | Testes were flabby, reddish-pink and smaller than in stage V. |
| **Stage VII:** Spent | Ovaries were flabby and loose with a few scattered residual Stage V oocytes. | | |

Macroscopic gonad staging was histologically validated. Tissue subsections were taken from the rostral, middle and caudal regions of the ovary, fixed in Bouin's fluid for 72 hours and transferred to 75% ethanol. Some samples were stored in buffered formalin. The tissues were processed for light microscopy, dehydrated in ethanol, cleared in xylol and embedded in paraffin. Sections were cut at 5 μm thickness and stained with Harris's haematoxylin and eosin counter staining. Some testis tissue samples were stained using Toluene blue, since it gave a better contrast in testicular tissue than H & E staining. The histological staging of ovaries was based on the stage of oocyte development, adopted and modified from Basiita et al. (2011). The oocytes of all stages of development, from posterior, middle, and anterior sections of the ovary were randomly examined, counted and the total number of oocytes was recorded. The frequency of oocytes at different stages of development was calculated as a percentage of the total number of oocytes counted from the anterior, middle, and posterior portion of the gonads and were related to the month of the year. The data were used in estimating the best time at which to collect ripe broodstock and the spawning seasonality in the population.

A total of 652 ovaries were weighed (GW) to the nearest 0.1 g and a tissue sample of approximately 2.0 g was removed from each gonad, dehydrated in ethanol, cleared in xylol and embedded in paraffin. Sections were cut at 5 μm thickness and stained with Harris's haematoxylin, followed by the eosin counter stain. Histological staging of ovaries was based on the stage of oocyte development and on the occurrence of post-ovulatory follicles (POF) and atretic oocytes (Hunter and Goldberg, 1980). The criteria for the identification of atresia stages and the atretic condition of the ovary were adopted from Hunter and Macewicz (1985) and Basiita et al. (2011) (Table 3.2).

**Table 3.2:** Histological characteristics of *L. niloticus* oocytes at various stages during gonadal recrudescence, as modified from Basiita et al. (2011)

| **Oocyte stage** | **Description** |
|---|---|
| Oogonia | Nucleus large (>75%) of entire cell; large nucleus to cell ratio, one distinct nucleolus |
| Chromatin nucleolus (CNS) | Polygonal in shape, large, centrally located nucleus and one large nucleolus, high nucleus to cell ratio |
| Peri-nucleolus stage oocyte (PNS) | Polygonal to oval oocytes with more than one nucleolus |
| Primary yolk vesicle oocyte (PYVO) | Appearance of lipid droplets around the nucleus, increase in number to fill the ooplasm |
| Secondary yolk vesicle oocyte (SYVO) | Appearance of yolk granules in the ooplasm, visible follicular layers |
| Tertiary yolk vesicle oocyte (TYVO) | Presence of yolk granules and migrating germinal vesicle. Coalescence of oil globules to one or two large oil globules |
| Post-ovulatory follicle (POFs) | Change from a squamous cell shape to a cuboid/columnar, follicular cell. Increased vascularisation of the thecal layer. |
| Type I: Atresia | Vitellogenic oocytes had fragmented *zona radiata* with intact yolk globules |
| Type II: Atresia | Vitellogenic oocytes yolk granules broken down to smaller granules and intact *zona radiata* |
| Type III: Atresia | Degeneration of non-vitellogenic oocyte. |

The gonad, liver and mesentery fat mass of each fish were weighed to 0.01 g. The sex of each specimen was recorded and gonads were macroscopically staged according to Hopson (1972) and Basiita et al. (2011) (Table 4.1). Sections of the gonad from its rostral, middle and caudal part were preserved in Bouin's fluid for 72 hours prior to transfer to 75% ethanol for storage. The mass of the gonad, mesenteric fat, and liver as a percentage of fish mass, were calculated to derive several indices. These indices included the gonadosomatic index (GSI), the hepatosomatic index (HSI) and the mesenteric fat index (MFI). An eviscerated condition factor was calculated as K (g /cm$^3$) = eviscerated weight / cube of total length. Eviscerated weight was used so as to remove the effect of gut fullness. Weather and hydrological data, rainfall, wind speed, and temperature were obtained from the Meteorology and Water Departments of Uganda.

*Size at sexual maturity*

Length-at-maturity is the length at which 50% of the sampled fish in a size class are sexually mature. Males at macroscopic gonad Stage 3 and females from Stage 2 and above were considered mature after histological validation. Length, at which 50% of individuals in a size class are sexually mature, was estimated using a logistic ogive described by the non-linear two parameter model (Booth, 1997).

$$P_l = \frac{1}{1+e^{(L-L_{50})/\delta}} \quad \text{..... (1)}$$

Where $P_l$ = the predicted percentage of mature fish at length, $L_{50}$ = the length-at which 50% of fish in a size class $L$ mature and δ = model parameter. The model parameters were determined by non-linear minimization of a negative binomial log-likelihood function (Equation 2).

$$-\mathrm{In}L = -\sum\left(Y_l \mathrm{In}\left(\frac{P_l}{1-P_l}\right) + (n_l - Y_l)\mathrm{In}(1-P_l)\right) \ldots\ldots\ldots\ldots (2)$$

Where $P_L$ is the predicted maturity from the logistic regression, $Y_l$ = the number of fish mature in length class i and $n_l$ = number of all fish sampled in length class $L$.

*Data analysis*

Variations of indices between months were analysed using the non-parametric Kruskal-Wallis test, if residuals were not normally distributed after data were transformed to the log of base ten. Non parametric Spearman's Rho correlation analysis of monthly mean gonadosomatic index (GSI) against monthly mean rainfall, temperature and wind speed were carried out. Length at which 50% of individuals in a size class were sexually mature was predicted by fitting a logistic ogive, using MS Excel Solver. The probability of finding ripe female broodstock in a given month was calculated using the Z-test on GSI and tertiary yolk vesicle oocyte frequency, based on the average value of GSI and TYVO oocytes of all ripe females in the respective monthly sample.

**Results**

*Monthly variations of various reproductive indices*

*Average fish total length*

The mean fish total length of fish sampled differed significantly between months ($F_{(11,186)}$ = 4.924, $p<0.001$ (high 84.8±3.2 cm in May, low 55.0±2.0 cm in February) in females while in males means length was F(11,192) = 3.254, p<001 (high 82.1±9.9 cm in May and low 57.8±1.9 cm January).

*Gonadosomatic index*

The Gonadosomatic index (GSI) did not differ between the two years. Thus, data for the two years was averaged. Significant differences between the monthly GSI, in both female and male *L. niloticus*, were observed; Kruskal-Wallis test, H (11, n = 652) = 125.5, $p < 0.001$, and Kruskal-Wallis test, H (11, n= 429) = 125.7, $p < 0.001$, respectively. In females the highest mean was observed in July, 2.2 ± 0.3% and ranged from 0.1 to 9.1% and the lowest was in February with a mean of 0.4 ± 0.04% (Fig. 3.1a) and the highest for males was 2.34 ± 0.45% in November (Fig. 3.1b).

*Condition factor*

In both sexes the average condition factor differed significantly between months, Kruskal-Wallis test, H = 125.7, n= 652, $p <0.001$, and Kruskal-Wallis test, H = 19.9, n = 474, $p < 0.025$, female and male, respectively. The highest average condition factor was observed in November for both sexes; mean and standard error 1.4 ± 0.2 in females ranged from 0.62 to 7.73% (Fig. 3.1a) and in males was 1.53 ± 0.14 ranging from 0.83% to 3.23%. It remained low throughout the other months (Fig. 3.1b).

*Hepatosomatic index (HSI)*

Significant differences in the mean hepatosomatic index between months were observed in both females and males, Kruskal-Wallis test, H = 47.4, n = 652, $p < 0.001$ in females and H = 99.3, n = 652, $p < 0.001$ in males. The highest HSI was observed during July (1.50 ± 0.18%) and the lowest (0.87 ± 0.49%) occurred in February in females (Fig. 3.1a).

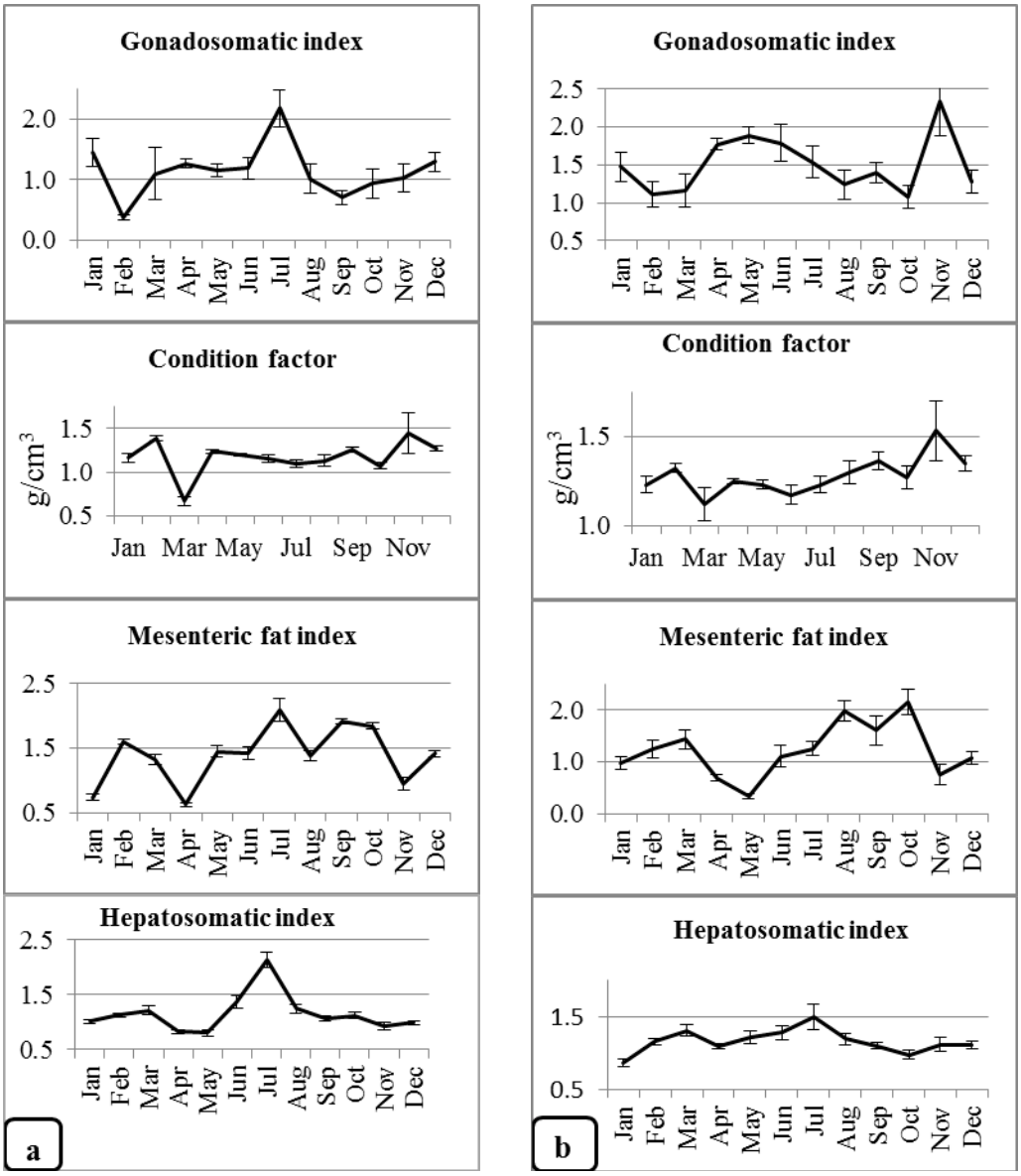


**Figure 3.1:** Monthly means with vertical standard error bars for gonadosomatic index, condition factor, mesenteric fat index, and hepatosomatic index in panel (a) female and (b) male *L. niloticus*

*Mesenteric Fat Index (MFI)*

Significant differences in the mesenteric fat index between months, in both females and males were observed, Kruskal-Wallis test, $H = 181.9$, $n = 652$, $p < 0.001$, and $H = 136.8$, $n = 652$, $p < 0.001$, respectively. The highest mesenteric fat index (MFI) was in September and the lowest values were in May, in both sexes (Figure 3.1 a, b).

*Length at 50% sexual maturity*

Of 675 female Nile perch collected in the period May 2007 to April 2009, 128 were immature and 547 were mature. Of the 523 male Nile perch, 165 were immature and 358 were mature. Maturity logistic ogives for females and males predicted the length at which 50% of individuals were sexually mature ($L_{50}$). Size at 50% sexual maturity for female Nile perch was represented by a size class of 59.4 cm total length ($\delta = 6.303$, $\lambda = 0.105$, $r^2 = 0.65$, $p < 0.001$, $n = 547$) (Fig 3.2a). The size at 50% maturity for male *L. niloticus* was 57.8 cm total length ($\delta = 6.000$, $\lambda = 0.100$, $r^2 = 0.76$, $p < 0.001$, $n = 358$) (Fig. 3.2b). Using the reproductive variable gonad maturity stages, the smallest recorded mature female *L. niloticus* was 45 cm long and the largest female immature fish had a total length of 110 cm. In males, the smallest mature male was 45 cm long and the largest immature fish was 103 cm in total length.

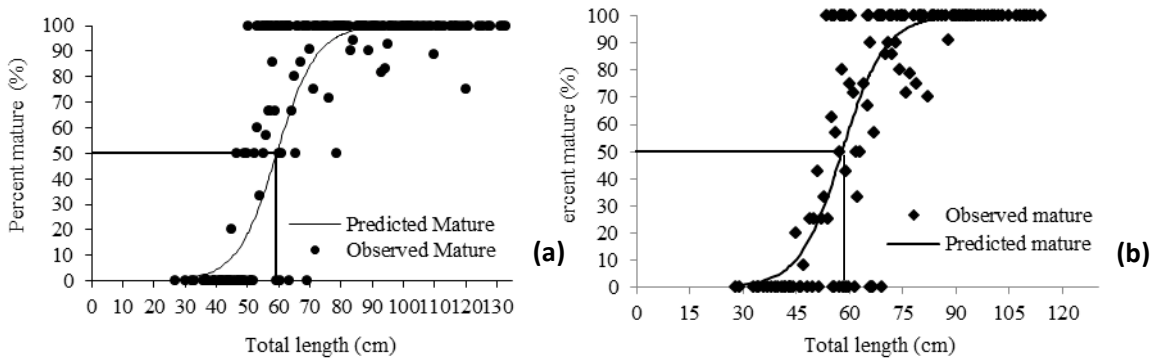


**Figure 3.2:** Observed and predicted percentage sexually mature (a) females and (b) males of *L. niloticus* from Lake Victoria, Uganda, collected between May 2007 and April 2009

*Monthly distribution of proportion of individuals at different gonad maturity stages*

In female *L. niloticus*, individuals at gonad Stage 3 were observed throughout the year while the highest number of fish with ovaries at Stage 5 was observed in July and August. Fish with spent ovaries were frequent in the month of September, albeit accounting for less than 5% of fish collected in that month (Fig. 3.3). Male *L. niloticus* with gonad Stage 5 persisted throughout the year, with relatively more fish observed in April, May, September and December. Females with gonads at Stage 7 were observed in September while males with Stage 7 gonads were observed in August, September and November and less than 5% in January and March (Fig. 3.4).

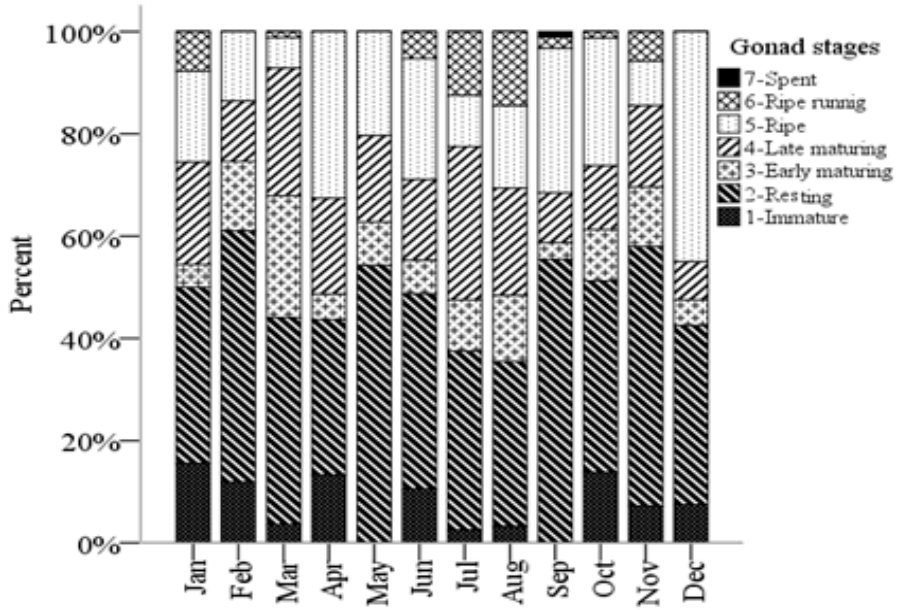


**Figure 3.3:** Temporal variations in maturity stages for female *L. niloticus,* collected from Lake Victoria from May 2007 to April 2009

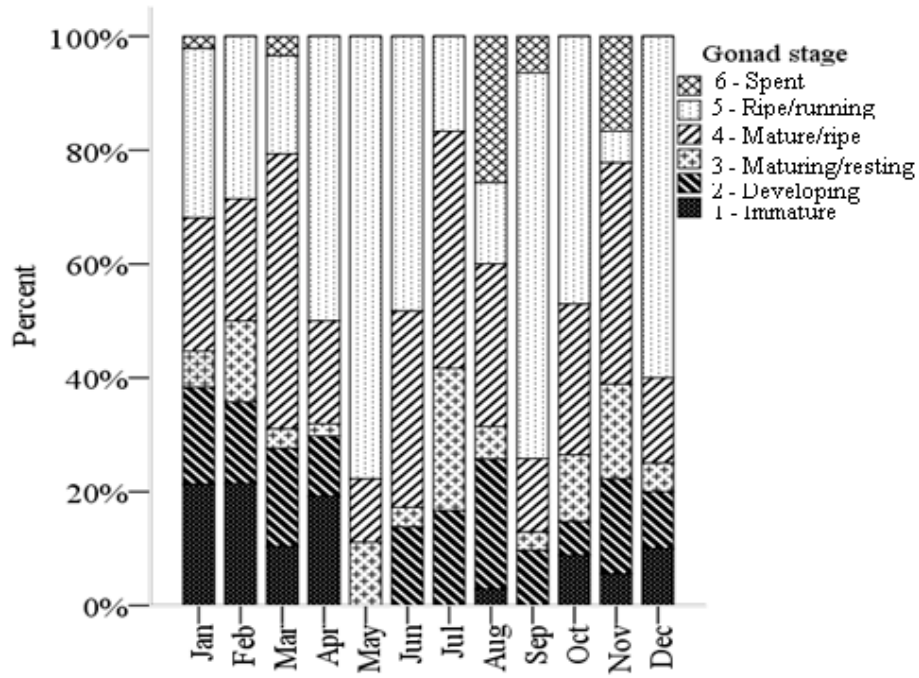


**Figure 3.4:** Temporal variations in maturity stages for male *L. niloticus,* collected from Lake Victoria from May 2007 to April 2009

*Monthly frequency distributions of oocyte stages*

Pre-vitellogenic oocytes maintained high relative frequencies throughout the year. Thus, they were not included in the analysis and only oocytes that had commenced vitellogenesis were considered, i.e., from primary yolk vesicle to atretic oocytes. The primary yolk vesicle oocyte (PYVO) remained above 25% of the sum of PYVO, SYVO, TYVO, atretic oocyte, and POFs, counted per fish and the highest frequency of 45% was in March. The number of secondary yolk vesicle oocytes was generally lower than the PYVO oocytes throughout the year (Fig. 3.4).

There was a positive correlation between TYVO and atretic oocyte numbers ($r = 0.5$, $p < 0.001$, $n = 146$). Type I atresia was commonly encountered in tertiary oocytes from March to June, and type III atresia was present from July to September and also between November and December.

Post-ovulatory follicles (POFs) made up a low percentage of the total oocyte count, starting in November (0.15 ± 0.12 %) and increasing to a maximum in March (2.29 ± 1.39%). There were no POFs from April to October (Fig. 3.5). Histological examination of the ovaries suggested that post-ovulatory and tertiary oocytes were present in various categories of gonad stages, even in fish that had been considered to be in gonad Stage II.

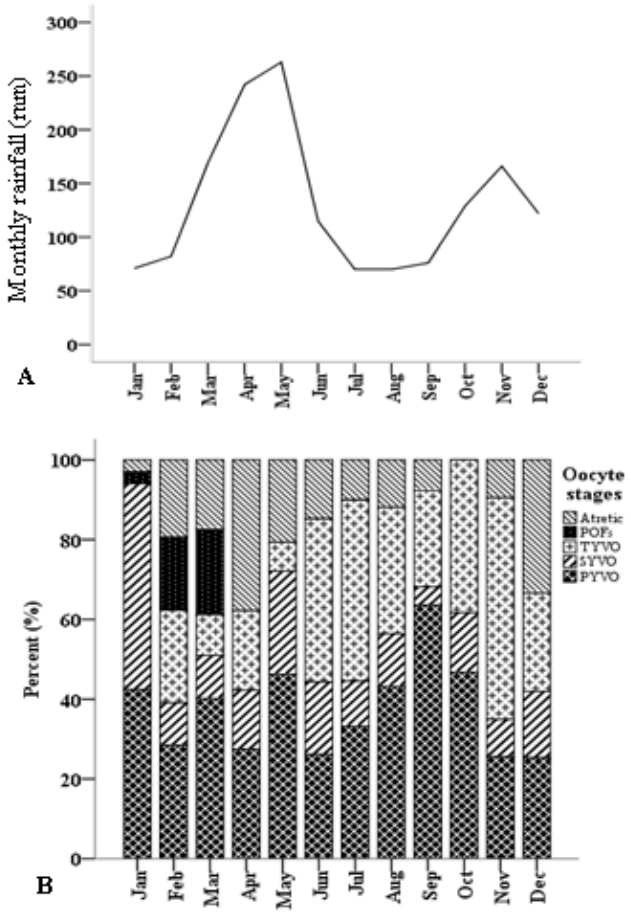


**Figure 3.5:** Top panel (A): Monthly rainfall variations and bottom panel (B): temporal variations in oocytes stages (PYVO = primary yolk vesicle oocytes, SYVO = secondary yolk vesicle oocytes, TYVO = tertiary yolk vesicle oocytes, POF = post-ovulatory follicles, n = 178) in *L. niloticus*

*Influence of rainfall, temperature, and wind speed on gonadosomatic index*

There was no significant correlation between female *L. niloticus* monthly mean GSI and prevailing environmental variables, monthly mean rainfall (Spearman's Rho, r = -0.32, p = 0.133, n = 22), mean temperature (r = -0.34, p = 0.115, n = 22), and mean wind velocity (r = -0.34, p = 0.116, n = 22). In males, monthly mean GSI was significantly correlated with ambient

monthly mean water temperature ((r = -0.71, p = 0.0001, n = 22) while there was no significant correlation between monthly mean GSI and monthly mean rainfall (-0.12, p = 0.214, n = 22) and mean wind speed (r = -0.27, p = 0.052, n = 22). Average monthly wind velocity and temperature conditions, recorded a month before sampling were, however, significantly correlated with the gonadosomatic index, Spearman's Rho, r = -0.51, p = 0.014, n = 22, and r = 0.61, p = 0.002, n = 22, respectively (Fig. 3.5).

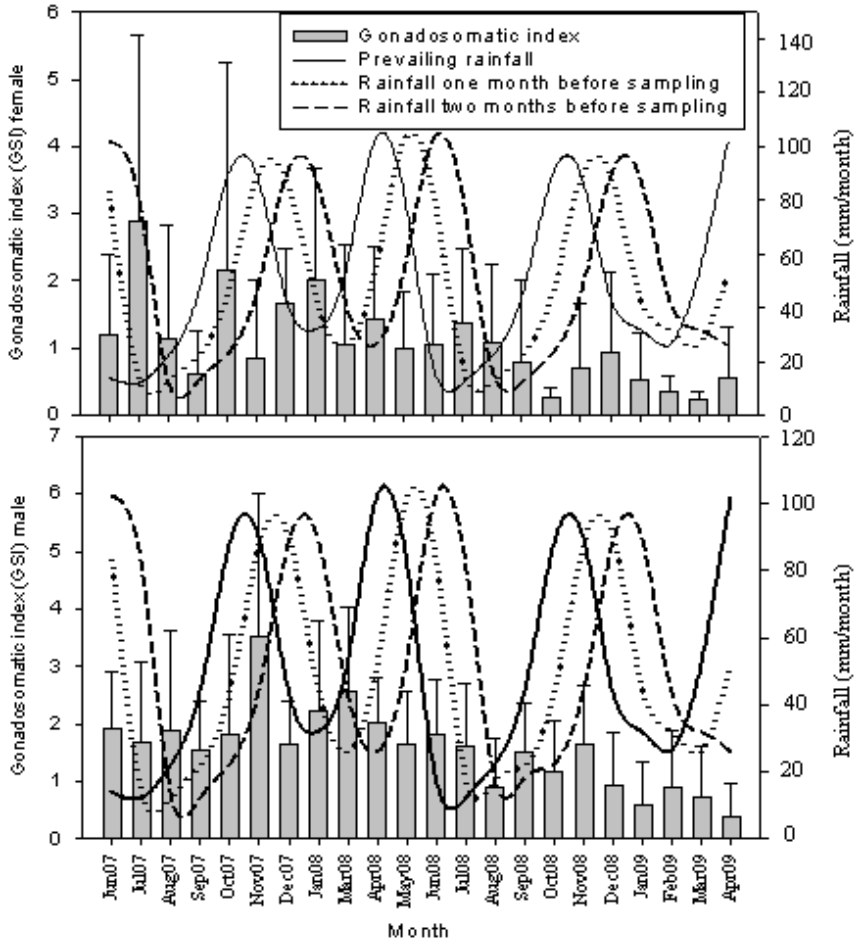


**Figure 3.6**: Monthly mean and standard error bars for gonadosomatic index and monthly mean rainfall at sampling time (prevailing), one and two months before sampling a) females and b) males of *L. niloticus*

*Influence of rainfall, temperature, and wind speed on the frequency of oocyte stages*

The percentage of primary yolk vesicle oocytes (PYVO) increased with increasing rainfall. There was a similar trend in secondary yolk vesicle (SYVO) and atretic oocytes (Fig. 3.7). The lowest percentage of tertiary yolk vesicle oocytes (TYVO) was when the rainfall was above 251 mm in the month of May. Most of post-ovulatory follicles (POFs) were only present when monthly average rainfall was between 151 and 250 mm and few were in the range 1to150 mm. There were none during the period of monthly rainfall averages of > 251mm (Fig. 3.7).

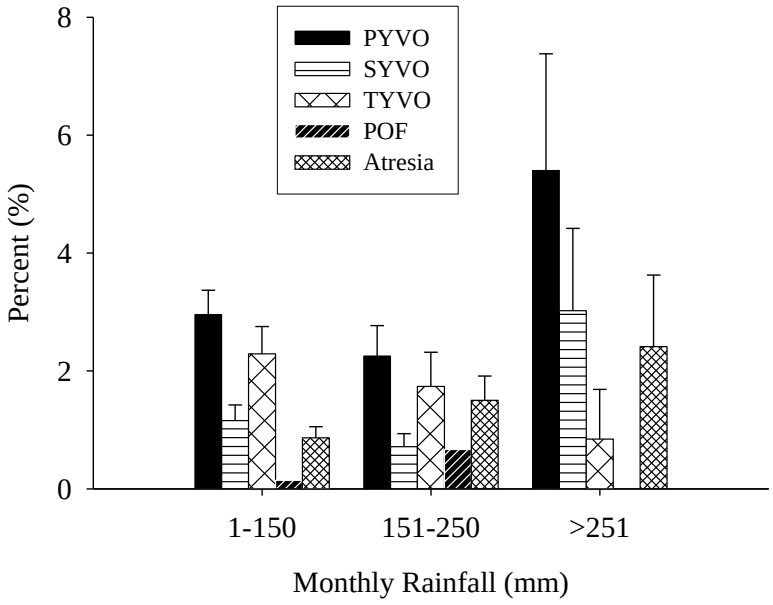


**Figure 3.7:** The frequency of five oocyte developmental stages in relation to rainfall in *L. niloticus*

Despite the observed trend, rainfall was not significantly correlated with the frequency of all oocyte types ($p > 0.05$), except for mean monthly atretic oocyte frequency, Spearman's Rho,

correlation coefficient, r = 0.65, p = 0.022, n = 12, also no significant correlation between monthly mean post-ovulatory follicle frequency and rainfall (p > 0.05).

Temperature was not significantly correlated with the mean percentages of all oocyte stages, including post-ovulatory follicles (PYVO, Spearman's Rho r = -0.090, p = 0.78, n = 11; SYVO, r = -0.366, p = 0.24, n = 11; TYVO, r = 0.651, p = 0.058, n = 11; POFs, r = 0.24, p = 0.449, n = 11; atretic oocytes, r = 0.27, p = 0.391, n = 11).

Wind speed had a significant relationship with average TYVO and POFs frequencies, Spearman Rho correlation co-efficient, r = 0.64, p = 0.024, n = 11 (Fig. 3.8c) and r = 0.57, p = 0.05, n = 11. Wind speed did not influence the frequency of PYVO, SYVO and atretic oocyte frequencies (p > 0.05).

*Probability of finding ripe female broodstock in a given month*

The mean gonadosomatic index of all females, observed and confirmed to be ripe, or ripe running, was calculated and was used as the minimum gonadosomatic value at which adult female Nile perch are considered ripe. The probability of finding ripe female broodfish with a gonadosomatic index greater than 1.25% was calculated using z-statistics. The probability (P) of finding ripe female fish was estimated for January (P = 0.73), April (P = 0.35), July (P = 0.99), and December (P = 0.08) (Table 3.3). Although April presented a probability of finding suitable broodstock for induction of spawning, the high frequency of atretic tertiary oocytes made it a less favourable month for the collecting broodstock.

**Table 3.3:** Probabilities of finding brood fish with GSI greater than 1.25% in a particular month using z-statistics

| **Month** | **Mean GSI ±STD** | **n** | ***Probability*** |
|---|---|---|---|
| Jan | 1.45 ± 1.41 | 39 | 0.730 |
| Feb | 0.37 ± 0.26 | 36 | 0.000 |
| Mar | 0.67 ± 0.72 | 51 | 0.000 |
| Apr | 1.26 ± 0.95 | 163 | 0.350 |
| May | 1.12 ± 0.95 | 89 | 0.000 |
| Jun | 1.10 ± 0.98 | 29 | 0.000 |
| Jul | 2.01 ± 2.13 | 46 | >0.99 |
| Aug | 1.01 ± 1.31 | 32 | 0.000 |
| Sep | 0.67 ± 0.87 | 59 | 0.000 |
| Oct | 0.94 ± 1.56 | 43 | 0.000 |
| Nov | 1.06 ± 1.25 | 29 | 0.000 |
| Dec | 1.24 ± 0.99 | 39 | 0.080 |

Based on the proportion of tertiary yolk vesicle oocytes in the gonad in histological sections of ripe and ripe running individuals, the probability of getting ripe or ripe running broodstock female fish with TYVO accounting for over 3.95% of the total oocytes in the ovary was observed during May (P = 0.29), July (P > 0.99), August, (P = 0.72) and November, (P = 0.10) (Table 3.4).

**Table 3.4:** Probabilities of finding brood fish with gonads having over 3.95% TYVO of the total sum of oocytes in a month

| Month | Mean ±SE | n | Probability |
|---|---|---|---|
| Jan | 1.70 ±3.80 | 65 | 0.00 |
| Feb | 0.50 ± 1.40 | 33 | 0.00 |
| Mar | 0.80 ± 1.50 | 35 | 0.00 |
| Apr | 1.50 ± 2.90 | 45 | 0.00 |
| May | 2.70 ± 4.00 | 66 | 0.00 |
| Jun | 2.40 ± 3.40 | 73 | 0.00 |
| Jul | 9.50 ± 12.20 | 35 | >0.99 |
| Aug | 5.10 ± 7.60 | 135 | 0.72 |
| Sep | 1.10 ± 1.90 | 92 | 0.00 |
| Oct | 1.80 ± 3.50 | 135 | 0.00 |
| Nov | 4.10 ± 8.30 | 67 | 0.10 |
| Dec | 3.70 ± 5.10 | 38 | 0.00 |

**Discussion**

The temporal pattern of mean monthly gonadosomatic, mesenteric fat and hepatosomatic index suggested that *L. niloticus* in Lake Victoria did not breed throughout the year. The highest gonadosomatic index, in July, coincided with the peaks of mesenteric fat and hepatosomatic indices. This suggests a hepatic and mesenteric tissue involvement in the gonad growth. Clearwater and Pankhurst (1994) reported the same trend in the red gurnard, *Chelidorichthys*

*kumu* before spawning. In yellowtail rockfish, *Sebastus fluvidus* a high proportion of accumulated lipid in the mesentery tissue was incorporated in the developing ovaries (MacFarlane et al., 1993). Lipids provide metabolic energy during gonad formation in female fish (Sargent, 1995; Rainuzzo et al., 1997) and are a major source of energy during spawning. The decline in the mesenteric fat index in April and November suggests that mesenteric fat deposits support reproduction in Nile perch.

The Nile perch spawning times (November to March) did not correspond with the GSI thus the results deviated from the paradigm. The observed mismatch can probably be attributed the sampling error in which it would appear the month October was dominated by young adults as shown by low mean total length compared to the month of May in both female and male.

Nile perch reproduction did not coincide with the two rainfall peaks, as was shown for many tropical equatorial fish species (Munro, 1990). Prevailing rainfall levels did not have a significant influence on reproductive variables, suggesting that rainfall did not function as a proximate cue for reproduction. Similar findings were observed in *L. calcarifer* were rainfall did not influence gonadal cycling in Tahiti (Guiguen et al., 1994).

In Lake Chad, where the temperature varies from $18^{o}$C to $31^{o}$C, spawning occurred mostly during the warm ($24^{o}$ C) parts of the year, with a peak between January and June (Hopson, 1972). In Lake Albert, spawning of *L. niloticus* occurred throughout the year (Hamblyn, 1962), and in Lake Mariout, *L. niloticus* spawned in the early months of the year, after the January minimum temperatures. The related species, Baramundi, *L. calcarifer,* exhibited one annual

spawning period from October to February, coinciding with warming and the onset of the wet season in Tahiti (Guiguen et al. 1994). Striped bass, *Roccus saxatilis*, spawned in early summer at temperatures above 15$^{o}$C and ovulation and spawning in *Plectroplies ambiguous* occurred when the temperature rose above 23.6$^{o}$C (Lake, 1967). Some tropical fishes breed throughout the year, often with a peak in activity during rainy seasons, as was documented for tilapia species (Philippart and Ruet, 1982; Trewavas, 1983). In *L. victorianus* of Lake Victoria, spawning peaks were observed in April to May and September to October, coinciding with the bi-annual rainfall pattern of the area (Rutaisire, 2003).

In this study, there was an increase in the relative frequency of POFs during periods of low rainfall (November to March), and no POFs from April to October, which coincided with the peaks of the rainy seasons. The presence of POFs and TYVO in most gonad stages in mature fish leads to the hypothesis that Nile perch is a protracted total spawner, spawning sequentially before exhausting all tertiary oocytes. Thus, the Nile perch has the ability for multiple spawning events within a spawning period. It is suggested that factors leading to the onset of rainfall, signal the end of the spawning period and the onset of the next reproductive cycle. However, as there were only two years of data available, a statistical analysis is limited to a maximum of four seasons, two dry and two rainy seasons, and further studies should focus on proximate cues, by quantifying variables such as food abundance for both broodstock and offspring.

The deviation of the Nile perch, *L. niloticus*, from a rainfall-dependent spawning strategy may be explained by the fact that this species occupies the highest trophic level in the lake. The reproductive phenology of a predator and its prey cannot be triggered by the same environmental

conditions (Forchhammer et al., 1998), to avoid a mismatch between a predator larval energy requirements and larval food (prey) abundance (Cushing, 1990; Durant et al. 2005, 2007), as any imbalance would result in low predator larval survival (Durant et al., 2005). It is suggested that *L. niloticus* in Lake Victoria started spawning in November and ended in March when most of the fish had spawned, as evidenced by the high frequency of post-ovulatory follicles in the ovaries and it is hypothesized that larvae prey abundance increases at the time when the offspring has the highest demand for food. This should be tested in future studies.

The beginning of the reproductive cycle was marked by the onset of vitellogenesis and this was reflected by an increased frequency of primary yolk vesicle oocytes. This was observed in April and May, when the rainfall levels were above 250 mm, suggesting rainfall might trigger the initiation of gamete recrudescence in Lake Victoria Nile perch. At this time, the frequency of atretic tertiary oocytes increased with increasing frequency of primary yolk vesicle and decreasing TYVO frequency. This suggests that atresia leads to the elimination of all tertiary yolk vesicle oocytes that might have remained from the previous spawning period.

Atresia is a frequently reported phenomenon in vertebrate ovaries (Saidapur, 1978), which can be induced by factors such as stress, fasting, changes in light intensity and day length, temperature, and confinement and the resultant changes in hormone levels. The high frequency of follicular atresia between March and June is indicative of the post-spawning period in *L. niloticus*, further suggesting that the species breeds during periods of low rains. Guraya (1994) reported that follicular atresia was common in all vertebrates, throughout the reproductive cycle and high frequencies of atretic oocytes were common after spawning, hence frequency changes can be used as an indicator of the post-spawning period.

Spawning was further inferred, based on the increased presence of post-ovulatory follicles from November to March. Lake Victoria begins to undergo gradual mixing from April and reaching a climax in July (Njiru et al., 2008) as the thermocline breaks down under the onset of south-east winds lowering the surface water temperature, thereby causing the lake to become isothermal with respect to depth (Talling, 1966). Complete mixing of a lake releases nutrients from the sediment which results in increased zooplankton abundance (Talling, 1966). This is vital for Nile perch larval survival. Isumbisho et al. (2006) reported an increase in plankton abundance, followed by deep mixing in the epilimnion in Lake Kivu. Sundby (1997), and Kiørboe and MacKenzie (1995) postulated that small-scale turbulent mixing by wind increased larval feeding success, due to increased larval contact with the planktonic prey. Food availability was not quantified in this study. There is thus a need for studies on food (zooplankton) abundance and availability and to examine its influence on the timing of reproduction in Nile perch.

The reproductive strategy of a species is the summation of a collection of traits that enable an individual to produce the maximum number of offspring (Stearns, 1992). These traits comprise of: size at which an individual first reproduces; size- and age-specific fecundity; reproductive effort such as egg weight, clutch size, weight of eggs, mean egg weight and the timing of spawning (Stearns, 1992).

Female size at 50% maturity was similar to that of males. This finding is similar to that reported by LVFO (2007) from a study of *L. niloticus* factory samples collected in 2006. Some authors suggested that females matured at a larger size than males (Hopson, 1972; Ogutu-Ohwayo, 1988; Hughes, 1992; Ogutu-Ohwayo, 2004). In the Gulf of Nyanza Lake Victoria, male *L. niloticus* matured at a size range of 50-55 cm at approximately two years and females at 80-85 cm total length, probably at four years old (Hughes, 1992). In studies before 2000, *L. niloticus* males

matured at a lower size than females (Okedi, 1970; Hopson, 1972, Ogutu-Ohwayo, 1988, Hughes, 1992, Ogutu-Ohwayo, 2004) (Fig. 3.10). In this thesis it is suggested that the difference between male and female size at maturity has become smaller and the size at which 50% of individuals sexually mature has declined since 1990, from a total length 94.5 ± 6.4 cm in female *L. niloticus*, to the smaller values of 59.4 cm, estimated here (Fig. 3.10). Similar results were obtained in the 1960s time when the fishing pressure was equally intensive (Fig. 3.10). By inference this trend shows that the Nile perch population can adjust its reproductive physiology to the pressures applied by capture fisheries.

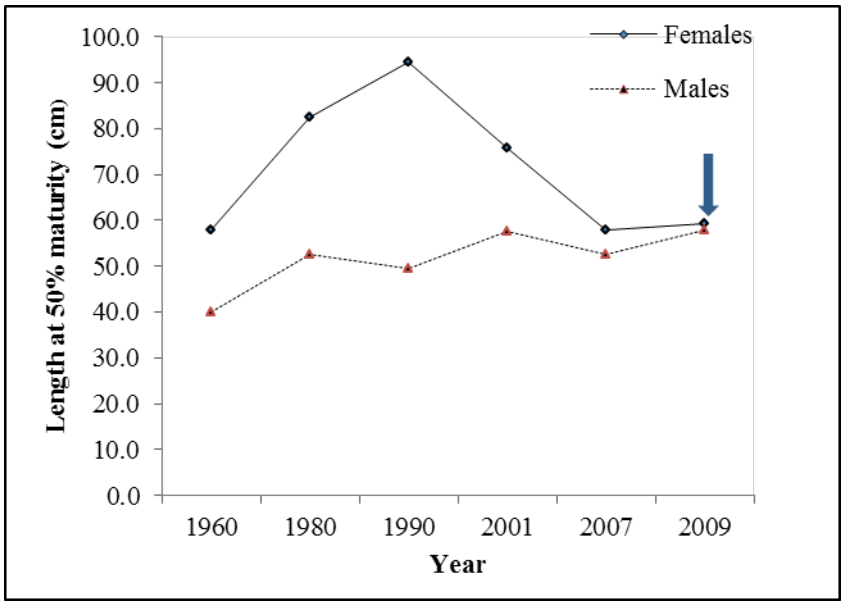


**Figure 3.8:** Temporal trends in Nile perch maturation: body total lengths at which the probability of 50% individuals mature in a size class, for female and male *L .niloticus* from Lake Victoria, from research done from 1964to 2009; Data Sources: (Okedi, 1970; Ogutu-Ohwayo, 1988; Hughes, 1992; Ogutu-Ohwayo, 2004; LVFO, 2007) and arrow indicates present study

The recommended minimum gear size for the Lake Victoria Nile perch fishery is 7.5 cm mesh size and the general fishery is 5 cm (MAAIF, 2004). This size potentially removes genotypes that mature at a larger size, hence exerting high fishing mortality on large female Nile perch. By inference this also explains why length at 50% for females of Nile perch has declined over years. I therefore hypothesise that the reduction in body length at which 50% of females mature is attributable to the gear size selection pressure which targets larger individuals of female Nile perch.

The findings in this study are similar to those found in heavily exploited fish populations in other environments (Jørgensen, 1990; Olsen et al., 2004). A trend towards smaller size at maturity was reported in heavily exploited fish stocks such as the Northeast Arctic cod, *Gadus morhua* and this was associated with the loss of large females as a result of fishing mortality (Jørgensen, 1990). Jørgensen (1990) suggested that the reduction in median age and size at maturity is primarily more of a compensatory response to the reduced stock size, as a result of higher fishing pressure on the late-maturing individuals, than on the early-maturing individuals. Such trends in maturation are indicative of rapid selection that precedes the collapse of a fishery (Olsen et al., 2004). Such changes may also account for the observed reduction in size at maturity in the Lake Victoria Nile perch population.

In conclusion, this chapter has shown that there was reproductive seasonality in Nile perch, with a long spawning season from November to March. Spawning is more frequent in drier months. It is hypothesised that the reproductive, i.e. ovarian, recrudescence was triggered by high rainfall averages. Female *L. niloticus* appears to be maturing at a smaller size than it matured before 1990.

# CHAPTER FOUR

## Gonad and gamete morphology and ultrastructure of the Nile perch, *Lates niloticus*: Scanning, transmission electron and light microscopy

### Introduction

In aquaculture and fisheries management, information about patterns of gonadal recrudescence, spawning seasonality, size at sexual maturity, fecundity, gonad and gamete type and structure of a target species play an important role in policy formulation. Histological studies of gonads provide information based on gamete developmental stages and their seasonal variability (West, 1990). Histology validates macroscopic studies (Booth and Weyl, 2000) and allows for comparisons to be made between reproductive stages at cellular level and at different times of the year. In *Lates niloticus* some reproductive studies were carried out, using macroscopic staging of the gonads, oocyte stages and size, size at maturity and gonadal indices (Hopson, 1972; Ogutu-Ohwayo, 1988, 2004). Basiita et al. (2011) described gonadal sex differentiation in the Nile perch, but no studies have provided information on the gonad and gamete ultrastructure of *L. niloticus.*

*Testis structure – types of testis*

According to Grier et al. (1980), two tubular testicular types exist in teleosts and they can be distinguished from each other by the spatial distribution of spermatogonia in the testis tubules or lobules. Tubules can be straight and basically un-branching, branching or anatomising, but the spermatogonia are indiscriminately distributed along the whole length of the tubule in *Salmoniformes, Cypriniformes* and *Perciformes*. Alternatively, they can be totally restricted to the distal end of the tubules as has been reported in *Atheriniformes*. Grier et al. (1980)

accordingly distinguished between unrestricted spermatogonia and restricted spermatogonia testis types respectively.

*Sperm structure – types of sperm*

Studies on the morphology of the teleost testis and spermatozoa have been conducted in many species (Afzelius, 1978; Grier et al., 1980; Jamieson and Leung, 1991; Jamieson, 1991; Mattei, 1991; Coward et al., 2002; Lo Nostro et al., 2003; Guo and Zheng, 2004; Gwo et al., 2005; Nóbrega and Quagio-Grassiotto, 2007; Psenicka et al., 2007; Psenicka et al., 2009). Various types of fish sperm have been described, based on their mode of fertilisation and flagella rotation axis in relation to the nucleus (Jamieson, 1991). There are two major categories of fish sperm; aquasperm and introsperm. Aquasperm are discharged into the water column where fertilisation takes place externally. Introsperm are not released in the water and fertilisation is internal. Aquasperm can further be divided into (a) acrosomal aquasperm, found in the lower fishes ranging from *Agnatha* to *Dipnoi* (Jamieson and Leung, 1991), and (b) anacrosomal aquasperm.

There are two types of anacrosomal aquasperm, type I and type II, which are unique to teleosts. Structural studies of spermatozoa have contributed to the taxonomy of fishes (Jamieson, 1991). The type of spermatozoa found in *Centropomidae* is, however, contentious (Gusmão-Pompiani et al., 2005). The description of *Lates calcarifer* spermatozoa (Jamieson, 1991) placed the species between Type I and Type II sperm. Gusmão-Pompiani et al. (2005), based on Jamieson's findings, described the spermatozoa of *L. calcarifer* as that of type II. They have further used the same analysis of the *L. calcarifer* sperm structure to classify *L. niloticus* as producing type II spermatozoa. This suggestion has been based on speculation rather than studying the sperm

structure of *L. niloticus*. There is therefore a paucity of information on *L. niloticus* sperm ultrastructure and spermatogenesis. Some aspects of testis histomorphology of *L. niloticus* were carried out by Basiita et al. (2011), but no studies on the testis and spermatozoa ultrastructure and gametogenesis have been published. As a result, gonadal recrudescence is poorly understood in this species. This information is vital in evaluating milt quality (Billard, 1978) for hatchery management and for the classification of a species (Mattei, 1991).

*Ovarian tissue, germ cell and oocyte structure*

Understanding the process of gamete formation and oocyte structure is essential to the successful commercial culture of fish, as much as it is to fisheries management. Such information can also be used in the morphological characterization of *L. niloticus* egg preservation and fertilization as well as egg quality assessment (Kjørsvik et al., 1990). Many authors have described female gametogenesis in teleost fish as going through oogenesis and oocyte primary and secondary growth, leading to final oocyte maturation. Few authors have however described ultra-structure of the germline stem cells and such studies are limited to a small number of species, mainly those of commercial importance (West, 1990; Guimarães and Quagio-Grassiotto, 2001; Nakamura et al., 2011). There are no detailed studies of species from the family Centropomidae, and considering the increasing aquaculture and commercial fisheries potential of the Nile perch, there is a need for information on male and female germ cells, spermatogonia, oogonia structures, and an understanding of primary and secondary growth of oocytes and spermatocytes in this species.

This chapter provides a description of gametogenesis in *L. niloticus* and serves as the first in depth description of testes and sperm ultrastructure as well as ovarian histo-morphology. It

attempts to highlight germ-line stem cells, oocytes, and gonad structure in Nile perch by using a combination of scanning and transmission electron microscopy and light microscopy.

## Materials and methods

Female *L. niloticus* (n = 246) were collected from Lake Victoria, Entebbe, Uganda. The fish were captured, using commercial gill nets and long-lines, for twelve months from May 2008 to April 2009. Fish total weight (g), standard length and total length (cm) were measured to a precision of 0.1 of a unit measure and recorded.

*Light microscopy*

A total of 178 ovaries were weighed to the nearest 0.1 g (GW) and a 2.0 g sample from each fish was preserved for histology as described in Chapter three. The occurrence of post-ovulatory follicles (POF) was used as an indicator that fish had spawned and the frequency of primary yolk vesicle oocyte and atretic oocytes were used to specify the start and end of the reproductive cycle in *L. niloticus* (Brown-Peterson et al., 2011). The criteria for identification of atresia stages and the atretic condition of the ovary were adopted from Hunter and Macewicz (1985).

*Scanning and transmission electron microscopy*

Seven males and six females of *L. niloticus* from Lake Victoria, Uganda were collected from the vicinity of Entebbe, Uganda (see Chapter two), transported to Makerere University, Faculty of Veterinary Medicine. They were euthanized by a high dose of clove oil. Morphometric measurements were taken and fish were dissected and the gonads removed, staged and weighed. Small sections (2mm blocks) of the gonads were fixed overnight at $4^{o}$C in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.2). After washing in 0.1 M phosphate buffer, tissues were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer for 90 minutes at room

temperature, serially dehydrated in ethanol and embedded in Epon 812 resin. Ultra-thin sections (1.20 μm) were cut, using a diamond knife, using Ultratome RMC MT-7 microtome and collected on 300-mesh copper grids, before staining with uranyl acetate and lead citrate. Tissue and cell types were examined using a JEOL 1210 transmission electron microscope at 120 kV and digital images were archived.

For scanning electron microscopy (SEM), 1 ml of sperm was collected by applying a gentle pressure on the male fish abdomen, and it was preserved overnight at $4^{o}$C in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2). The sample was diluted using 0.1 M phosphate buffer (pH 7.2) air-dried overnight and sputter-coated with gold. The sperm was examined using a JEOL 840A scanning electron microscope at 30.00 kV and the digital images were archived. Photographs were scanned and the dimensions of various cell types and their inclusions, i.e. the centriolar system, mitochondria and nuclei were measured using the AnalySIS Soft Imaging System software (www.softimaging.net).

## Results

*Testes structural arrangement*

In *L. niloticus*, the testes were situated dorsally to the posterior gut and ventral-laterally to the swim bladder. As sperm maturation and recrudescence proceeded, the two elongated organs became thick and swollen, changing colour and shape, from a triangular form with sharp edges, to round edges in cross section. The main sperm duct was located within the testes, from which a system of branching lobules extended to the testes periphery (Fig. 4.1). The main duct was present in this particular arrangement, extending from the anterior end of the testis to the

posterior, where a short ejaculatory duct was located close to the cloaca, not shown in the figure. Lobules close to *tunica albuginea* had a very small lumen.

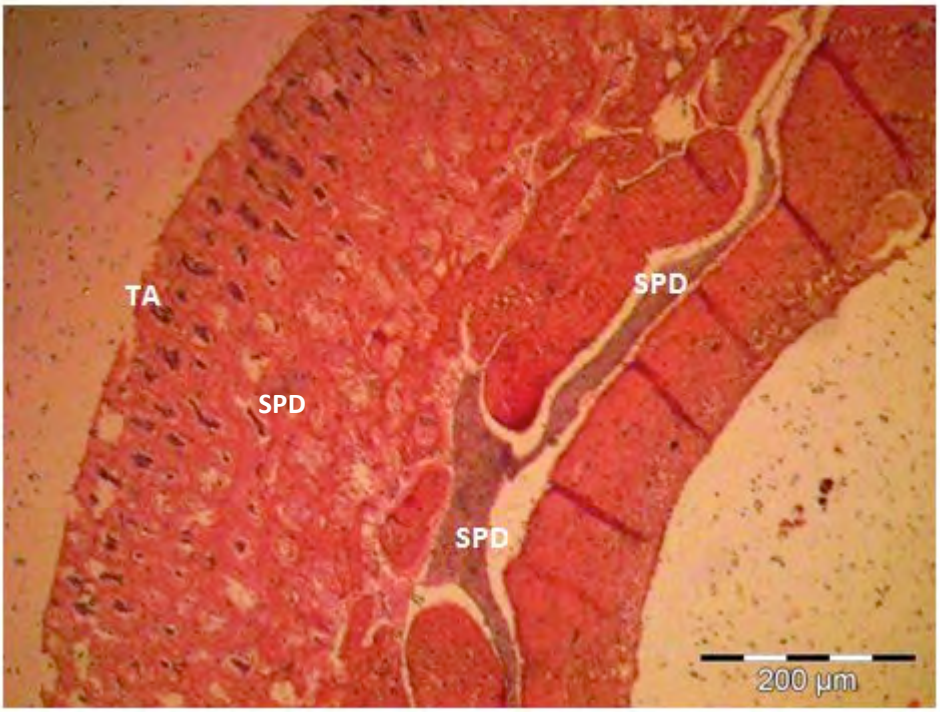


**Figure 4.1:** Testicular arrangement in Nile perch, *L. niloticus*, testis longitudinal section showing sperm duct. SPD = sperm duct, TA = *Tunica albuginea*

As spermatocysts matured and voided spermatozoa into the lobular lumen (Fig. 4.2), a network of branching lobules was also forming. Valve-like structures were observed at a magnification x 400 (scale of 500 µm) (Fig. 4.3).

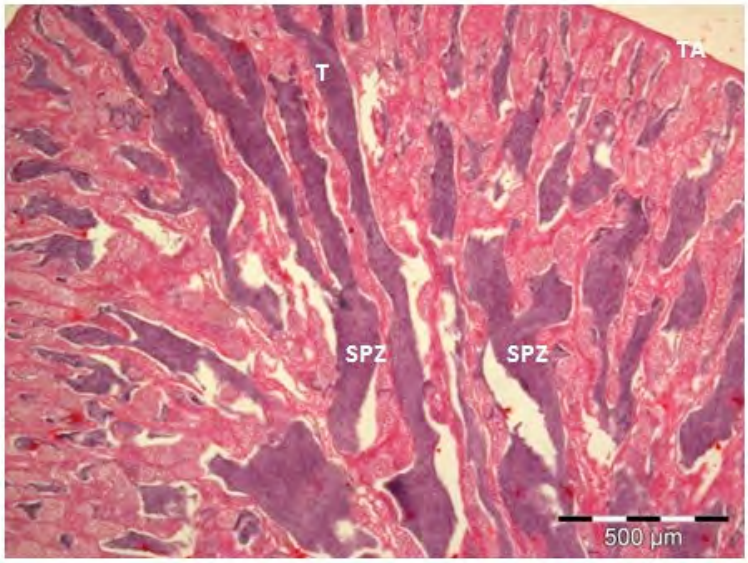


**Figure 4.2:** Testicular arrangement in Nile perch, *L. niloticus*, transverse section showing branching network of tubules, T = tubule, TA = *tunica albuginea*, SPZ = spermatozoa

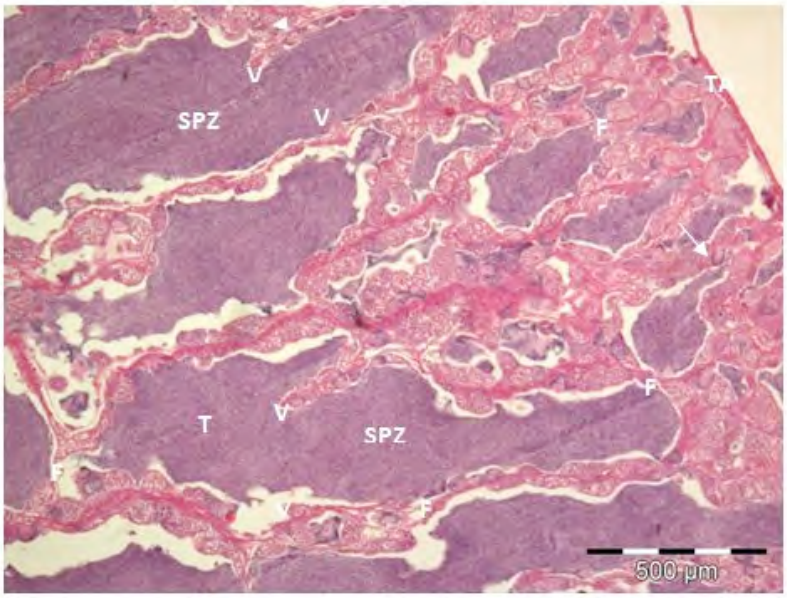


**Figure 4.3:** Section of Nile perch testes lobules; SPZ = spermatozoa, L = lobule lumen, F = fault; point of weakness, TA = *tunica albuginea*, V = valves, black arrow heads = maturing spermatocysts

*Ultrastructure of the testes*

The testis in *L. niloticus* was made up of compartments which included a) the interstitium, and b) the germinal epithelium, with germ and Sertoli cells within the spermatocyst. It was composed of a number of seminiferous lobules that were lined by Sertoli cells and germinal spermatocysts that were filled with germ cells. In addition, the lobular walls were lined with spermatocysts made of Sertoli cells within which male gametes were at different stages of development. Each individual spermatocyst contained germ cells at the same stage of development (Fig. 4.4a). In addition, the seminiferous lobules were separated by a line of collagen fibres, with the blood capillaries forming the base for the lobular structure. In some cases, the spermatocyst walls directly juxtaposed with the blood vessel and were separated by a basal membrane (Fig. 4.4a). The myoid/fibroblast cells did not form a continuous layer around the lobules and, as a result in some cases, other interstitial cells (Leydig cells, macrophages and blood capillary wall cells) bordered the basal membrane (Fig. 4.4a).

Spermatogenesis occurred within the spermatocysts, which contained isogenic germ cells, and the borders of the spermatocyst were formed by multiple Sertoli cells. At an advanced stage, the spermatids arranged themselves with the head facing to the lobule wall and the flagella facing to the lobule lumen (Fig. 4.4b). At the peak of spermatogenesis all lobules were devoid of cysts and adjacent lobules were joined, forming an apparently continuous passage of the sperm cells to the main sperm duct.

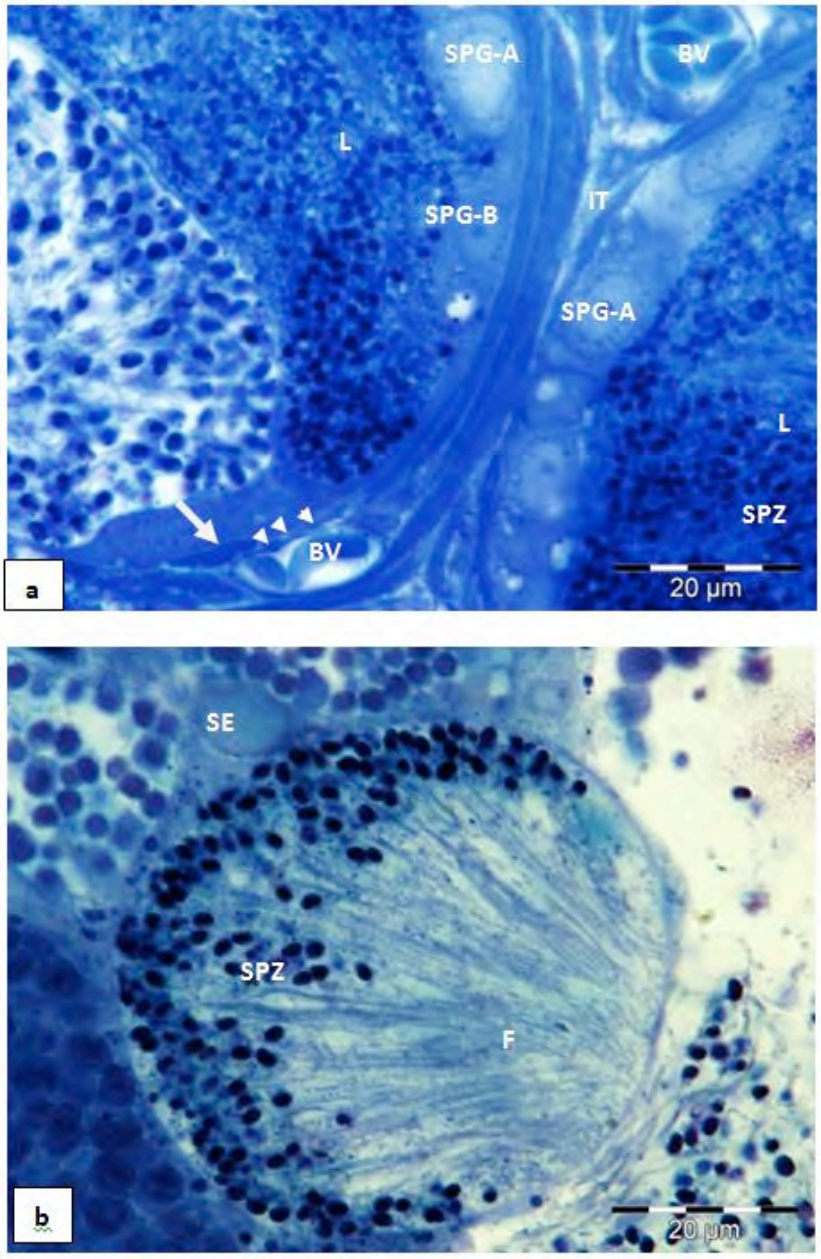


**Figure 4.4:** Light microscope micrographs of testes tissue a) cross section of two adjacent lobules separated by the interstitium, b) mature spermatocyst before voiding its content: SPZ = spermatozoon, SPG-A = primary spermatogonia, SPG-B = secondary spermatogonia, F = flagella, IT = interstitium, SE Sertoli cell, BV = blood vessels, and L = lobular lumen. Spermatocyst adjoining blood vessel = arrow heads, Leydig cell = white thick arrow

*Testis somatic tissue ultrastructure: Interstitium*

The lobules were ensheathed in a layer of cells of the interstitium. This tissue was compact, with limited extra-vascular space (Fig. 4.5a). It was composed of the cell types mentioned above. The boundary cells (myoid cells or fibroblasts, mean and standard error, 1.28 ± 0.26 μm, n = 20, wide at the nuclear region), were elongated, mean and standard error, 12.22 ± 1.93 μm (n = 12) long, with a tapering end, arranged alongside, overlapping one another and forming a wall made of one to four cell layers, depending on the stage of gonad maturation. In the gonads at stage three, and above, the testes had a thin interlobular wall and few interstitial cells. Their cytoplasm was made up of numerous bundles of microfilaments/microfibrils that were usually parallel to the long axis of the cell, occasionally attaching to the plasmalemma of the cell. Mitochondria and glycogen granules were abundant and appeared black in the entire cytoplasm where they surrounded the mitochondria at the cell periphery (Fig. 4.5b, c). The myoid/fibroblast cells in some fish had pinocytotic vesicles that were fringing the cytoplasm. These cells usually maintained close contact with the collagen fibres that were abundant in the intercellular spaces (Fig. 4.5b). The collagen fibres were arranged perpendicular to one another.

The other cell type is the endothelial cell that aligns the blood capillaries. Nucleated red blood cells, monocytes and granulocytes were also evident, though scarce, and were located near blood vessels. One or more microphages were observed, often isolated, but close to the blood vessels (Fig. 4.5d). Macrophages had an irregular nucleus and condensed chromatin. The cytoplasm was electron-dense, with mitochondria and Golgi apparatus highly developed.

Leydig cells were oval, with a large, elongated electron-dense nucleus (3.75 ± 0.20 μm long and 1.06 ± 0.10 μm wide, n = 6), coupled with numerous mitochondria that appeared elongated with tubular cristae, Golgi apparatus and glycogen granules in their cytoplasm (Figures 4.5f and 4.6). In two of the fish, a clear distinction between the myoid/fibroblast cells and the Leydig cells could not be made. A basal membrane separated the somatic and germinal epithelia. The germ cells were, however, secluded from the interstitial epithelium by purely the basal membrane and an extensive Sertoli cell cytoplasmic network. The Leydig cells were located around the blood vessels and sometimes in isolation in the extra-vascular space of the interstitium. Leydig cells had Golgi apparatuses, mitochondria and vesicles that were observed pinching off the Golgi apparatus and a large nucleus occupying about 50% of the cell volume. Evidence of vesicle transportation to the cell periphery and fusion with the cell membrane was observed (Fig. 4.6).

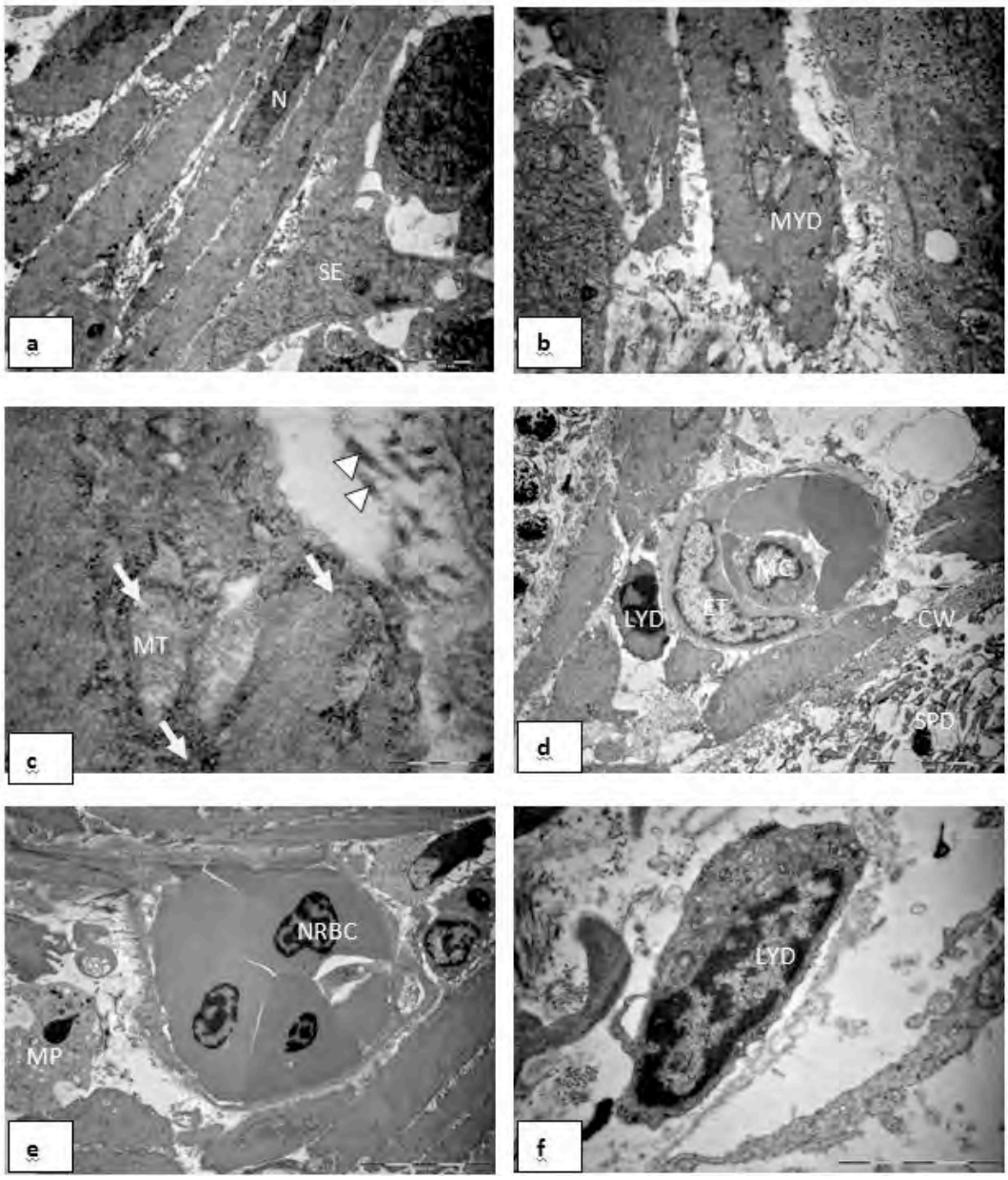


**Figure 4.5:** Electron microscopy images of interstitial tissue cells; a) interstitial wall arrangement, b) myoid/fibroblast cell, c) myoid cell, d) blood vessel showing monocytes, endothelial cell, Leydig cell, cyst wall, and spermatids, e) blood vessel with nucleated red blood cells, and macrophage close, f) Leydig cell, LYD = Leydig cells, NRBC = Nucleated red blood cells, MP = macrophage, ET = endothelial cell, MC = monocytes, CW = cyst wall, SPD = spermatid, MT = mitochondria, MYD = myoid cell, SE = Sertoli cell, N = nucleus, thick arrow = glycogen granules, arrow head = collagen fibres

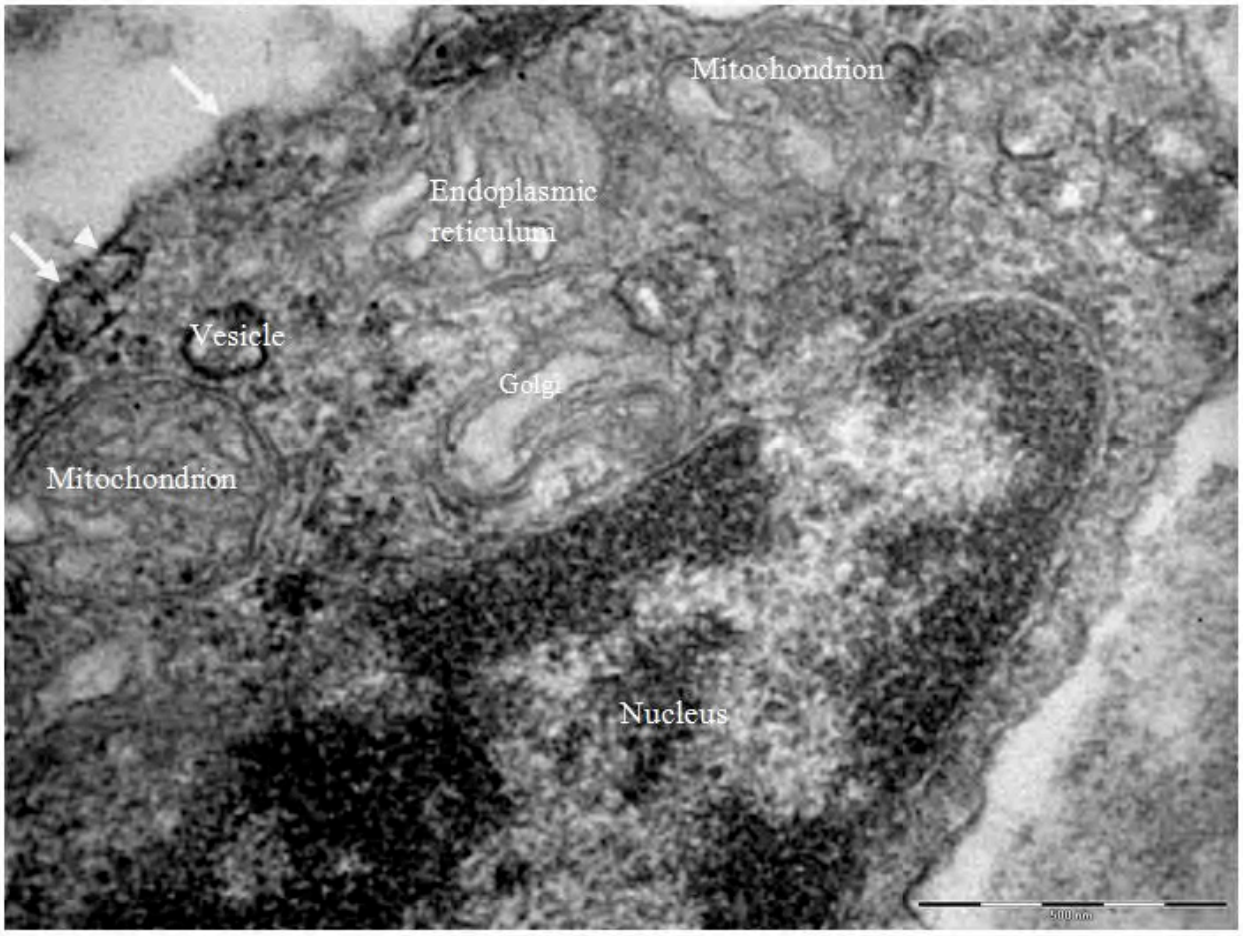


**Figure 4.6:** Leydig cell with various cytoplasmic inclusions, white arrows = undischarged vesicles and vesicle discharging its content (white arrow head)

*Germ cell tissue fine/ultrastructure*

The ultrastructure revealed primordial germ cells and Sertoli cells inside the lobule. One or more Sertoli cells made up the entire wall of the cyst. The Sertoli cells had a large nucleus, usually triangular in form, and their cytoplasm spread around a spermatogonium or several spermatogonia. They had low electron density and were devoid of nuclear nuages that were common in the germ cells (Fig. 4.7a). Approximately 26 mitochondria were surrounding the nuclear membrane (Fig. 4.7c). Mitochondria also surrounded individual nuage structures (Fig. 4.7c).

*Spermatocytogenesis*

The germ cells included spermatogonia, spermatocytes, spermatids and spermatozoa. Primary spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids were found in the spermatocysts, but only spermatozoa occurred in the lumen of the lobule. The primary spermatogonia were the largest cells in the testes and they were always associated with the Sertoli cell, whose cytoplasm surrounded one or a cluster of them. The diameter of the primary spermatogonia was 11.13 ± 1.73 μm (n = 7) and the nuclear diameter was 4.60 ± 1.47 μm. The mitochondria occurred in the cytoplasm near the nuclear membrane. Their number ranged from 28 to 32 (n = 4). The spermatogonia had a high nucleo-cytoplasmic ratio, with one large nucleolus and numerous nuages around the nuclear membrane and heterochromatin granules (Figures 4.7 a, b). Pores in the nuclear membrane (pore size of 0.08 ± 0.01 μm, n = 5) were placed at the point where the nuages associated with the nuclear membrane (Figures 4.7 c, d). Groups of globule-like structures occurred frequently in the primary spermatogonia.

Secondary spermatogonia were spherical to oval with a nucleus devoid of a nucleolus in some cases and, if present, it was at the periphery of the nuclear membrane. They were smaller than primary spermatogonia, with a cell diameter of approximately 9.84 ± 1.07 μm and a nucleus of 5.62 ± 0.28 μm diameter (n = 6). The nucleus was more electron-dense than that of primary spermatogonia (Fig. 4.7c). Oil globules were absent.

*Spermatogenesis*

Spermatogenesis began with the first meiotic cell division, leading to the formation of spermatocysts. Associated with this was a gradual growth of the spermatocyst size. The two stages of cell development included primary and secondary spermatocytes. Primary spermatocytes (nucleus diameter 3.33 ± 0.29 μm, n = 8) were spherical cells, characterized by a regular light nucleus, sometimes with a single nucleolus and a rim of cytoplasm. They were present along the entire lobular length, but maintained a large population close to the *tunica albuginea*. The primary spermatocytes were marked by a gradual condensation of chromatin material (leptotene) (Fig. 4.7d). During the pachytene stage chromosomes were formed, as evidenced by the parallel electron-dense chromatin strips. The final stage of chromosomal maturation, the diplotene, was characterized by bivalent, short, and thicker chromosomes that tended to coil (Fig. 4.7e).

Secondary spermatocytes were spherical with a high nucleus to cell ratio. Their nuclei (diameter 3.82 ± 0.28 μm, n = 9) were more electron-dense than those of the primary spermatocytes. The nucleus diameter in this cell type was larger than that of primary spermatocytes (Fig. 4.7f).

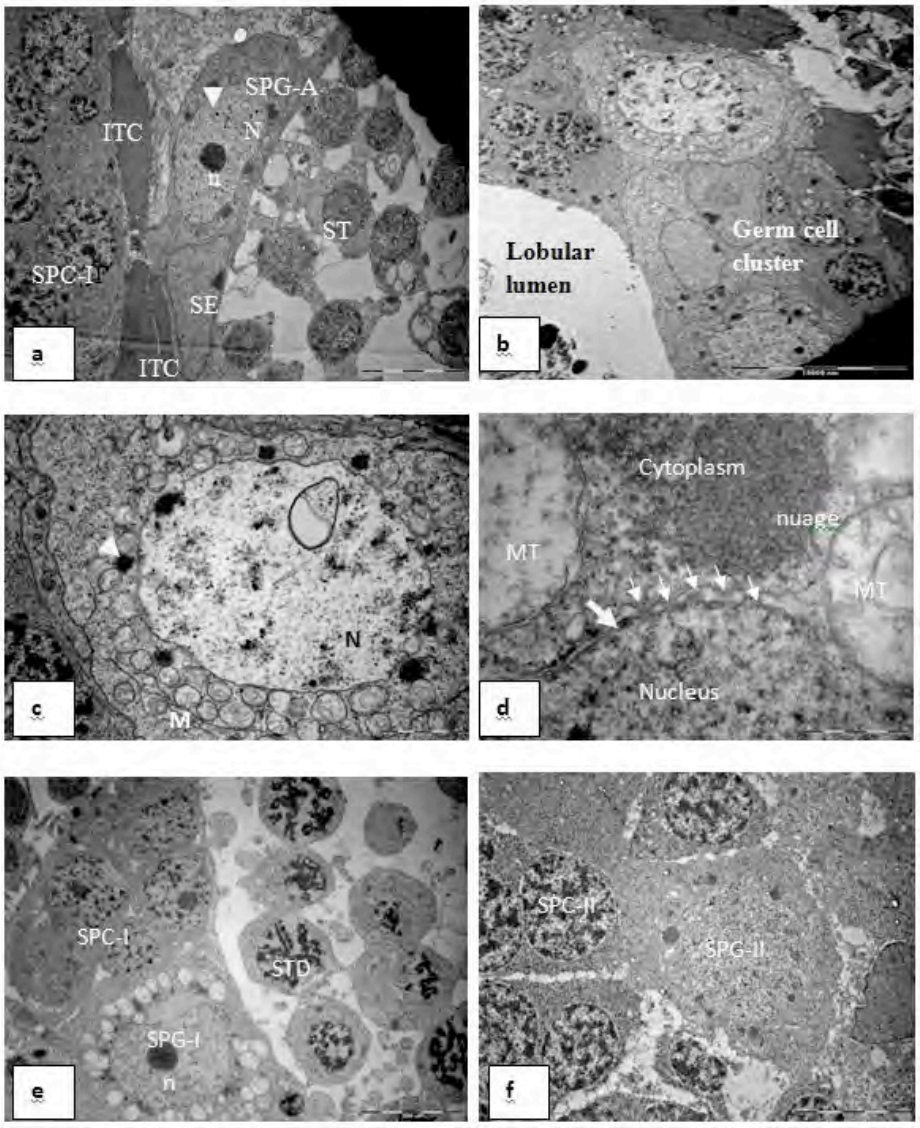


**Figure 4.7**: Electron microscopy images of spermatogenesis in *L. niloticus*, a) one celled interstitial wall separating two lobules, b) primary spermatogonia), c) secondary spermatogonia - condensing chromatin material and nuages (arrow heads), d) association of mitochondria, nucleus, and nuages, and small arrows = nuclear membrane pores, e) cyst with $1^o$ spermatocytes, spermatids, f) SPCII = $2^o$ spermatocytes. SPG-A = $1^o$ spermatogonia, SPG-B = $2^o$ spermatogonia, SPC-A = $1^o$ spermatocytes, SPC- B = $2^o$ spermatocytes, ST = spermatid, ITC = interstitial cell, SE = Sertoli cell, N = nucleus, n = nucleolus, MT = mitochondria, arrow head = nuages, thick arrow = nuclear wall, L = lobular lumen

These kinds of cells were the most abundant in the late-maturing testis and were common in spermatocysts whose cells were in their transition to the spermatid stage. By meiotic division of the secondary spermatocytes, spermatids were produced, marking the beginning of spermiogenesis.

*Spermiogenesis*

During spermiogenesis, the spermatids underwent final cell modification in preparation for the free independent life form. This entailed development of accessory structures like flagellae and chromatin condensation and granulation. This process began after haploid spermatids had formed. Parallel to this, the enclosed sex cells decreased in size and increased in number as final maturation of spermatozoa within the spermatocysts progressed. The spermatids went through five stages of development, spermatid I to spermatid V. The nucleus diameter averaged 2.79 ± 0.12 μm (n = 15) and it became more electron-dense and granulated as spermiogenesis progressed (Fig. 4.8a). At the posterior end of the germ cell, the cytoplasm was wide and irregular and contained small mitochondria forming the spermatid II-stage (Fig. 4.8b). The nucleus continued to decrease in size, revealing more condensed chromatin and granulation. Most of the cell cytoplasm, together with the mitochondria, was now located in the posterior part of the cell. The mid-piece started to form the stage known as spermatid III (Fig. 4.8c). At this stage, the mid-piece contained a flagella base and the mitochondria. Cytoplasmic bridges, formed earlier, continued to connect two or more spermatids. In addition, a centriolar system and flagella were discernible while the cytoplasmic canal was not yet formed. In spermatids at stage IV, the cytoplasm began to form a channel that resulted in a fully formed mid-piece formation. The other two cellular changes were chromatin material condensation and nuclear granulation commencement (Fig. 4.8d).

In the spermatid V-stage, the nuclear granulation continued and spread throughout the entire nucleoplasm. At this stage, residual bodies began to form. These structures housed excess cytoplasm and other cell organelles that would be discharged prior to spermiation (Fig. 4.8e). By this stage, the nuclei with a diameter of 2.20 ± 0.10 μm (n = 8), were strongly condensed and formed granules. The plasmalemma was closely attached to the nucleus. The cytoplasm now narrowed around the flagella and the surrounding collar continued to form and to accommodate the mitochondria and the residual body (Fig. 4.8e). The process of spermatogenesis ended when the spermatids lost their residual bodies, followed by a loss of the cytoplasmic bridges that had connected the spermatids (Fig. 4.8f).

At this stage, all structures that afford the sperm cell a free-living life, such as the flagella and its extensions, the mid-piece housing the mitochondria and the sperm head, were fully formed with no more intercellular connections. This was immediately followed by the rupture of the spermatocysts that resulted in voiding their contents, the spermatozoa, into the lobular lumen.

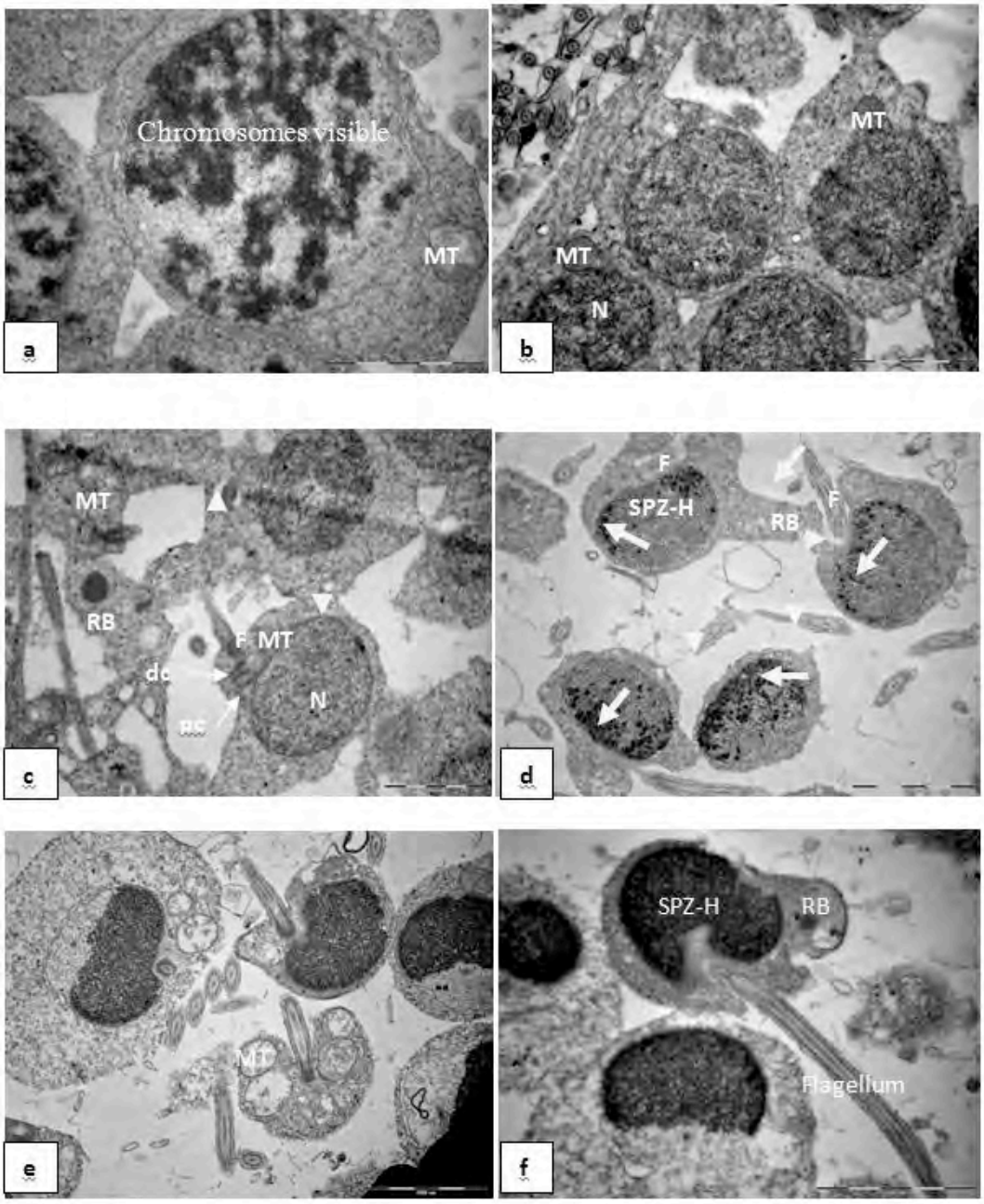


**Figure 4.8:** Changes in the nuclear electron density as chromatin condensation progresses during type II spermiogenesis in *L. niloticus.* a) spermatid I, b) spermatid II, c) spermatid III, d) spermatid IV, e) spermatid V, and f) spermatid before spermiation; MT = mitochondria, N = nucleus, pc = proximal centriole, dc = distal centriole, F = flagella, SPZ-H = spermatozoon head, and SPZ = spermatozoon, RB = residual body, and white arrows = changing chromatin electron density, white arrow heads = cytoplasmic bridges

*Testis regression, sperm cell necrosis*

Three of the seven fish, captured in May, had regressed testes with spermatozoa that were undergoing cell (chromatin) necrosis (Fig. 4.9a to c). The necrotic sperm cells were characterized by nuclear changes such as chromatin fragmentation, dissolution and swelling of the nucleus. The chromatin content in the sperm head had become necrotized, which resulted in cells becoming less electron-dense and hypertrophied (Fig. 4.9d). Necrotic sperm cells ruptured and released their contents into the lumen (Fig. 4.9e).

The Sertoli cells became phagocytotic as they engulfed the necrotic sperm cells. These modified Sertoli cells contained germ cells, mitochondria and other organelles, apparently from the engulfed sperm cells, all packed into the cytoplasm of the cell. Electron-dense spherical structures, which may have been lysosomes and glycogen-like granules, were numerous in the Sertoli cell cytoplasm (Fig. 4.10b). Once the Sertoli cell had turned into a phagocytic cell, all cells that must have been in contact with it, were engulfed, including spermatogonia. Furthermore, lumps of necrotic spermatozoa and other organelles occurred in the lobule lumen of two of the Nile perch testes (Fig. 4.10a).

In some fish, the entire lobule contents had been disintegrated and free lysosomes appeared in the lumen (Fig. 4.11a-b). In the interstitial wall tissue, structural changes occurred, with almost all cells having lost integrity and with granulocytes becoming visible. Only a few structures in the interstitium, such as fibroblasts, blood vessels and granulocytes were left intact (Fig. 4.11c).

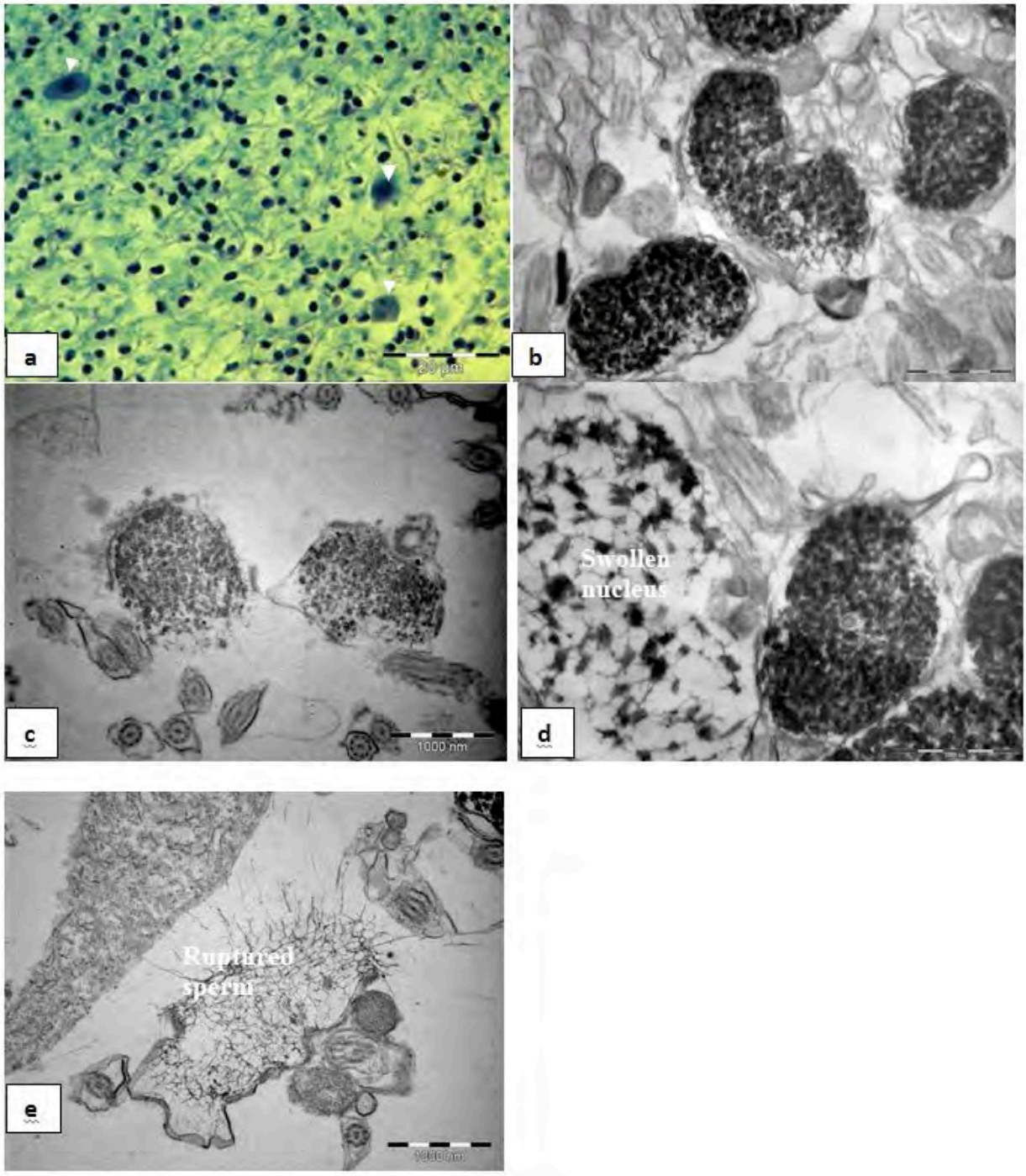


**Figure 4.9:** Degeneration and necrosis of spermatozoa in the testes of a Nile perch, *L. niloticus* from Lake Victoria a) arrow head = pyknosis, b) cells begin nuclear desolution, c) karyorrhexis (cell fragmentation, d) karyolysis (nuclear disolution), e) karyorrhexis, sperm cell nuclear rupture

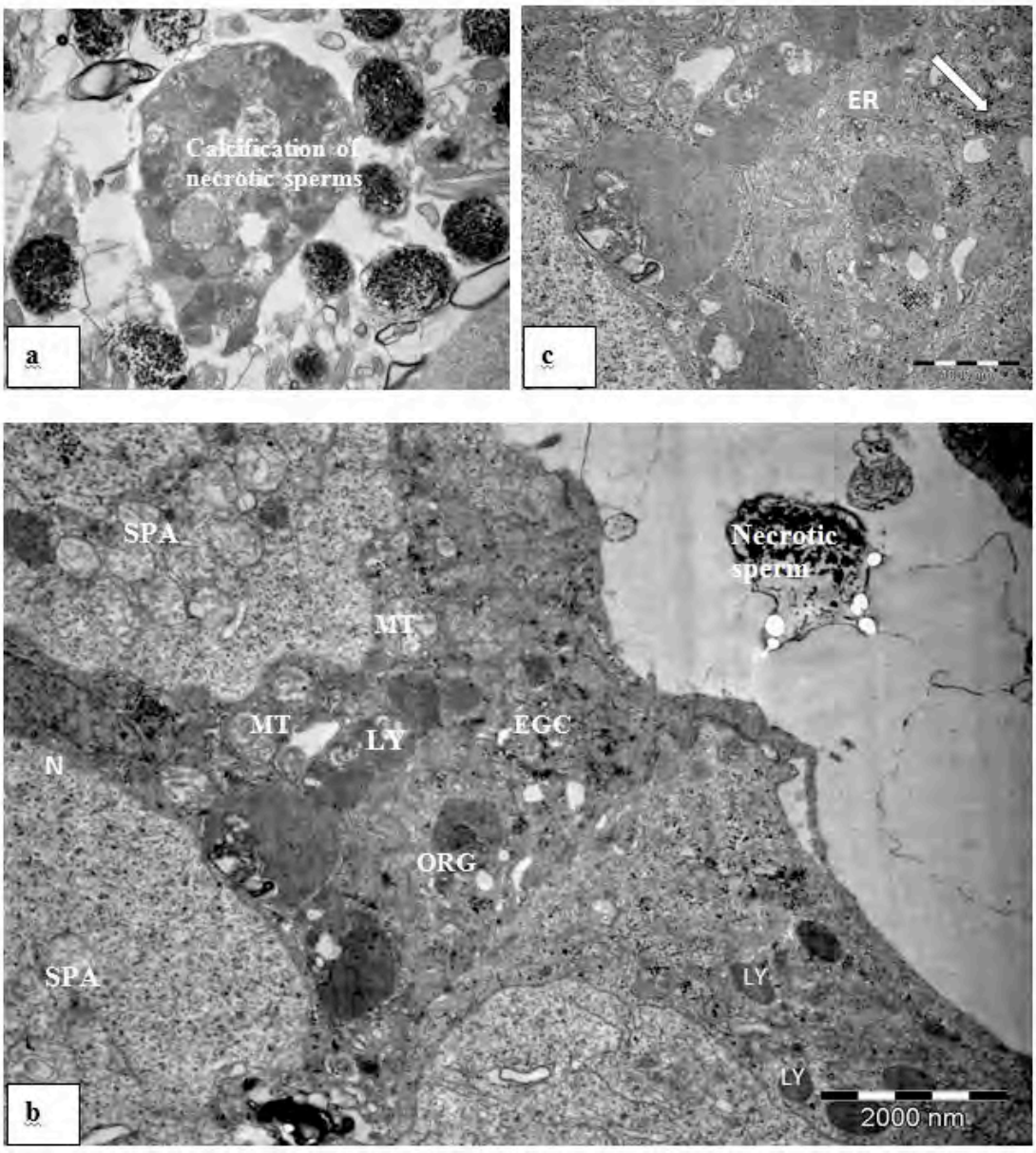


**Figure 4.10:** Phagocytosis of necrotic sperm cells by a Sertoli cell in *L. niloticus* a) dystrophic calcification, the deposition of mineral salts in dead cells, b) Sertoli cell engulfing a necrotic sperm cell, c) a Sertoli cell at a higher magnification, SPA- spermatogonia undergoing digestion, EGC = engulfed cell. MT = mitochondria, ER = endoplasmic reticulum from digested cells, LY = lysosomes in the cytoplasm of the Sertoli cell, ORG = organelles, glycogen (white arrow)

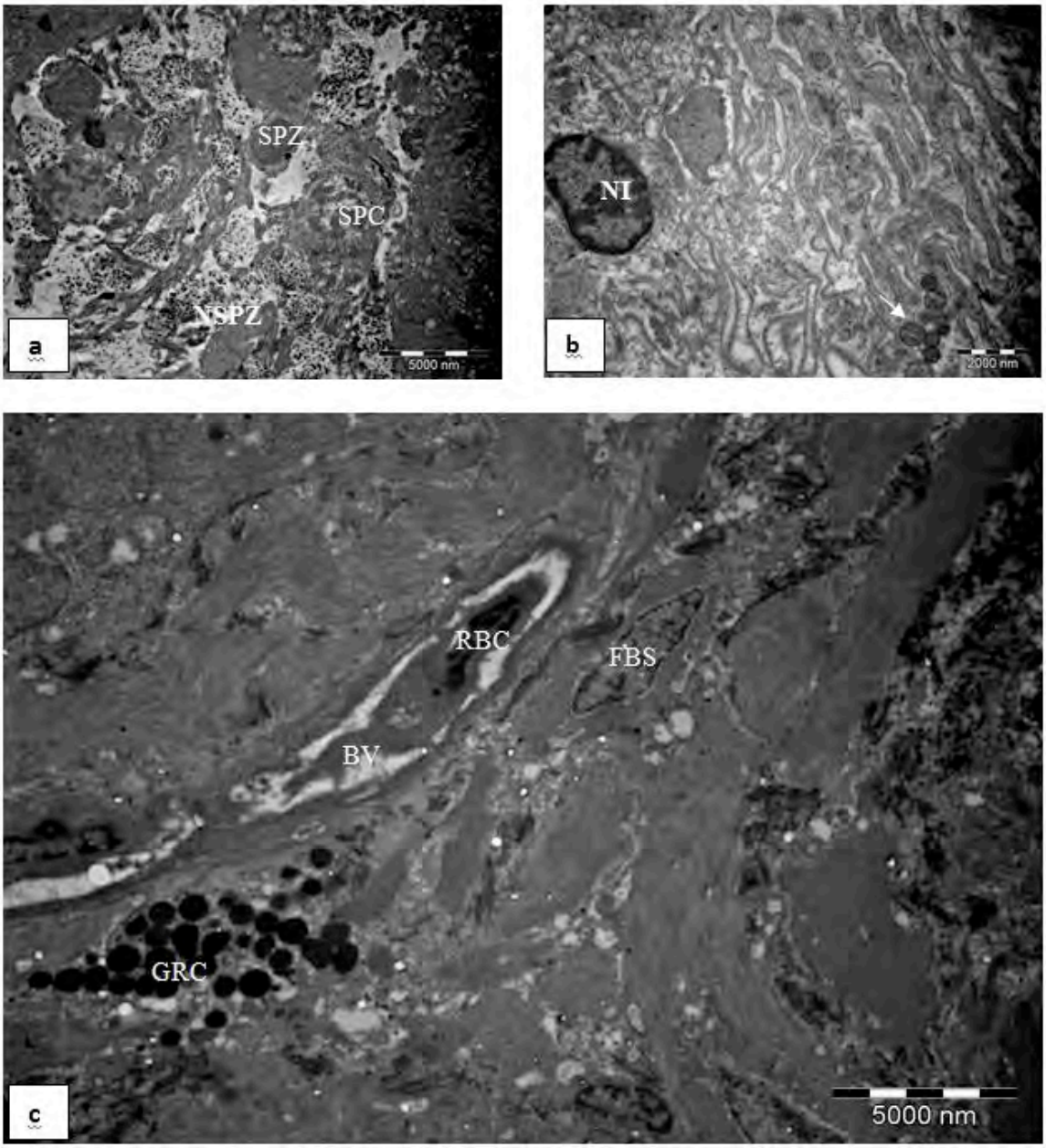


**Figure** 4.11: Germ and interstitial cell necrosis: a) necrosis of germ cells at different stages in the lumen, b) lysosomes (black arrows) in the lumen, c) interstitial cell necrosis; RBC = red blood cells, BV = blood vessel, GRC = granulocyte, FBS = fibroblasts, NI = nucleus from an identified cell, F = flagellae, SPZ = intact spermatozoa, SPC spermatocytes, NSPZ = un-necrotic spermatozoa

*Sperm ultrastructure*

The spermatozoa of *L. niloticus* had a round head-shape without an acrosome. There was a short mid-piece and one flagellum (Fig. 4.12). The sperm head measured 2.57 ± 0.47 μm (n = 11) in diameter and was 2.07 ± 0.07 μm (n = 11) thick. The nucleus was electron-dense with granulated chromatin that occupied the head region, which was surrounded by a film of cytoplasm. The nucleus, with a diameter of 2.13 ± 0.04 μm (n = 7), and 1.29 ± 0.05 μm thick, had two pits; one shallow and the other narrow, but deep, the latter measuring 0.57 ± 0.04 μm (n = 3). In the centre of the round head in the flattened region, there was a nuclear implantation fossa to which the distal centriole was attached, using extended fibril structures (Fig. 4.13a to b).

The centrioles were arranged at an angle of $90^{o}$ to each other, forming a T-shape, parallel to the nucleus. The proximal centrioles were 0.37 ± 0.02 μm long and 0.25 ± 0.04 μm (n = 5) wide, while the distal centrioles were 0.44 ± 0.04 μm long and 0.27 ± 0.03 μm wide, (n = 7). The two centrioles were held to each other and to the nucleus by electron-dense material, micro fibrils (Fig. 4.13b). Electron-dense structures, 'arms', extended from each doublet, extending outwards (Fig. 4.13c). Both the distal and the proximal centrioles were not inserted into the nucleus fossa. The sperm-head region contained the nucleus, the centriolar system, the proximal part of the flagellum and the cytoplasmic channel (Fig. 4.13d).

The mid-piece was 1.68 ± 0.01 μm long, with an external diameter of 0.85 ± 0.05 μm (n = 6), cylindrical, loaded with cytoplasm, spherical mitochondria, and a part of the proximal flagellum. Mature sperm had five to seven spherical mitochondria with an average diameter of 0.49 ± 0.09 μm (n = 16). They were sometimes found lying tightly packed at the base of the nucleus in the

short middle piece of the spermatozoon (Fig.4.13b to d). The mitochondria had a tubular type of cristea. There were glycogen granules in the cytoplasm. The flagellum was located in the cytoplasmic channel, which was formed by an invagination of the plasmalemma (Fig. 4.13d), with an average inside diameter of 0.38 ± 0.04 μm (n = 6).

The average length of the flagella was 29.4 ± 2.5 μm, (n = 5) with a typically eukaryotic organization of one pair of central singulates and nine peripheral pairs of doublets of microtubules (9 + 2 axoneme pattern), with an extension of the flagella's plasma membrane that formed one lateral wing, measuring 1.08 ± 0.28 μm in width (n = 13). In some spermatozoa, the second wing was rudimentary. In some cases the flagellum wing formed a bulb at its edge, while in others it was plain (Fig. 4.14a). Also, the wing was parallel to the plane of the doublet of the central microtubules (Fig. 4.14b). The diameter of the flagellum, perpendicular to the plane of the doublet of the central microtubules, was 0.33 ± 0.03 μm (n = 8). Peripheral doublets of microtubules and the central doublet of microtubules were 0.044 ± 0.004 μm and 0.025 ± 0.001 μm (n = 12) wide, respectively. The longitudinal section of the flagellum had an osmiophilic strip running along the entire length of the flagella (Fig. 4.14c).

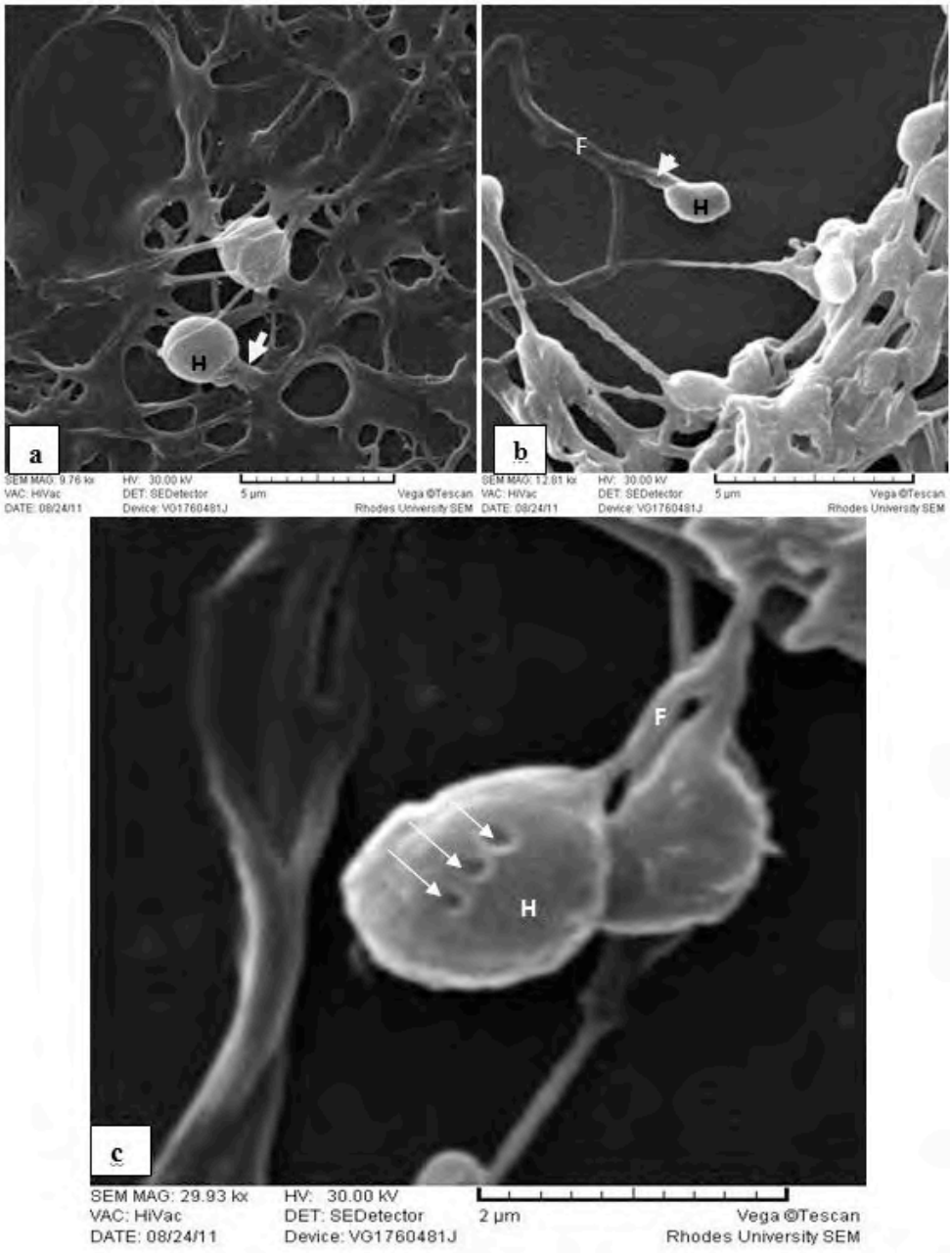


**Figure 4.12:** Scanning electron microscope micrograph of *L. niloticus* sperm head: a) mid-piece = white arrow heads, b) side view showing the squashed shape of the sperm head and c) high magnification of sperm head with surface depressions; H = sperm head, F = flagella, and surface depressions = white small arrows

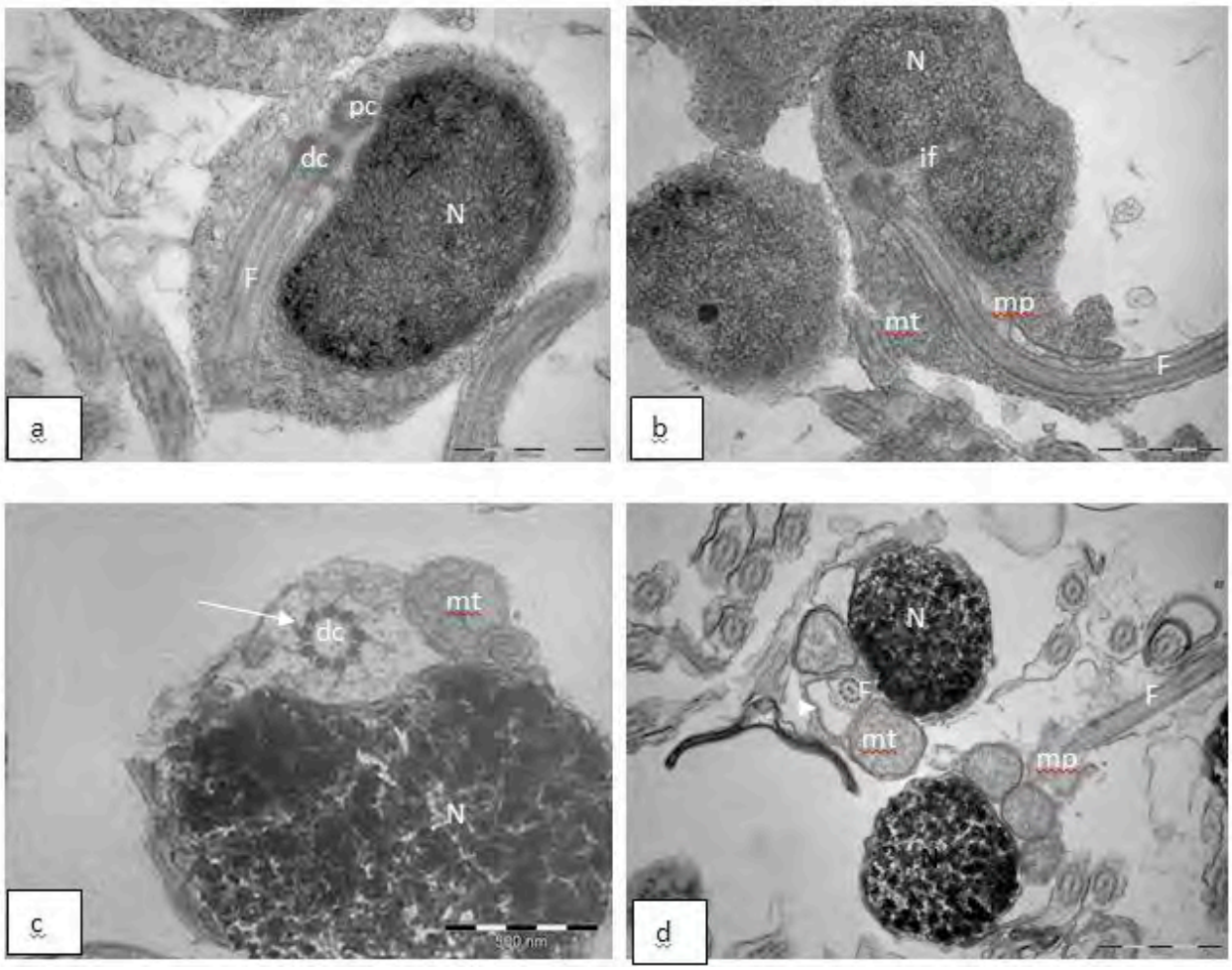


**Figure 4.13:** Nile perch, *L. niloticus* sperm ultrastructure; Top row, head oval medio-laterally flat: a) circular end, b) lateral view; Bottom row: F = flagella, dc = distal centriole, pc = proximal centriole, if = implantation fossa, c) distal centriole, showing osmiophilic satellite lamellae (white arrow ), mt = mitochondria, d) a) cross section of sperm head – proximal end of flagella, N = nucleus, mt = mitochondria, cytoplasm channel (white arrow head) mp = mid piece, F = flagella

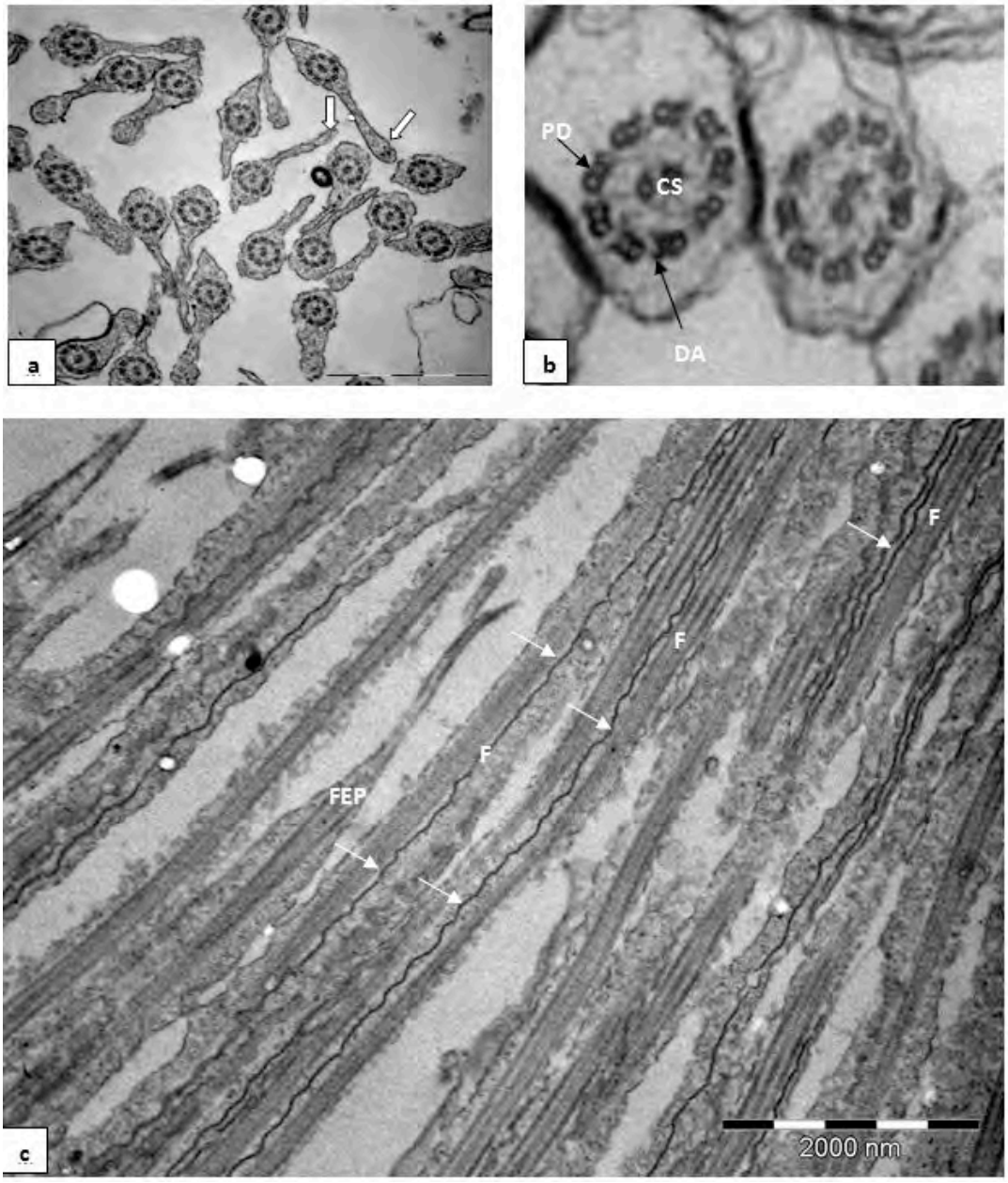


**Figure 4.14:** Nile perch, *L. niloticus*, sperm flagellum ultrastructure: a) cross section of flagella, showing lateral wing extensions, white arrows pointing at the wing, b) cross section showing 9+2 axoneme structure, dynen arms of microtubules, c) transverse section through flagellum, arrows pointing at osmiophilic fibre running along the flagellum length. PD = periphery doublets, DA = dynen arm, CS = central singulets, F = flagella, FEP = flagellum end piece

*Description of oocyte structure and development and ovarian organisation in* L. niloticus

The ovary of the Nile perch is made of two lobes, left and right, poitioned side by side beneath the swim bladder, separated throughout their length. The ovaries in maturation Stages four to six, appear yellow with a prominent blood vessel running along the whole length. In all specimens, the ovarian lobes were not equal in length, for example, the right hand lobe was consistently longer than the left hand lobe. The two lobes joined at the posterior end close to the cloaca. At this point, the urinary bladder joined the short canal that measured 10.0 ± 2.0 mm long (n = 42) (Fig.3.15).

The ovary of *L. niloticus* was made up of myoid cells that were separated by a packed fibrous structure of collagen fibres. The somatic epithelium comprised of two structures; the ovarian cavity on the ventral side and the stromal layer in the dorsal region of the ovary. The ovarian germinal epithelium separated the ovarian lumen from the stroma (Fig. 4.16). The stroma encompassed an extravascular space, which was large and fluid-filled, depending on the stage of gonad development. During advanced stages of oocyte development (tertiary stage), the extravascular space was filled with vitellogenic oocytes, leaving a small space between them. The germinal epithelium was made of epithelial cells that became pre-follicle cells (Fig. 4.16). Only epithelial cells close to the germ cell became follicle cells. The germinal epithelium folded into the ovarian lumen to form projections. Oocytes at developmental stages, from oogonia to the tertiary oocyte stage, occurred in the ovary, especially during the gonadal recovery phase (Fig. 4.16).

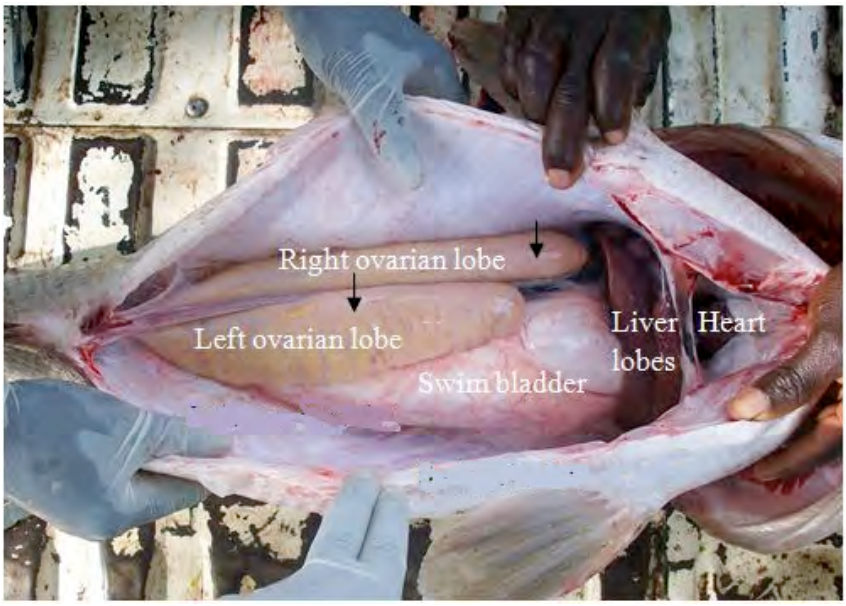


**Figure 4.15:** Location and appearance of mature ovaries in *L. niloticus*

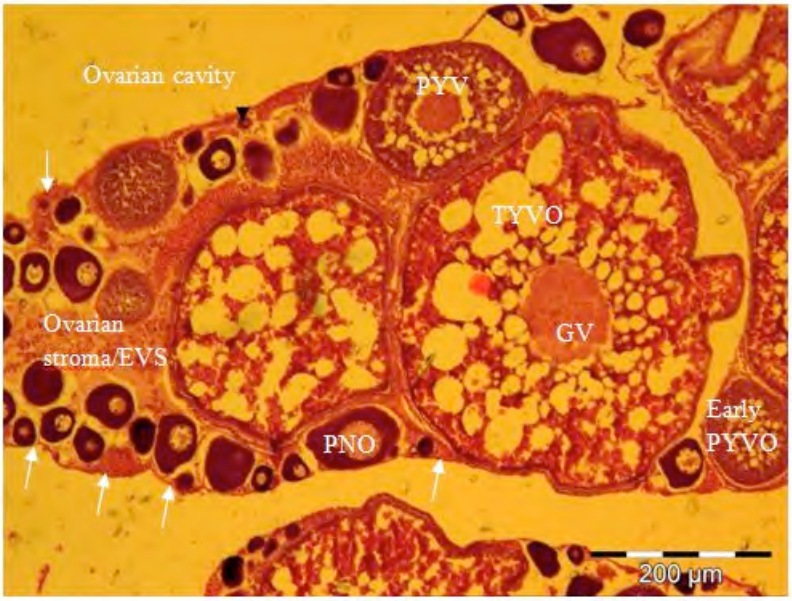


**Figure 4.16:** Asynchronous Nile perch ovary, showing all stages of oogenesis, EVS = extra-vascular space, PNO = peri-nucleolus oocyte, PYVO = primary yolk vesicle oocyte, SYVO = secondary yolk vesicle oocyte, TYVO = tertiary yolk vesicle oocyte, black arrows = germinal epithelium, arrow head = oogonia

*Ovarian germ line stem cells and oogonia and oocyte development in the Nile perch*

The phases of oogenesis included oogonial proliferation, oogenesis, primary folliculogenesis and secondary oocyte growth. Secondary growth entailed vitellogenesis, a process by which external yolk material is incorporated into the growing oocyte, followed by oocyte maturation. Germ cells were spherical to oval-shaped, characterized by less electron-dense cytoplasm that was usually found in clusters within the germinal epithelia. They had a large nucleus in relation to cell volume at the centre of the cell. The outline of the nuclear envelope was regular and the nucleoplasm contained fine granular chromatins that were widely dispersed within the nucleoplasm. A single nucleolus was situated eccentrically and it consisted of a small number of fibrils (Figure 4.17b). In addition, a few isolated ribosomes were dispersed throughout the cytoplasm, making it less electron-dense. The mitochondria were round and scattered around the nuclear membrane in association with masses of electron-dense nuages. The nuages were located outside the nuclear membrane, either in contact with, or separated from the nuclear membrane. Furthermore, the nuages decreased in size and number as oogenesis progressed towards formation of diplotene oocytes. Amorphous fibrous structures were present in the cytoplasm of the oogonia. Other cytoplasmic inclusions, such as lipid droplets or glycogen, were absent at this stage of development (Figure Fig. 4.17 b to c).

*Transformation of oogonia into oocytes*

Transformation of oogonia into oocytes occurred through a series of mitotic to meiotic cell divisions that formed large cells with condensed chromosomes (Fig. 4.17b). Folliculogenesis was observed in diplotene oocytes after entering the stroma and this resulted in an oocyte enveloped in a follicle, thus marking the end of folliculogenesis. At this time, pre-follicular cells were prominent and they appeared in close contact with the germ cell (Fig. 4.17b).

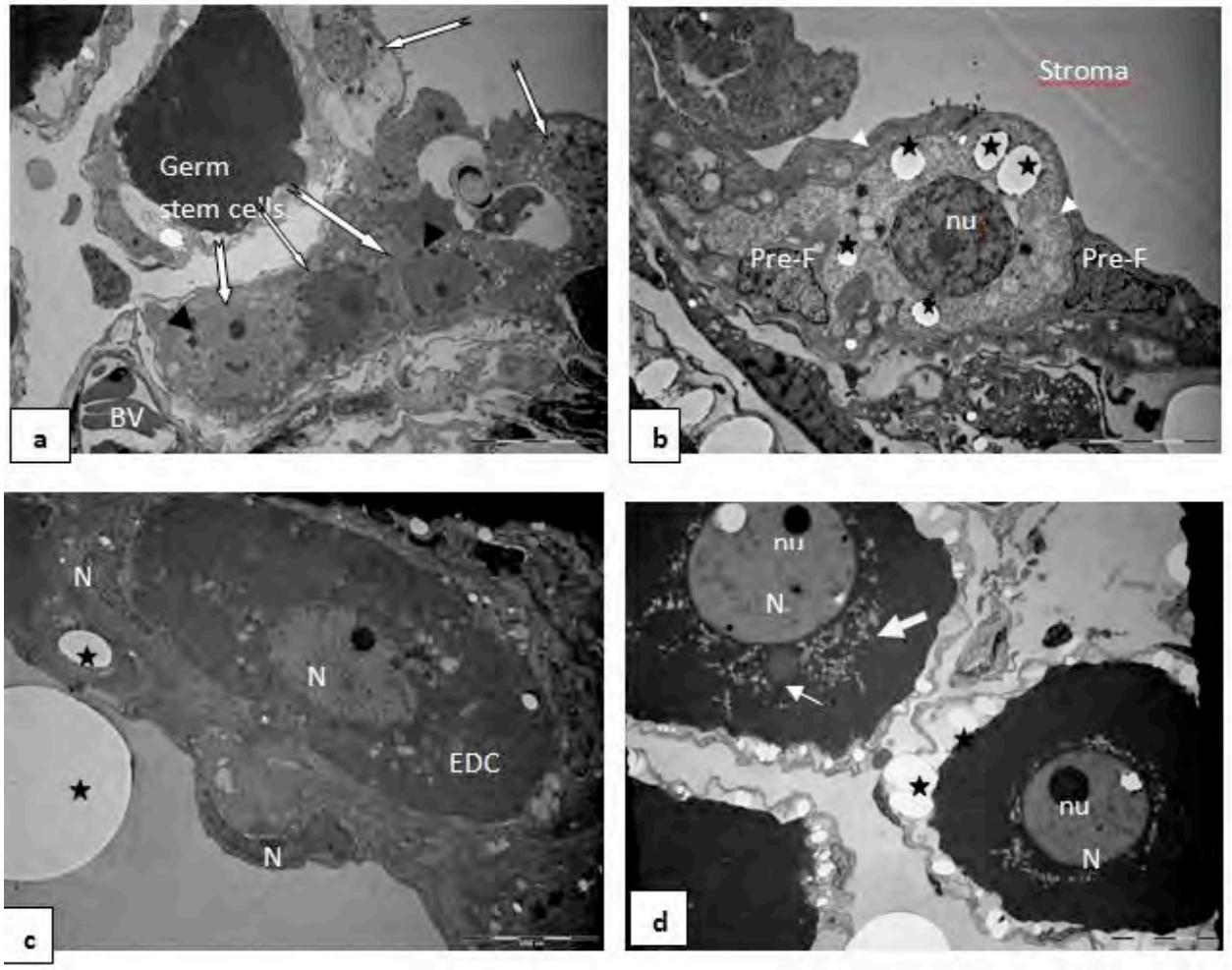


**Figure 4.17:** Gametogenesis from germ stem cells through oogonia to early oocyte growth: a) mitotic germ cells nest, b) diplotene oocyte and two pre-follicular cells = Pre-F, c) four follicular cells surrounding the oocyte, d) primary oocyte growth - appearance of electron-light structures (granular) around the nucleus, ( ★ ) (artefacts) holes made by electron bombarding the section. BV = blood vessels, nuages (arrow head) PreF = pre-follicular cells, N = nucleus, nu = nucleolus, EDC = electron dense cytoplasm, Balbiani body (thin white arrow)

During this stage of development, the chromatin material condensed and became visible as electron-dense, fibrous structures in the nucleoplasm of the oocyte. There was one large nucleolus at the centre of the oocyte (Fig. 4.17b). At this stage, the oocytes entered the primary growth phase as the cytoplasm became more electron-dense. At the same time, Balbiani bodies appeared in the ooplasm. The Balbiani body appearance differed from the nuages in the germ cells in the ovaries and also from those in the testes (Fig. 4.17d).

*Oocyte development stages*

Oocyte development commenced at the end of folliculogenesis when the diplotene germ cell was completely encompassed by follicular cells, forming the chromatin nucleolus oocyte. This was followed by the formation of perinucleolus oocytes that were characterized by numerous nucleoli along the periphery of the nucleus. During the primary yolk vesicle oocyte (PYVO) stage, no yolk cortical alveoli were observed. The developmental stages of the oocytes for this species were classified as follows:

1. Oogonia stage (Figure 4.18a): Nucleus large, i.e. >75% of cell area, large nucleus: cell ratio, one distinct nucleolus. Once the oogonium entered meiosis it became an oocyte and progressed to the diplotene stage.

2. Chromatin-nucleolus stage (Figure 4.18b): Oocyte size ranged from 17.9 to 43.2 μm (n = 24) in diameter with a spherical nucleus occupying a large part of the oocyte.

3. Peri-nucleolus stage oocyte (PNS) (Figure 4.18c). The oocytes 54.9 to 121.6 μm (n = 30) in diameter were polygonal to oval, with several nucleoli. The cytoplasm was strongly stained with Harris haematoxylin and eosin and the nucleus was less basophilic, with two to sixteen nucleoli spread around its periphery. The cytoplasm had a strong affinity for haematoxylin. The oocyte

stages preceding the pre-vitellogenic phase were always present. After the diplotene stage, the oocyte entered a new growth phase (pre-vitellogenesis phase) that was characterized by the appearance of oil droplets, presumably cortical alveoli around the nuclear membrane (Fig. 4.18d).

4. Primary yolk vesicle / oil-droplet stage (ODO) (Figure 4.19a). The oocytes were 124.9 to 210.2 μm in diameter (n = 36) and their cytoplasm continued to become less basophilic. The ooplasm stained light purple basophilic in haematoxylin and eosin. The oil-droplets in the cytoplasm appeared as unstained, empty vacuoles around the nuclear membrane and the diameter of the nucleus was similar to that of oocytes in Stage 3.

5. Secondary yolk vesicle stage or the early yolk globule stage (Figure 4.19a). This stage was characterized by oocytes of a diameter ranging from 212.54 to 426.31 μm (n = 45) with oil-droplets distributed in the cytoplasm. Yolk globules formed in the oocyte, initially in the peripheral cytoplasm as small granules that stained red with haematoxylin and eosin and then accumulated in the cytoplasm, thereby filling the spaces between the oil droplets. This was followed by the tertiary yolk vesicle stage, which was characterized by the formation of cytoplasmic yolk.

6. Tertiary yolk vesicle stage (Fig. 4.19b). The oocytes measured 406.4 – 569.9 μm (n = 75) in diameter and their size increased rapidly with increasing yolk accumulation to form large yolk globules. The nucleus (germinal vesicle) occupied the centre of the oocyte. This was followed by the oocyte entering the final oocyte maturation phase, which was characterized by oil globule coalescence, resulting in a larger globule at the centre of the oocyte, followed by germinal vesicle migration to the periphery (animal pole) of the cell. At this advanced stage of oocyte

maturation, before germinal vesicle breakdown, the diameter of the oil globule accounted for 30.5% (n = 20) of the diameter of the egg.

The nuclear shape changed from oval to doughnut shape during the final stage of oocyte maturation and the nucleus was at the animal pole, where it became attached to the *zona radiata*. At this stage, a depression was discernible at the point where the germinal vesicle juxtaposes the *zona radiata* (Fig. 4.19c). The germinal vesicle migration to the polar region of the oocyte coincided with the coalescence of the oil globules forming one large centric oil globule, while displacing the yolk granules and the germinal vesicle to the periphery. No yolk globule dissolution was observed. All oocyte stages occurred in one ovary and post-ovulatory follicles were observed as a result of asynchronous growth (Fig. 4.19d).

7. Atretic oocytes: There were two types of atretic oocytes namely, pre-vitellogenic oocytes and vitellogenic atretic oocytes. The signs of atresia manifested as disintegration of the nucleus, followed by the loss of the follicular layer integrity in the vitellogenic oocytes (Fig. 4.20).

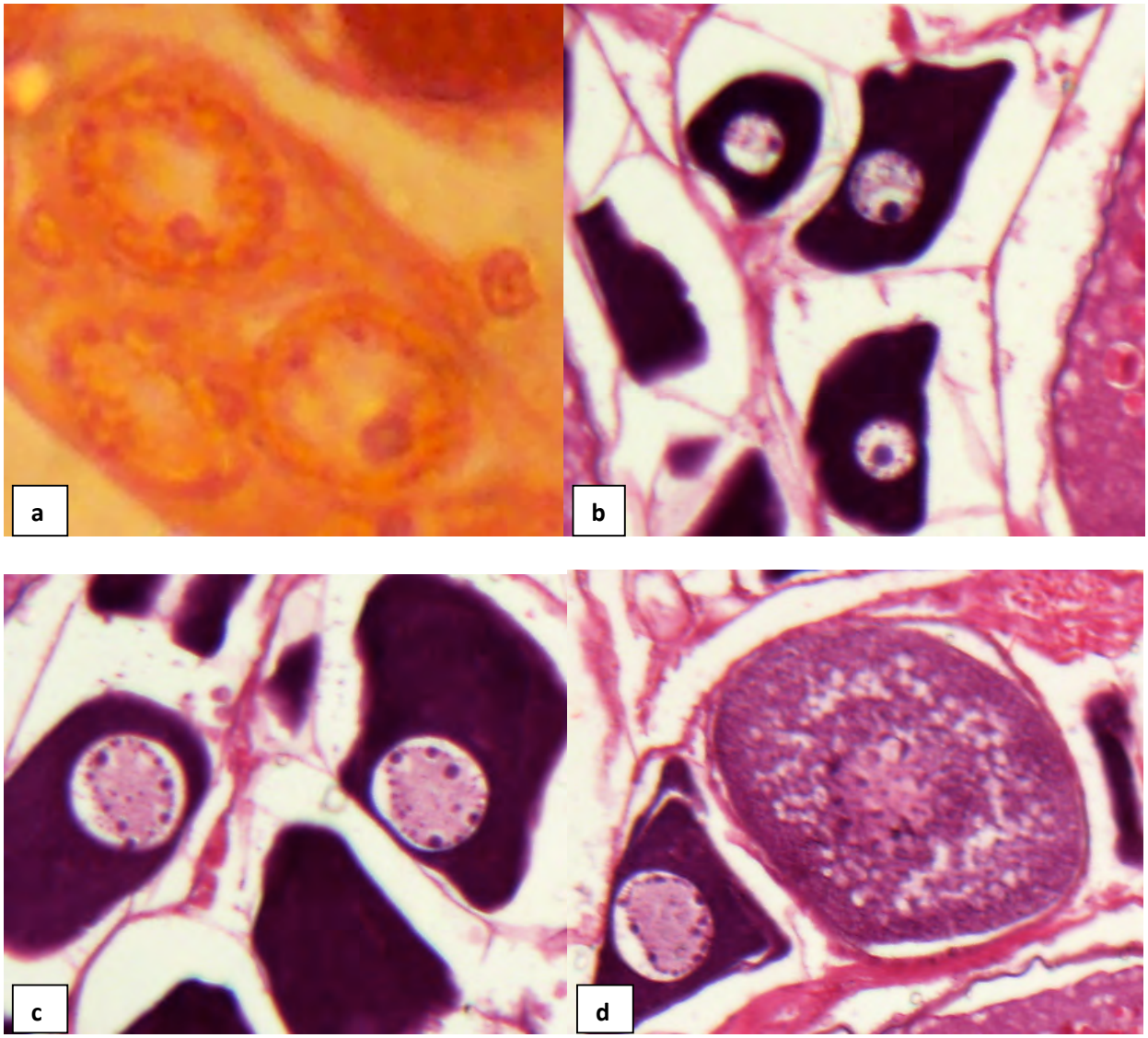


**Figure 4.18:** Primary oocyte growth in *L. niloticus:* a) oogonia cells under light microscope (x1000), b) chromatin nucleolus stage oocyte with one prominent nucleolus (x400), c) perinucleolus oocyte characterised by multiple nucleoli assembled at the nuclear periphery (x400), d) oil droplets forming around the nucleus of secondary yolk vesicle oocyte, N = nucleus, nu = nucleolus

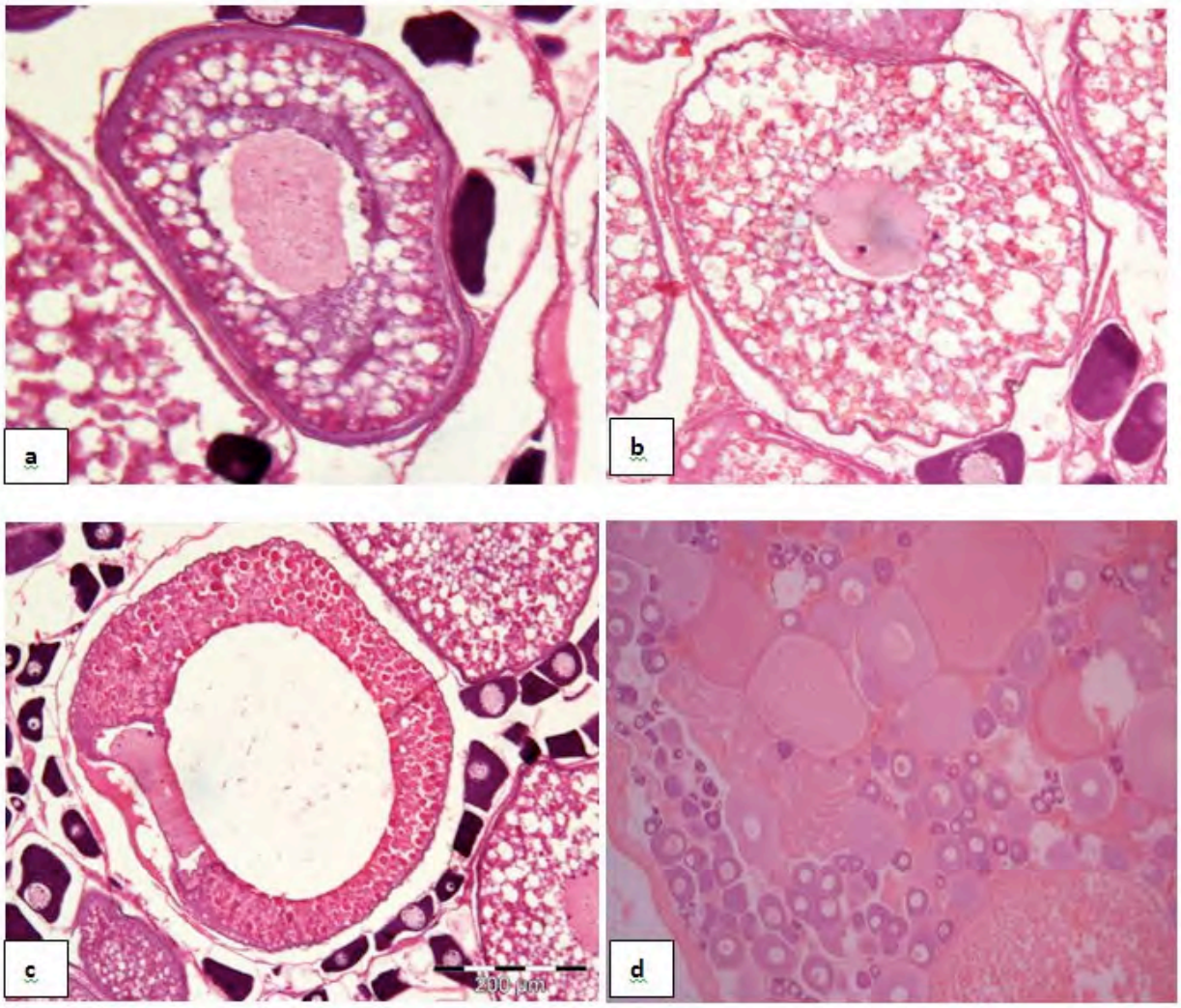


**Figure 4.19:** Secondary oocyte growth (vitellogenic oocytes); a) oil droplets distributed throughout the oocyte, SYVO yolk vesicles appear at the oocyte periphery (x 400), b) TYVO with yolk granules distributed throughout the ooplasm, nuclear in central position and oil droplets begin to coalesce marking the beginning of oocyte maturation (x 200), c) TYVO in final oocyte maturation stage, nucleus at the periphery, central oil globule, and granulated yolk in the periphery (x200), d) a gonad with POFs surrounded by developing oocytes, oocytes at various stages of development (x 40)

*Spawning strategy of* L. niloticus

A ripe running female has an ovary running along the entire abdominal cavity length, with pouches containing ovulated eggs. Egg ripening starts at the anterior portion of the ovarian lobes and is characterised by the development of pouches that contain the ovulated eggs. A prominent blood vessel is discernable, running the whole length of the ovarian lobes (Fig. 3.21). A ripe running female, weighing 40 kg, has an ovary length of more than 30 cm and weighing more than 1.5 kg. Two to three pouches containing ovulated eggs were located at the anterior end of the ripe ovary (Fig. 3.21).

Histological examination indicated that oocytes of all stages were present in an ovary at Stage five of ovarian development. Spawned individuals, for example those females that contained a population of post-ovulatory follicles had oocytes at all stages of development (Fig. 3.22). Oocytes at different stages of development appeared in groups of oocytes at the same stage of development in a ripe ovary. A group of tertiary oocytes appeared to mature as a cohort in one portion of the ovary and ovulated at the same time, as evidenced by the presence of the groups of post-ovulatory follicles occupying a portion of the ovary (Fig. 3.22).

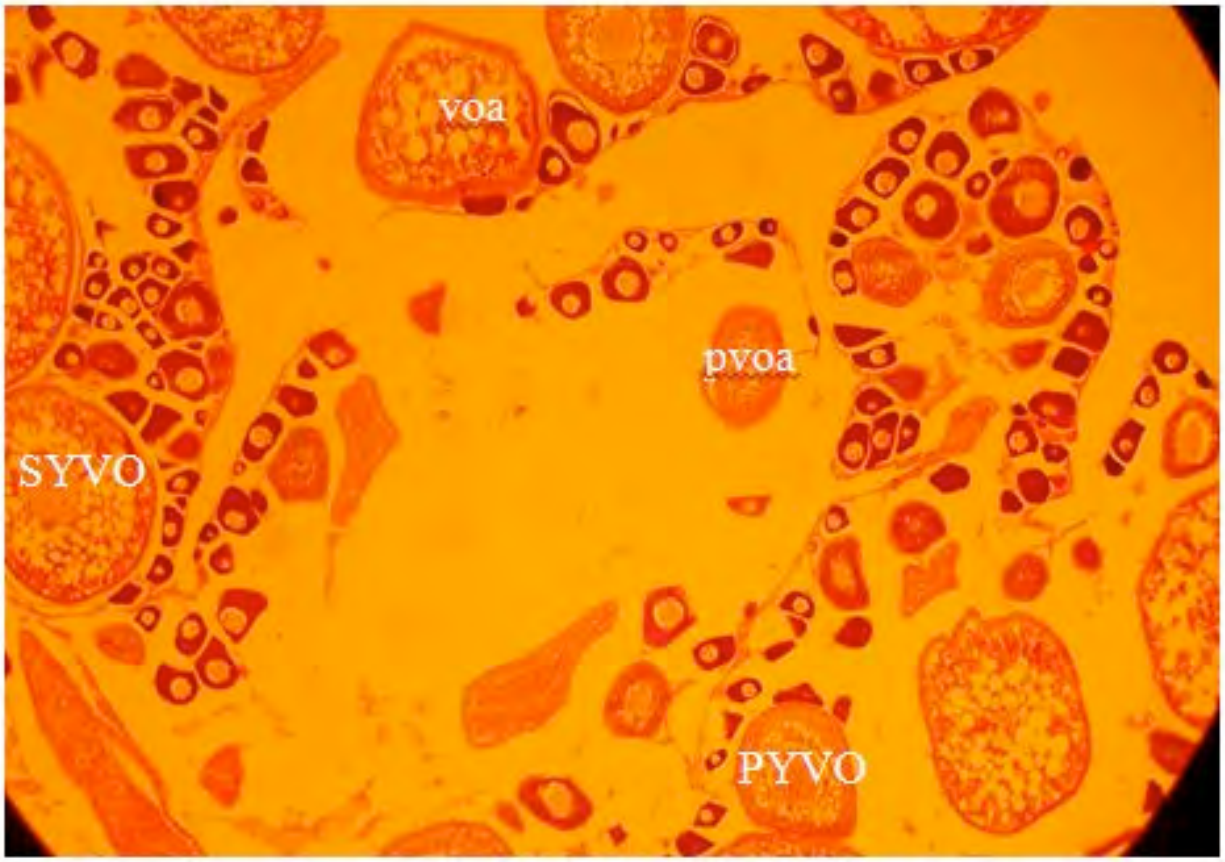


**Figure 4.20:** Atretic oocytes; pvoa = pre-vitellogenic oocyte atresia, voa = vitellogenic oocyte atresia, PYVO = primary yolk vesicle oocyte, SYVO = secondary yolk vesicle oocyte (Fish caught in July 2008)

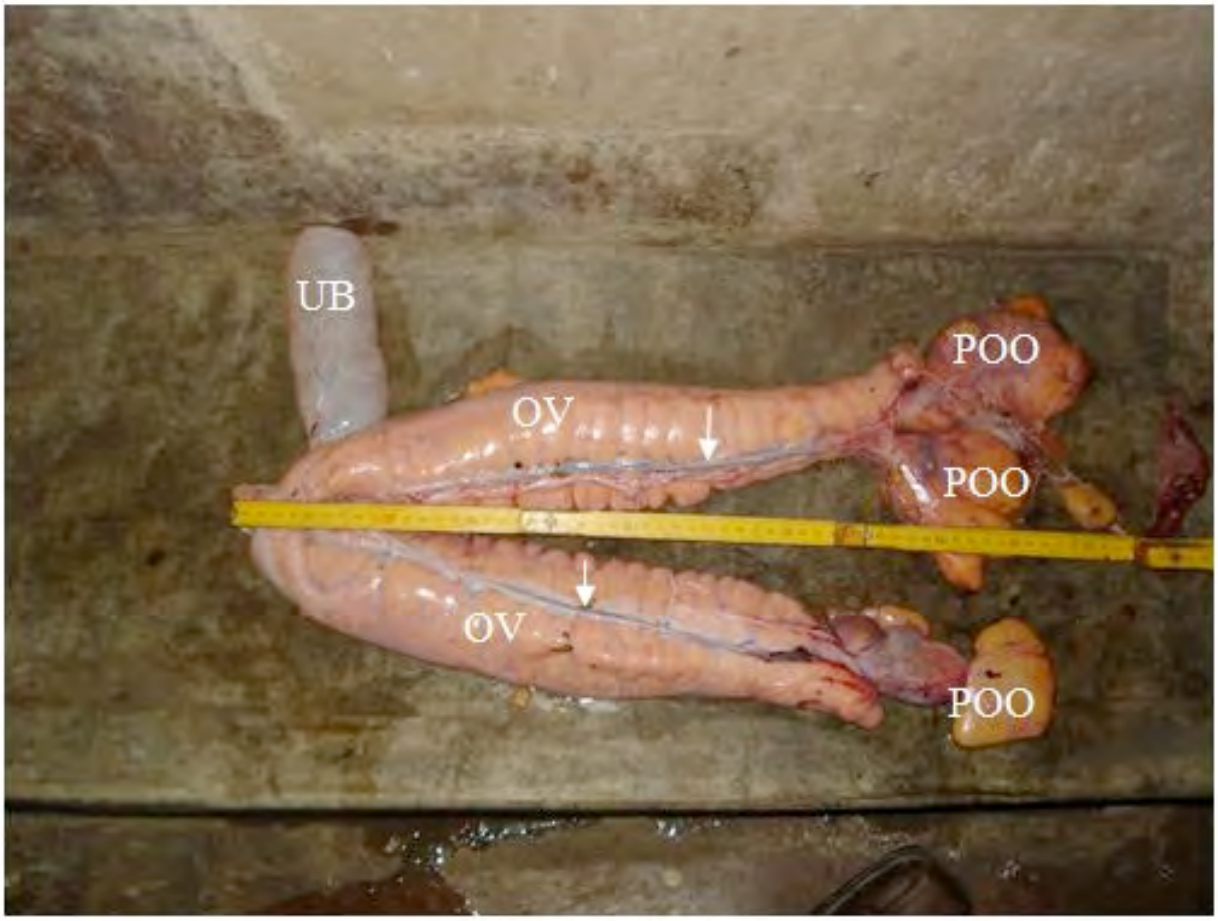


**Figure 4.21** An ovary with ovulated oocytes, UB = urinary bladder, OV = ovarian lobe, POO = pouches within the ovary with ovulated oocytes, arrows = blood vessels

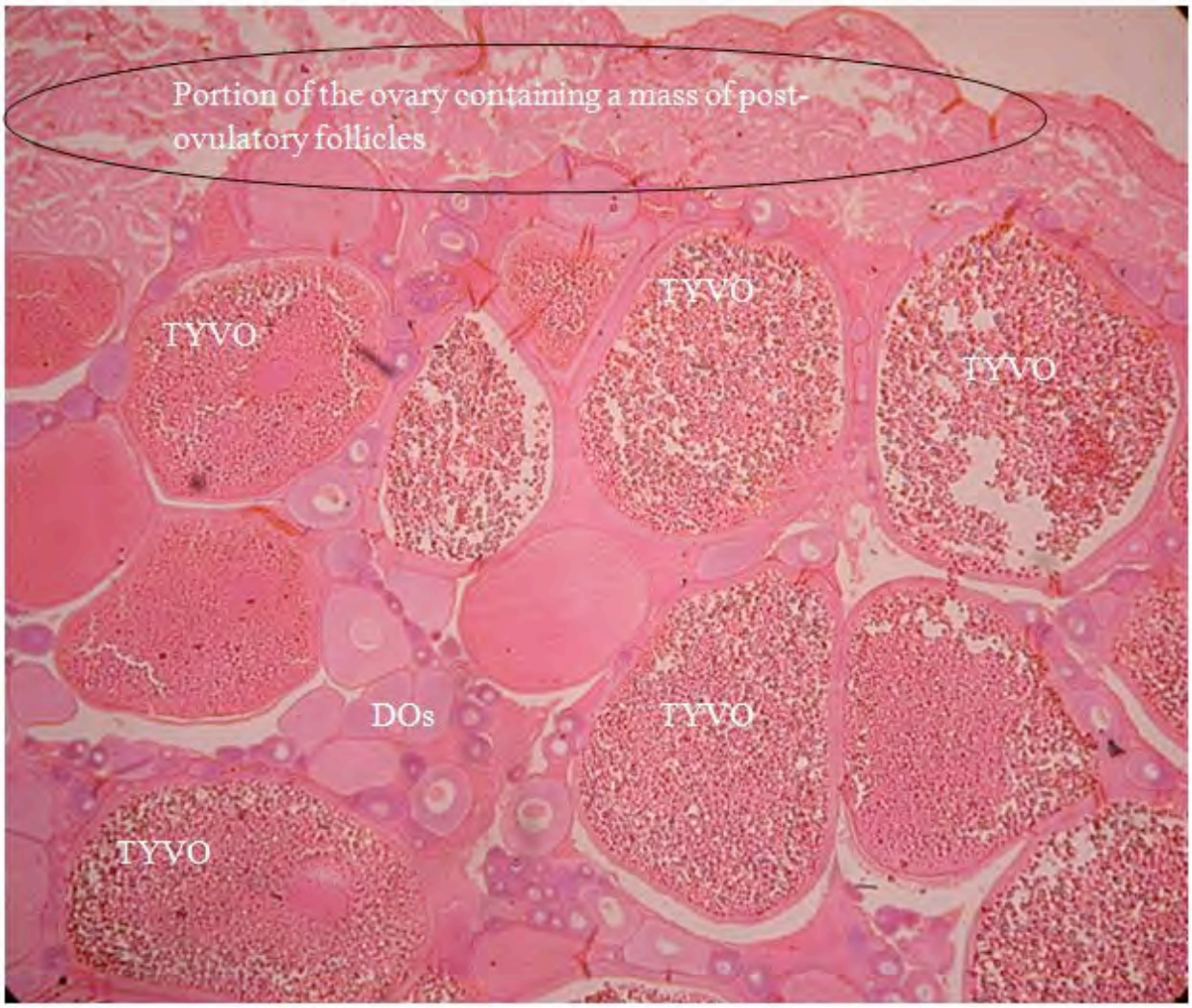


**Figure 4.22**: Micrograph of Nile perch ovary, highlighting different developmental stages, i.e. post-ovulatory follicles, a batch of tertiary yolk vesicle oocytes (TYVO) and a group of developing oocytes (DOs) (magnification x50)

## Discussion

*Testicular structure and type*

Gonad morphology and gametogenesis at gross anatomical and histological levels in *L. niloticus* was similar to what has been observed in other fishes. The testicular arrangement of the lobules and main sperm duct in *L. niloticus* comprised of the main duct located within the testes, from which a branching system radiated to the *tunica albuginea*, forming a blind end at the periphery. *Lates niloticus* had an unrestricted lobular, cystic-type of testes (Grier et al., 1980; Parenti and Grier, 2004, Basiita et al., 2011). Lobular testes are typical in neoteleostei fishes (Parenti and Grier, 2004). This structural arrangement is similar to what Grier et al. (1980) observed in *Perciformes* such as in *Lepomis microlophus* and *L. maculatus.* This structural arrangement is known as "type B" testes (Grier et al., 1980).

In this study, spermatogenesis occurred exclusively in spermatocysts made of Sertoli cell cytoplasm and only the mature sperm cells were found in the lobular lumen. The act of spermatozoa release into the duct during spermatogenesis was probably to reduce backward pressure on the young spermatocysts, thereby creating room for more spermatozoa from the spermatocyst in the lobules. Testes in advanced stages of maturity had valve-like structures which may give a unidirectional flow of sperm cells towards the main duct during spermiation. Similar structures were observed in other species such as, *Labeo victorianus* (Rutaisire and Booth, 2004). The valve-like structures in *L. niloticus* probably explains why milt in ripe running male Nile perch exudes easily on applying slight pressure to the abdomen. This implies that it will not be necessary to induce males in order to obtain sperm/milt for artificial fertilisation in this species and it will thus reduce the costs of hormones.

*Testicular ultrastructure*

Interstitial epithelial tissue of *L. niloticus* was made up of boundary cells (myoid/fibroblasts), Leydig cells, macrophages, blood vessels containing nucleated red blood cells, monocytes, granulocytes and intercellular collagen fibres. Nerve fibres were not observed. Analysis of the ultrastructure of the boundary cells suggested that these cells have contractile characteristics, similar to those found in mammals (Bloom and Fawcett, 1994). The presence of intracellular extensively linked microfibrils, large sized mitochondria and the association of both mitochondria and intracellular microfibrils with the glycogen globules, suggests contractile abilities within these boundary cells. The presence of collagen fibres between the myoid cells and their adhesion to the myoid cell plasmalemma to form a vast continuous structure, further strengthens the argument about their ability to contract. This suggests that contraction may facilitate sperm movement during spermiation, eventhough these myoid cells did not make a continuous layer around the lobule surface. Studies using histochemical tools are required to understand the nature of these cells. Grier et al. (1989) found a similar cellular arrangement in *Esox niger* and *E. lucius*. The same arrangement was also reported in the anurian amphibian, *Xenopus laevis* by (Unsicker, 1975) in specimens that had been kept under both, natural and experimental conditions. The collagen fibres' connection with interstitial cells and other structures were presumed to play a structural support role to the entire lobular system in the *Esox sp*. (Grier et al., 1989). I therefore hypothesise that the microfibrils play a role of structural support and tissue contraction during spermiation in *L. niloticus*.

*Fibroblasts*

Fibroblasts occupied the inner part of the interstitium. Fibroblasts can synthesise extra-cellular matrix and collagen, the structural framework for animal tissues, and play an important role in wound healing (Pfisterer et al., 2011). Fibroblast activity may thus explain the presence of collagen fibres in the testicular lobules of *L. niloticus*. They exist in two states, fibroblasts, the activated state and fibrocytes, which are less active, the latter being responsible for wound healing, as in essence they are stem cells.

Leydig cells were often found closely associated with blood vessels. They were found in the interstitium between lobules, especially at the point where the blood vessel passed through the interstitium between two to three lobules. These cells were common at the periphery of the testes lobules. Although Grier (1981) suggested that Leydig cells are never found between testes lobules, the results in the present study agrees with García-López et al. (2010) who, using toluidine blue-stain, an *insl3* antisense cRNA probe, and *3β-Hsd* enzymatic reaction in zebra fish testes, reported the presence of Leydig cells in the interstitium between lobules. In *L. niloticus* they were characterised by numerous Golgi apparatuses, endoplasmic reticulum, mitochondria, and numerous vesicles. These cell inclusions are characteristics of excretory cells hence, Leydig cells. Leydig cells are steriogenic, synthesising androgens and excrete these hormones into the vascular system (Gresik et al., 1973; Grier, 1981). This may explain their close association with the lobular blood vessels.

Spermatogenesis appeared to take place in an enclosed structure, i.e., in the spermatocysts within a lobule that contained germ cells at the same developmental stage. The spermatocyst wall was made up of one or more Sertoli cells that resided on the basement membrane. As in

other teleosts like Atlantic Cod, *Gadus morhua* (Almeida et al., 2008), the germ cells were not attached to the Sertoli cell, but appeared with their head facing the lobular wall, away from the lumen.

Two types of spermatogenesis have been described in teleosts; cystic and semi-cystic (Mattei et al., 1993). The cystic type is common in teleost fishes (Grier et al., 1980; Gusmão-Pompiani et al., 1999). Spermatogenesis in the Nile perch conformed to the cystic type. The germ cells were at various stages of development, thus this species may employ asynchronous activation of the germinal cell development, as has been reported in other teleosts such as *Cichla intermedia* (Quagio-Grassiotto et al., 2003).

The primary spermatogonia were the largest cells. Each cell was surrounded by the Sertoli cell plasma, and they were less electron-dense than other germ cells. These cells were characterized by the presence of electron-dense bodies like nuages (Eddy, 1975; Hamaguchi, 1987; Guimarães and Quagio-Grassiotto, 2001) that surrounded the nucleus. They were also surrounded by mitochondria. The nuclear membrane had pores at the point where the nuages were associated with the nuclear membrane, suggesting that they may be of nuclear origin. It was proposed that the nuages moderate the developmental versatility of germ cells (Hamaguchi, 1987).

According to Eddy (1974, 1975), these dense bodies are ribonucleic protein structures which come from the nucleus. In this study, germ cell electron-dense bodies (nuages) were observed throughout the cytoplasm of the primary spermatogonia, up to the early secondary spermatogonia stage. The findings in the present study concur with Hamaguchi (1987) who reported similar findings in the male germ line of *Oryzias latipes*. Eddy (1974) found the nuage

material in most development stages of the germ line of rats, where it always occurred in the cytoplasm of primordial germ cells and primary spermatogonia, while being absent in secondary spermatogonia and zygotene spermatocytes. Chromatoid bodies were also found in mammalian spermatocytes, but in rats, Söderström (1981) regarded them as organelles, different from nuages. These bodies were also reported in *Xenopus laevis* (Kerr and Dixon, 1974). In the spermatogonia of *Poecilia reticulata,* nuages were present (Billard, 1984). The electron-dense materials (nuage) in all organisms in which they have been observed, appeared to degenerate in spermatocytes, as has been observed here in *L. niloticus*.

In the present study, spermatogonia decreased in size, the nucleolus relocated to the periphery and chromatin and cytoplasmic density increased and mitochondria were less visible. Similar trends have been reported in *Poecilia reticulata* during spermatogenesis (Billard, 1984). Cysts at all stages of germ cell development were found throughout the lobule length. Both the nuclear and cell size continued to decrease. Of particular interest, however, was a sudden increase in nuclear size in secondary spermatocytes, which preceded an increase in the amount of chromatin material and the appearance of chromosomal structures. Billard (1984) observed similar structures during metaphase I in *P. reticulata*. Like in other teleosts, mitochondria and associated nuages were not discernible at this stage. The frequency of mitochondria decreased as spermatogenesis progressed to form spermatozoa from about 25 mitochondria in spermatogonia to five to seven mitochondria in Nile perch spermatozoa. The fate of other mitochondria observed in primary spermatogonia cannot be explained. Perhaps some of the mitochondria are lost with the excess cytoplasm as they are being shed as residual bodies, as numerous mitochondrial bodies have been observed in the phagocytotic Sertoli cell in the Nile perch. Hecht

(1995) and Meinhardt et al. (1999) reported that mitochondria of the germ cells modify their morphological organization, numbers and location during spermatogenesis, and others leave the developing spermatozoa in the residual bodies. While in invertebrates, several mitochondria undergo fusion that results into large derivative mitochondria (Nicotra and Mura, 1985). Meinhardt et al. (1999) suggested that the mitochondria shed in the residual bodies, are phagocytised by the Sertoli cells.

In this study, spermiogenesis followed an increase in number of cell inclusions and their arrangement. The centriolar and flagella systems were established and organelles collected in one location. At this time, the cytoplasm moved towards the centriolar point, leaving the nucleus at one end, soon after the second meiotic cell division. There was a gradual condensation of the chromatin material (granules) that started at the point adjacent to the centriole system in the nucleus and spread, gradually filling the entire nucleus. This type of spermiogenesis is typical of advanced teleosts, the acanthopterygians and is a character unique to *Perciformes* (Mattei, 1988; Jamieson and Leung, 1991; Gusmão-Pompiani et al., 2005). It has also been reported in African striped grunt, *Parapristipoma octolineatum* (Jamieson and Leung, 1991). Therefore, spermiogenesis in the Nile perch, *Lates niloticus*, conformed to Type II spermiogenesis. This provides more information vital in the control of the Nile perch sperm quality during artificial breeding of the species. It also provides more information on the classification and evolution of the species.

Intercellular connections, via cytoplasmic bridges, occurred throughout gametogenesis, up to the end of spermiogenesis. Frequently, one cell maintained cytoplasmic contact with more than two other cells in the same cyst, suggesting that cells within a spermatocyst are clones of one

spermatogonium. This phenomenon occurs in multi-cellular organisms and is well conserved in both plants and animals (Guo and Zheng, 2004). These intercellular bridges have been suggested to allow genetically segregated haploid spermatids to share diploid gene products (nucleotides) (Haqlund et al., 2011). They also allow for rapid transfer of important signals or nutrients, and they play a role in fertility in both insects and animals (Guo and Zheng, 2004; Haqlund et al., 2011).

*Sperm necrosis*

At the end of spermiation, the Sertoli cells' wall ruptured, releasing spermatozoa into the lobular lumen. In spent fish, Sertoli cells changed form and function. Lysosomes became prominent in the cytoplasm and the Sertoli cells became phagocytotic. Several cells, including both necrotic and non-necrotic sperm cells, spermatogonia and residual bodies were observed inside a Sertoli cell. Sertoli cells have been reported to be involved in the formation of phagosomes that come from degenerating spermatids and sperm cells by phagocytosis (Chung, 2008, *Boleophthalmus pectinirostris;* Chung et al., 2010, *Pampus argenteus*). It is thus hypothesised that Sertoli cells participated in the removal of unspawned sperm cells and other residues, during and at the end of the spawning season in Nile perch.

*Morphology and ultrastructure of spermatozoa of Nile perch*

Sperm structure of several species in the suborder *Percoidei* suggests that the structure of the spermatozoa, showed considerable variation within families (Jamieson, 1991). A review of the descriptions of sperm structure from different families in this suborder (Gusmão-Pompiani et al., 2005), revealed new information and corroborate variations in spermatozoa structure in the suborder. However, the type of spermatozoa found in Centropomidae has been controversial

since Jamieson published the sperm structure of *L. calcarifer* in 1991. This is attributed to the fact that most of the phylogenetic descriptions in this suborder have been based mainly on schematic drawings, rather than ultra-structural studies (Gusmão-Pompiani et al., 2005).

In this study, ultrastructure of *L. niloticus* sperm has been described for the first time, using scanning and transmission electron microscopy. *Lates. niloticus* has primitive, simple, uniflagellate, anacrosomal aquasperm, typical of teleosts employing external fertilisation mechanisms. In the order *Perciformes*, suborder *Percoidei,* there are two types of spermatozoa. These are sperm of Type I and II (Jamieson, 1991), and this is based on whether during spermiogenesis, a nuclear rotation in relation to the axoneme takes place (Mattei, 1991). Spermiogenesis that involves nuclear rotation produces Type I sperm, and if rotation does not occur, the species produces Type II spermatozoa (Mattei, 1991). In *L. niloticus,* no nuclear rotation occurred and therefore a Type II spermatozoon was produced, confirming a hypothesis put forward by Gusmão-Pompiani et al. (1999, 2005) that the spermatozoa of the *L. niloticus* are Type II.

Despite being placed in the same genus, *L. niloticus* sperm differs slightly from that of the Australian Centropomid, *Lates calcarifer,* especially in the centriole arrangement and nuclear shape, length of mid-piece and the number of lateral wings. Jamieson (1991) described *L. calcarifer* sperm as “oval sperm with a short mid-piece and a flagellum with two lateral wings”. *L. niloticus* spermatozoon has an oval head, but a medio-laterally flattened nucleus, and a moderately short mid-piece with a flagellum with one lateral wing. It is likely that some of these differences might be due to the cryo-preservation effects, which may have led to structural

changes in *L. calcarifer* sperm, or it may be due to species-specific differences (Jamieson, 1991; Gusmão-Pompiani et al., 2005).

Nile perch spermatozoa share similarities with the spermatozoa of Australian *Percichthyidae*, in particular *Macquaria sp.* and *Macculochella* sp., although there are differences in the number of mitochondria and flagella extensions (Jamieson, 1991). In *Macculochella* sp., one to two large mitochondria were reported, while in *Macquaria ambigua*, there were three to six mitochondria. Round bodies appeared to be absent in *L. niloticus* sperm cells, in contrast to *M. quariensis* sperm which had three to four round bodies (Jamieson, 1991).

This study revealed a peculiar structure that was osmiophilic and electron-dense which appeared as a dark strip in the flagella, running side by side with the axoneme, along the entire flagella length. Axonemes are involved in motility of sperm flagella (Inaba, 2007). This cytoskeletal structure has also been observed in mammalian spermatozoa (Eddy et al., 2003; Turner, 2003). In Nile perch this structure is not understood. Miki et al. (2004) suggested that energy required for sperm motility is generated by glycolysis, a process catalysed by a sperm-specific glycolytic enzyme, bound tightly to the cytoskeletal structure along the flagella's main piece. It is thus recommended that further studies involving histochemistry be conducted using glycogen markers. These characteristics, such as mitochondria numbers, mid-piece size, and axoneme extensions, appear to be genus- and species-specific characters (Jamieson, 1991), and this study made a contribution to understanding the histology and ultrastructure of Nile perch sperm cells. This information can be used as a basis for the assessment of the sperm structure in cryo-preserved sperm cells. The presence of karyotic and karyorrhetic sperm cells in *L. niloticus* milt

should act as an indicator of poor sperm quality and I suggest that such milt should never be used in the artificial fertilisation of eggs.

*The ovary and oocyte structure in Nile perch*

Some aspects of the development of Nile perch oocytes are similar to that in other teleosts, (Wallace and Selman, 1981; Begovac and Wallace, 1989; Coward et al., 2002; Brown-Peterson et al., 2011). However, the yolk stage (cortical alveoli), frequently described in teleosts was absent. In teleost ovaries, germ cells occur in nests in the germinal epithelium (Tyler and Sumpter, 1996; Grier, 2000, 2002; Nakamura et al., 2011). Many organisms generate their oogonia from germ line stem cells, capable of almost unlimited asymmetric, self-renewing, mitotic divisions. In *L. niloticus,* germ cells were small and round in nests of the ovigerous lamellae. These cells were characterised by a large nucleus, usually exhibiting one nucleolus, and electron-dense bodies (nuages) around the nuclear membrane. Cytoplasm was limited, containing mitochondria in the endoplasmic reticulum that were poorly developed, suggesting little involvement of these cell types with protein synthesis (Guimarães and Quagio-Grassiotto, 2001). These morphological characteristics have been reported in cells of *Oryzias latipes* (Hamaguchi, 1987; Nakimura et al., 2011), and *Serrasalmus spilopleura* (Guimarães and Quagio-Grassiotto, 2001).

The presence or absence of nuages in both male and female germ cells has been used to study the evolution of germ cells in metazoans and the mechanisms of germ cell formation in all animal phyla (Extavour and García-Bellido, 2001; Extavour and Akam, 2003). Two mechanisms of germ cell formation have been described; preformation and epigenesis. In some species, germ

cells are specified very early in embryogenesis. These cells differentiate as germ cells before, or immediately following, fertilisation, hence preformation. In other species, germ cells have not been observed until later in development, and their formation is induced by signals from niche cells in the surrounding tissues during epigenesis (Extavour and García-Bellido, 2001; Extavour and Akam, 2003; Smith et al., 2010). The presence of nuages in the germ cells implies preformation of germ cells (Johnson et al., 2003). In *L. niloticus* the presence of nuages in both male and female germ cells suggests that the germ cells in this species are specified early in embryogenesis, or immediately after fertilization, hence the preformation mechanism aids in germ cell formation.

In the current study, during diplotene, one or two squamous pre-follicular cells surrounded the primary oocyte and these gave rise to primordial follicles. These cells (diplotene oocytes), left the nest and entered primary growth in the stroma. A similar growth pattern was observed in *Mugil auratus*, (Bruslé, 1980), *Syngnathus scovelli*, (Begovac and Wallace, 1989), *S. spilopleura*, (Guimarães and Quagio-Grassiotto, 2001), *Rattus rattus* (Pepling, 2006), *O. latipes* (Nakamura et al., 2011), and in the rat. Follicular development in *L. niloticus* started after the diplotene stage, in the inner part of the ovarian germinal epithelium and was followed by an increase in oocyte size and an increase in the number of granulosa cells forming a single layer arround the oocyte. Similar patterns have been reported for other teleosts (West, 1990). In the mammalian ovary, this occurs at birth, with the formation of multiple layers of granulosa cells (Pepling, 2006).

At the end of folliculogenesis, lipid droplets formed around the oocyte's nuclear membrane, followed by primary oocyte growth. Primary oocyte growth was characterised by an increase in the size of the oocyte. Cytoplasm volume also increased and became more electron-dense than in

earlier stages. The increase in the cell size of pre-vitellogenic oocytes can be attributed to membranous organelles becoming larger and numerous, an increase in the amount of ribonucleic acid molecules (RNA), and accumulation of protein in the cytoplasm (Wallace and Selman, 1981; West, 1990; Guimarães and Quagio-Grassiotto, 2001).

In *L. niloticus* at the beginning of the perinucleolus stage, Balbiani bodies occurred in the cytoplasm, close to the nucleus and later dispersed into the cytoplasm. These structures were observed in fresh water teleost fish (Bazzoli and Godinho, 1995), in Medaka, *Oryzias latipes* (Yamamoto, 1964), and in oocytes of sturgeon, *Acipenser gueldenstaedtii* (Zelazowska et al. 2007). This structure varies between species, ranging from invertebrates to vertebrates (Kloc et al., 2004), and its function is not well understood in most species. However, in the amphibian toad, (*Xenopus sp*.), the Balbiani body was reported to play a role in transferring germ cell determinants such as germinal granules and localized RNA to the oocyte animal pole (Kloc and Etkin, 1995).

At the end of folliculogenesis, as the oocyte developed into the perinucleolus oocyte stage, cytoplasmic lipid globules appeared close to the nuclear membrane. In the common snook, *Centropomus undecimalis*, this occurred, together with the appearance of peripheral cortical granules (Grier, 2000, 2002). No cortical granules were observed in any of the stages of oocyte development, which is different from a recent report by Basiita et al. (2011), who described primary yolk vesicle oocytes as having cortical alveoli. In the present study and in the micrographs available in Basiita et al. (2011), no distinction by colour or appearance could be made between oil globules and the presumed cortical alveoli, as has been demonstrated in other teleosts. Similar oocyte developmental stages were reported in guppy, *Poecilia reticulata* (Takano, 1964) and mosquito fish, *Gambusia affinis* (Koya et al., 1998). Coward et al.(2002)

hypothesised that the contents of the cortical alveoli serves to harden the vitelline envelope after ovulation, to prevent polyspermy. It is therefore likely that other mechanisms of minimising polyspermy might be present in *L. niloticus*. Thus, based on the proposed lack of cortical alveoli, it is hypothesised that other mechanisms of preventing polyspermy might be present in *L. niloticus* oocytes. Further studies are required to fully understand the mechanism employed in preventing polyspermy in this species. Other polyspermy control mechanisms i.e. change in the electrical charge across the surface of the egg, have been reported in sea urchins (Jaffe, 1980), and in amphibians (Iwao, 2000).The change of egg surface charge from negative to positive is caused by the fusion of the first sperm with the egg (Jaffe, 1980).

The oocytes contained granular yolk. During vitellogenesis, protein yolk formed spherical globules that stained red, in contrast to the ooplasma, which stained purple in haematoxylin and eosin. In this study, vitellogenesis, a process that involves uptake of hepatically derived glycolipophosphoprotein by receptor-mediated endocytosis (Perazzolo, et al., 1999), began in the secondary yolk vesicle oocyte. This process coincided with the enlargement of the oil droplets and peaked in the tertiary yolk vesicle oocytes, i.e., the largest oocytes. The oocytes in mature follicles possessed cytoplasmic lipid and protein yolk globules, but no peripheral cortical granules. The presence of large amounts of lipids in Nile perch eggs warrants the large size of oil globules observed during final oocyte maturation.

Aquatic organisms have evolved several mechanisms of attaining buoyancy of demersal and pelagic eggs. Some marine teleosts, for example, have overcome sinking by accumulating high amounts of water of up to 90% of the egg's weight, and others accumulate a large quantity of lipid in their eggs (Craik and Harvey, 1987). In *L. niloticus,* during final oocyte maturation, the yolk protein globules were displaced to the periphery of the ooplasm by the centrally enlarging

oil globule which had formed one large oil droplet. The oil globule diameter was more than 30% of the egg's diameter. Lipids in pelagic and demersal eggs have a hydrostatic function that is responsible for buoyancy in some marine teleost eggs (Craik and Harvey, 1987), in addition to its nutritional value. High egg lipid content >30% has also been reported in buoyant eggs of Grenadier fish (Craik and Harvey, 1987). It is thus suggested, in this thesis that the main reason for accumulating large quantities of lipids in the Nile perch eggs is to assist with buoyancy in both the eggs and the larvae. This information is vital in the management of the *L. niloticus* eggs during incubation and hatchery design. It is further suggested that egg and larval buoyancy may assist in the dispersal of Nile perch eggs and hatched larvae to nursery grounds and also to avoid the anoxic water conditions at the bottom and in the water columns of a highly eutrophic water system.

*Lates niloticus* oocytes developed asynchronously, with all stages of oocyte development present in the ovary at one time. Wallace and Selman (1981) based on the dynamics of organisation of the ovaries, described three categories of ovarian development organisation, namely, synchronous, group synchronous, and asynchronous oocyte development. Results from histological sections corroborate the field macroscopic observations that revealed the presence of pouches containing ovulated eggs. This supports the suggestion that Nile perch oocytes develops asynchronously and are being spawned in batches. Similar oocyte development strategies have been reported in the European hake, *Merluccius, merluccius*, Atlantic mackerel, *Scomber scombrus*, and anchovies, *Engraulis spp.* (Hunter and Leong, 1981; Murua and Saborido-Rey, 2003).

The Nile perch exhibited a protracted spawning pattern. A portion of yolked oocytes was spawned in each batch, as evidenced by the group of post-ovulatory follicles and yolked oocytes in one ovary. Two types of spawning patterns, namely total spawning and batch spawning have been described by Tyler and Sumpter (1996). Batch spawning is a strategy seen to release eggs over a long time, during the spawning season. This has been reported to increase the probability of offspring survival (Lambert and Ware, 1984). It was also found among highly fecund species and is a strategy employed by fish species with physical limitations in body cavity space, due to increasing volumes of eggs (Fordham and Trippel, 1999) as is the case in Nile perch, where fecundity ranges from 2.0 to 15 million eggs (Ogutu-Ohwayo, 1988).

The knowledge generated on germ cell line specification in male and female *L. niloticus* ovarian follicle growth, maturation, ovulation, and gamete structure, is vital to many aspects of fisheries science and aquaculture, where gamete quality is paramount. This is also relevant in fisheries management and environmental science and conservation (Patiño and Sullivan, 2002). It also underlines the importance of using histology to understand reproductive cell biology and the reproductive strategies a species employs for maximum survival of the offspring. The identification of the stages of development and the quantifying of the reproductive investment that involve structural changes can only be done using histological investigations. The knowledge on the Nile perch sperm structure can be used in further taxonomical classification of the members of the family *Centropomidae*, by describing the type of sperm produced by *L. niloticus.*

# CHAPTER FIVE

## The use of clove oil to induce anaesthesia and its effects on blood plasma cortisol, glucose lactate and chloride levels in Nile perch, *Lates niloticus*

### Introduction

The handling of fish in aquaculture is one of the major causes of stress in fish. Stress can manifest as a result of handling and transport, injury or changes in water quality, often causing suppression of the immune system and mortality (Barton and Iwama, 1991). Some anaesthetics have been tested for their ability to reduce the magnitude of the stress response during capture, handling and transportation of fish (Marking and Meyer, 1985).

Stress is a non-specific response that can be divided into a primary, secondary and tertiary phase. The primary response involves activation and secretion of corticosteroids and the catecholamine adrenaline into the blood plasma via the hypothalamus-pituitary-interrenal axis and chromaffin cells (Reid et al., 1998; Mommsen et al., 1999; Flik et al., 2006). The secondary response can lead to the release of glucose and lactate into the blood plasma, generally followed by an increase in heart rate, blood flow to the gills and metabolism, and changes in haematocrit value (McDonald and Milligan, 1997). The tertiary stress response is a whole-body response in an attempt to maintain homeostasis.

The excretion of hormones associated with the stress response can reduce growth, disease resistance, or gamete quality and effect behavioural changes (Pankhurst and van der Kraak, 1997). Stress related endocrinological changes influence reproduction in fish (Pankhurst and van der Kraak, 1997). Hormones can interfere with vitellogenesis and oocyte development and

reduce the survival rate of eggs and larvae. Both acute and chronic stress can reduce the blood plasma level of androgens or oestrogens. For example, male brown trout exposed to handling and confinement for one hour, had changes in blood plasma concentrations whereby testosterone and 11-ketotestosterone levels decreased and cortisol levels increased (Sumpter, 1997). Androgens are precursors of oestrogens. Oestrogen, i.e., 17β-estradiol, stimulates hepatic synthesis of the yolk precursor vitellogenin, which is taken up into growing oocytes under the influence of gonadotropin hormones. Thus, factors that interfere with the metabolism of androgens can influence egg quality. Therefore, there is a need to reduce the stress response when immobilising fish during handling or spawning. This may be particularly relevant for large broodstock animals that have been taken from the wild and where handling is difficult and time-consuming.

Clove oil, with its active ingredient eugenol, or isoeugenol, tricaine methane sulfonate (MS 222), metomidate, benzocaine, and tobacco extracts have been tested as fish anaesthetics. Anaesthetics, i.e. benzocaine, MS-222, metomidate and isoeugenol can elicit a stress response in fish (Zahl et al., 2010). For example, Atlantic salmon, *Salmo salar*, Atlantic halibut, *Hippoglossus hippoglossus* and Atlantic cod, *Gadus morhua*, which had no association with stress from confinement or handling, generated a significant increase in cortisol levels excreted into the water after anaesthetics such as MS-222, metomidate, benzocaine, and iso-eugenol were added to the holding facilities, while the magnitude of the stress differed between species (Zahl et al., 2010).

Clove oil is a viscous liquid, extracted from the leaves, buds and stem of the *Eugenia caryophyllata* tree (Isaacs, 1983). The active ingredient is eugenol (4-allyl-2-methoxyphenol). It

is approved as a food substance, generally recognized as safe, and it has been approved as a fish anaesthetic in New Zealand, Australia and Chile (FDA, 2002, 2007; Wagner et al., 2002). It is easy to acquire at low cost, has a good efficacy, and it is not toxic to humans at the concentrations used for fish (Soto and Burhanuddin, 1995; FDA, 2007). Eugenol is rapidly metabolized and excreted by fish, and thus does not require a withdrawal time (Wagner et al., 2002). Concentrations of 0.02 to 20 μl $ml^{-1}$ clove oil have been reported to have antimycotic (Pinto et al., 2009) and antibacterial properties (Karapinar and Aktuğ, 1987; Hussein et al., 2000).

*Lates niloticus* is a valuable aquaculture species and food fish. Following studies of its biology and the exploitation of its natural population (Hopson, 1972; Ogutu-Ohwayo, 1988; Hughes, 1992; Ogutu-Ohwayo, 2004; Balirwa, 2007), efforts to farm this species have increased. *Lates. niloticus* is nocturnal and its glaring eyes suggest that it may be sensitive to light. It may thus become stressed by exposure to sunlight and air as a result of handling. It has not been tested whether anaesthetising Nile perch with clove oil can reduce the magnitude of the stress response. In addition, Nile perch broodstock is large and difficult to handle. The aim of this study was to establish the efficacy of clove oil as an anaesthetic agent and its effect on the magnitude of the stress response of *L. niloticus*.

**Methods and materials**

Fish were captured, using long line hooks, from Lake Victoria, as described in Chapter two of the thesis, transported in plastic tanks (1000 litre), and stocked in 1835 $m^2$ earthen ponds at Kajjansi Aquaculture Research and Development Centre, where they were kept for four months,

until the start of the experiments. Their weight ranged from 0.4 – 12.5 kg $fish^{-1}$ at an average of 2.08 ± 0.21 kg $fish^{-1}$ (n = 93) and total length ranged from 300 – 950 mm (524 ± 14.9 mm, n = 93).

The pond was drained and fish were captured, using a pond seine net. They were transferred into indoor concrete tanks (30 $m^2$), maintained at a water temperature of 25.4 ± 0.5°C and dissolved oxygen concentration of 6.1 ± 1.8 mg $L^{-1}$ using diffused medical oxygen (Oxygen (U), Ltd.). All fish were purged for 12 hours before being used in an experiment.

Experiment 1 was designed to estimate the correlation between the clove oil concentration and the time required to reach defined stages of anaesthesia. Fish were exposed to four concentrations of clove oil with eight to nine fish per concentration. Fish within a treatment were randomly selected and tested individually to ensure independence of data. Because of low solubility in cool water, clove oil (Bell, Son and Co., Ltd, Southport, England) was dissolved in 95% ethanol at a ratio of 1 volume of clove oil to 9 volumes of ethanol. Kaiser et al. (2006) reported ethanol concentrations as high 30µl/L had no anaesthetic effect in Lake Victoria cichlids. The stock solution was made daily and kept dark at room temperature, to avoid photo degradation of the active ingredient (Soto and Burhanuddin, 1995). Aliquots of the stock solution were used to achieve the desired concentrations of clove oil of 25, 50, 75 and 100 µl $L^{-1}$ in the water, before the fish were placed into the tank. Observations made prior to the experiments suggested that exposure of *L. niloticus* to the concentration of ethanol, that was used in the experiments, did not sedate fish or induce anaesthesia, or cause any noticeable modifications in the behaviour of the fish. Immediately after a fish was placed into the tank, the time to reach each of the anaesthetic stages defined in Table 1 was recorded.

The time it took for fish to recover from anaesthesia, for example, as the fish gained total balance, was recorded. Control fish were placed into a tank with the same volume of water without clove oil. Fish that reacted to handling attempts were left in the anaesthetic solution and touched on the caudal peduncle every 15 seconds until no reaction was noticed. Length and weight were measured on anaesthetised fish and the fish were placed into an aerated tank to recover. No fish was used twice, as previous exposure to anaesthesia may bias results due to potential habituation to the anaesthetic (Weyl et al., 1996).

Experiment 2 was designed to test the efficacy of clove oil and its effect on blood values at 10, 25, 50, 75 and 100 μl $L^{-1}$ clove oil. Fish in the control treatment were kept and treated in the same way, but without the addition of clove oil. To take blood samples, anaesthetized fish were removed from the tanks and blood was drawn within 60 seconds from the caudal vein complex, using 5-ml syringes. Blood was transferred to 2-ml Eppendorf tubes and plasma was separated by centrifugation (5000 rpm), for five minutes. Blood plasma was kept at -20°C until used for analyses. In the control fish, after capture with a scoop net, a wet towel was placed on the head to cover the eyes, as a way restraint.

**Table 5. 1:** Behaviour of fish at different stages of anaesthesia (Summerfelt and Smith, 1990; Stoskopf, 1993)

| **Stage of anaesthesia** | **Description** | **Behaviour** |
|---|---|---|
| 0 | Normal | Reacts to external stimuli; no change to opercular movement and muscle tone |
| 1 | Light sedation | Slight loss of reaction to stimuli; slight decrease in opercular movement; equilibrium normal |
| 2 | Deep sedation | Total loss of reaction to all but strong stimuli; small decrease in opercular movement; equilibrium normal |
| 3 | Partial loss of equilibrium | Partial loss of muscle tone, swimming erratic; increased opercular beat; reactive only to strong tactile stimuli |
| 4 | Total loss of equilibrium | Total loss of muscle tone and equilibrium; slow opercular rate; loss of spinal reflexes |
| 5 | Loss of reflex reactivity | Total loss of reactivity; opercular movements slow and irregular; heart rate slow; loss of all reflexes |
| 6 | Medullary collapse | Opercular beat ceased; cardiac arrest and death. |

The aim of Experiment 3 was to test the effect of prolonged exposure to low concentrations of 2.5, 5, and 10 µl $L^{-1}$ clove oil on the blood chemistry of *L. niloticus*. Fish were randomly assigned to twelve 400-L tanks. Blood was taken from each fish at the peduncle blood system using a hyperinised 5 ml syringe and a needle (gauge 22) at 0 hours, i.e., before application of clove oil, and at two 4-hour intervals thereafter.

*Quantification of plasma glucose, lactate, Chloride and cortisol concentrations*

Glucose concentration was measured, using a blood glucose system (COBAS INTEGRA400 PLUS). A two-step reaction series was carried out; first step hexokinase was used to catalyse the phosphorylation of plasma glucose. This was followed by second enzyme reaction glucose-6-phosphate dehydrogenase (G6PDH) to catalyse oxidation of glucose-6-phosphate by $NADP^+$ to form NADPH. The concentration of NADPH in the plasma is directly proportional to the glucose concentration. NADPH was determined by measuring increase in the absorbance at 340 nm.

Anaerobic glycolysis increases blood lactate and caused some increase in pyruvate levels especially in prolonged exercises. Plasma lactate levels were determined using an assay (Lactate Gen.2 using a COBAS INTEGRA400 Roche Germany). Plasma lactate was oxidised to pyruvate by a specific enzyme lactate oxidase. Peroxidase was used to generate a coloured compound using hydrogen peroxide during the first reaction. The intensity of the colour formed is directly proportional to the lactate concentration. The increase in absorbance was measured at 552 nm using COBAS INTEGRA400. Plasma chloride concentration was determined using automated Cobas u 411 analyzer from Roche, Germany using the above laboratory.

Blood plasma cortisol concentration was quantified, using competetive enzyme-linked immunosorbent assay validated for fish by Velaso-Santamaria and Casallas (2007). Plasma cortisol assay was conducted using Elecsys Cortisol assay at Ebenezer Clinical Laboratory Kampala Uganda using automated Elecsys and cobas e 411 immunoassay analysers Germany. According to the specification of the Cortisol ELISA kit; cobas e 411, the cross-reactivity with other steroids for every substance added per 10µg/ml was 5.8% with corticosterone, 0.04% with cortisol-21-sulfate, 0.30% with cortisone, 0.69% with 11-deoxycorticosterone, 4.1%, with 11-deoxycortisol, 0.08% with dexamethasone 1.50% with 17-α-hydroxyprogesterone, estradiol, 0.28% with prednisone and 0.35% progesterone. The intra- and inter-assay precision was 2.45% (n = 10) and 3.58% (n =10) (coefficient of variation, CV) respectively, and the limit of sensitivity (minimum detectable dose) was 0.51 nmol $l^{-1}$and values above detectable range were 1750 $nmoll^{-1}$ for ten-fold diluted samples. The analysis was carried out in compliance with the assay procedure of the kit.

The time required to reach a particular stage of anaesthesia (Experiment 1) was estimated as a function of clove oil concentration and fish weight, using least-square regression analysis. In some cases, variables were log-transformed to achieve normality of residuals and reduce heterostadicity. A combination of the t-statistics used to test the contribution of independent variables to the model, the coefficient of variation ($r^2$) and the distribution of residuals, were used to decide on the best model. Least-square regression analysis was used to relate blood values to clove oil concentration (Experiment 2). In order to test the combined effects of exposure duration and concentration (Experiment 3) of clove oil on blood chemistry levels, multiple analysis of variance, including a test for interaction between the two main effects

"concentration" and "duration of exposure", was used with fish weight as a covariate. Effects were considered significant at a p-value < 0.05.

**Results**

Immediately after the addition of clove oil, fish exhibited a short period of burst swimming, before settling. This was followed by a stage during which the fish calmed down as they reached a light sedation. As the fish entered the phase of deep sedation, a coughing reflex was observed in all fish exposed to clove oil, irrespective of the concentration.

All fish exposed to different clove oil concentrations reached total equilibrium loss within 10 minutes of exposure time, with the exception of those exposed to a low concentration (10 µl $L^{-1}$), in which instance the fish remained in the normal position beyond 13 minutes of exposure to the anaesthetic. Induction times were more rapid at high clove oil concentrations, with fish treated with concentrations of greater than 25 µl $L^{-1,}$ exhibiting significantly shorter induction times of less than 180 seconds (Tukey's test; $F_{(3, 29)} = 33.7$, $p < 0.0001$) (Fig. 5.1). No fish in the clove oil concentration of 10 µl $L^{-1}$ reached stage five of anaesthesia; the fish remained stable and did not lose their equilibrium.

The time required to reach recovery was independent of the clove oil concentration (least-square regression analysis, $p = 0.17$) and the duration of anaesthesia stage 5 ($p = 0.37$). On average, fish recovered within 673 ± 58 (n = 32) seconds, with values ranging from 274 – 1653 seconds. Two of the 32 fish recovered after 1200 seconds. Averages and confidence intervals of times to reach stages of anaesthesia, are given in Figure 5.1, while the relationship between these variables and clove oil concentration was modelled, using least-square regression analysis (Table 5.2).

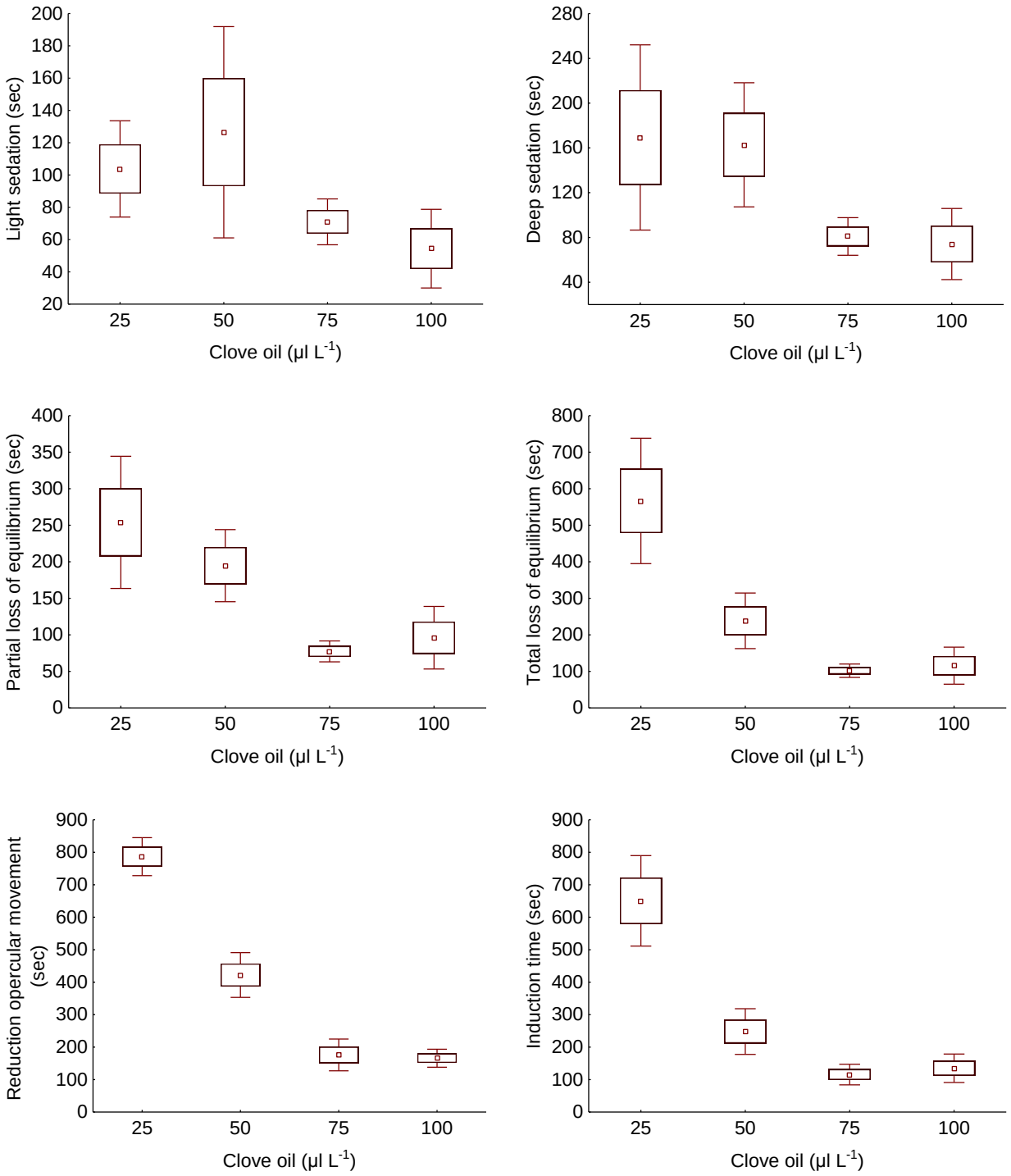


**Figure 5.1:** Average, standard errors (box) and 95% confidence intervals (bars ± standard error) of the time to reach any of six stages of anaesthesia at four concentrations of clove oil

**Table 5.2:** Time (seconds) to reach the six stages of anaesthesia, i.e., light sedation (LS), deep sedation (DS), partial loss of equilibrium (PL), total loss of equilibrium (TL), time to reduction of opercular movement (OP), and induction time (IT), could be estimated as a function of clove oil concentration (C, μl $L^{-1}$) and fish weight (W kg $fish^{-1}$)

| Least square regression model | Coefficients of the slope of the model (Ho: $\beta = 0$, Ha: $\beta \neq 0$) | |
|---|---|---|
| | p-value | Coefficient of correlation |
| Log (LS) = 2.15 – 0.005 C | p = 0.01, | $r^2$ = 15.6% |
| Log (DS) = 2.31 – 0.006 C + 0.30 log (W) | p = 0.0001, | $r^2$ = 36.0% |
| Log (PL) = 2.54 – 0.008 C + 0.31 log (W) | p < 0.00001, | $r^2$ = 61.6% |
| Log (TL) = 4.41 – 1.24 log (C) | p < 0.00001, | $r^2$ = 64.2% |
| OP = 2217 – 1078 log (C) + 146.3 log (W) | p < 0.00001, | $r^2$ = 89.0% |
| Log (IT) = 4.667 – 1.39 log (C) + 0.03 W | p < 0.00001, | $r^2$ = 77.3% |

Data within each treatment were subjected to statistical test and no significant differences (P = 0.067, n = 12) were observed within treatment implying that the net may have not effected the blood parameters studied in first, second and so on fish scooped out of the tank. The blood plasma cortisol concentration (Ct) was negatively correlated to the clove oil concentration (C) ($r^2$ = 26%, Figure 5.2) and could be estimated using the model Ct = 262.0 - 1.91 C. Fish weight did not contribute significantly to this model (multiple regression, p = 0.79). The regression model was L = 3.38 + 0.031 C ($r^2$ = 28%, p = 0.024, Figure 5.2), with fish weight not significantly contributing to the model (p = 0.31). The correlation between the level of blood glucose (Gl) and

clove oil concentration was ($r^2$ = 36%, p = 0.0087) and could be estimated using the model Gl = 6.41 + 0.067 C (Figure 5.2). This estimation was independent of fish weight (p = 0.34). There was no correlation between blood chloride levels and the clove oil concentration (p = 0.59) and therefore these data are not shown in Figure 5.2.

Results from Experiment 3 indicate that tests of the combined effect of the two main effects; “low clove oil concentration” and “duration of exposure” and their interaction on the blood plasma level of cortisol, glucose, lactate and chloride concentrations, using fish weight as a covariate, suggested no effect of clove oil concentration on any of these variables (cortisol, p = 0.35; glucose, p = 0.51; lactate, p = 0.71, and chloride, p = 0.46).

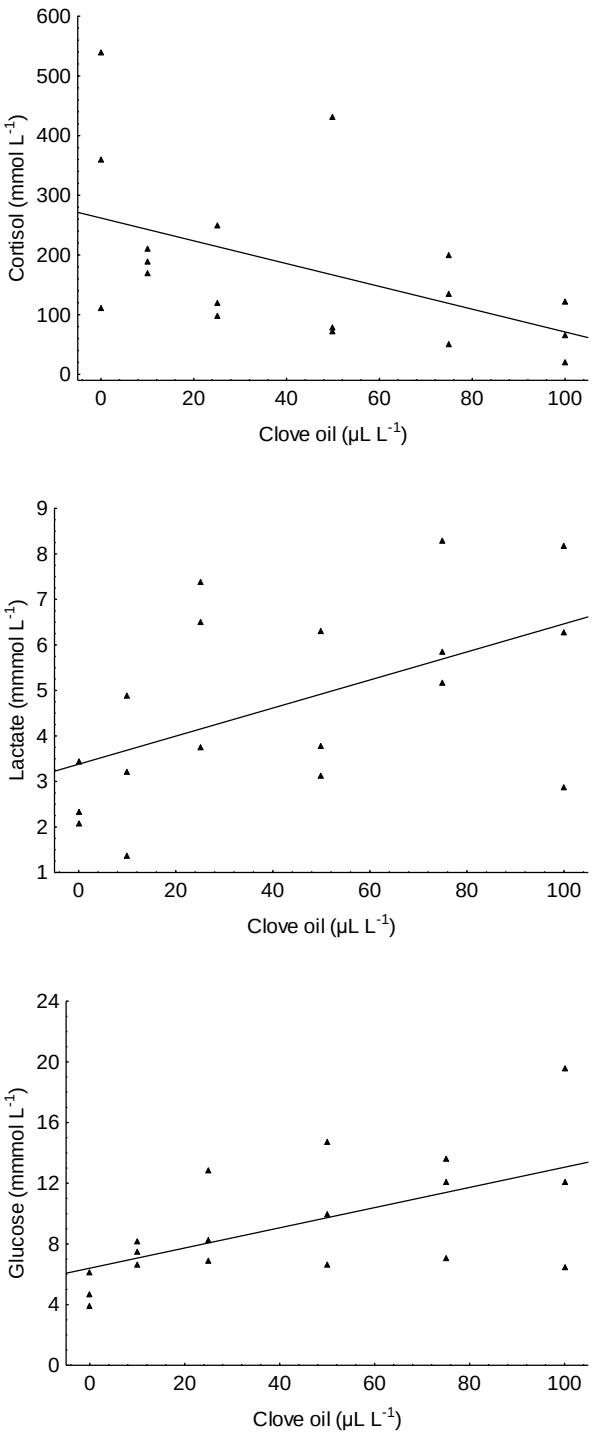


**Figure 5.2:** The relationship between clove oil concentration and blood chemistry in *L. niloticus*. Regression models and their coefficients are given in the text

Fish weight was not a significant covariate in any of the tests ($p > 0.5$), and there was no significant interaction between the main effects for any of the variables tested ($p > 0.5$). Only lactate and chloride concentrations changed over time, independent of clove oil concentration ($p = 0.0006$, and $p = 0.0001$, respectively). Post-hoc, multiple comparisons of means of lactate concentration showed a significantly increased average (11.41 mmol $L^{-1}$) after eight hours, while this value averaged 8.8 and 7.0 mmol $L^{-1}$ at 0 and 4 hours, respectively. Chloride concentrations were highest ($p < 0.00002$) at 0 hours (98 mmol $L^{-1}$) and significantly lower after 4 and 8 hours (85.5 and 79.8 mmol $L^{-1}$), respectively.

**Discussion**

Clove oil was an effective anaesthetic for the handling of Nile perch, *L. niloticus*, of sizes varying from 0.5 - 12 kg $fish^{-1}$. Concentrations that met the efficacy criteria for handling within three minutes and recovery in 10 minutes (Son, et al., 2001) were 75 and 100 µl $L^{-1}$. Fish anaesthetized with 25 µl $L^{-1}$ could be measured or weighed easily, although these fish sometimes exhibited reflexive action during handling. A clove oil concentration of 50 µl $L^{-1}$ appeared to be sufficient for fish measurements and the stripping of gametes.

Clove oil was an effective sedative for *L. niloticus* at a concentration greater > 25 µl $L^{-1}$. The fish entered behavioural Stage 4 within four minutes in concentrations of 50–100 µl $L^{-1}$ clove oil. Deep anaesthesia in *L. niloticus* could be induced in less than four minutes, and clove oil concentrations of 75-100 µl$L^{-1}$ may be required to induce surgical anaesthesia in *L. niloticus*. In red pacu, *Piaractus brachypomus*, approximately five minutes were needed to enter stage 4 at 50 mg $L^{-1}$ eugenol (Sladky et al., 2001). For rainbow trout broodstock, Iversen et al. (2003) reported 35-40 mg $L^{-1}$, and Keene et al. (1998) reported that a dose of 40–60 mg $L^{-1}$ eugenol induced

rapid anaesthesia in *Onchorynchus mykiss*. Roubach et al. (2005) reported that in *Colossoma macropomum,* 65 mg $L^{-1}$ eugenol sufficed to induce anaesthesia, while Yamanaka et al. (2011) suggested 50 mg $L^{-1}$ of clove oil to be adequate to induce anaesthesia in a 1.5 kg common carp, *Cyprinus carpio*. Prince and Powell (2000) recommended low doses (30–40 mg $L^{-1}$) to anaesthetise adult rainbow trout. Differences in clove oil efficacy reported in this study and other studies by different workers, might be due to differences in the ontogenetic stages used, the species of fish or the weight of the test fish. For example, results from the present study suggested an influence of fish weight on the time required to reach anaesthesia. This implies that larger fish would require a longer time to reach anaesthesia than smaller fish.

Clove oil concentrations influenced blood plasma, glucose, lactate and cortisol in *L. niloticus.* The inverse correlation between the clove oil concentration and the blood cortisol concentration suggests that clove oil did not elicit a physiological stress response in this fish. Fish will exhibit an increase in the plasma cortisol level in response to stressors, and this is generally followed by an elevation in the plasma glucose levels (Iwama et al., 2004). In this study, clove oil caused a reduction in the blood cortisol level, while there was slight increase in the blood glucose level as clove oil concentrations increased. This may be attributed to the continued confinement of the experimental fish in tanks. Costas et al. (2008) also observed an increase in plasma glucose concentration whilst cortisol was impaired in Senegalese sole, *Solea senegalensis* exposed to crowding stress. Future studies should test to what extent the fasting of fish prior to the experiments can have on glucose levels. Costas et al. (2008) suggested that the increase in

plasma glucose may be due to high energy required to cope with the synthesis of vital metabolites that are related to stress response.

The second part of this study was to evaluate the effects of prolonged exposure to clove oil anaesthetic on plasma glucose, lactate, and chloride and cortisol concentrations. Increase in plasma cortisol concentration has been used as a sign of stress in fish (Barton, 2002). Exposure of *L. niloticus* to clove oil, for up to eight hours, did not influence the blood plasma cortisol, glucose and lactate concentration. Similarly, Iversen et al. (2003) showed that Atlantic salmon did not have increased plasma glucose levels when exposed to a range of concentrations of clove oil. Palićе et al. (2006) reported that eugenol did not induce a stress response in fathead minnows, *Pimephales promelas.* Maricchiolo and Genovese (2011) reported a blood glucose concentration increase in greater amberjack anaesthetised with clove oil. Sladky et al. (2001) reported that the blood glucose concentration increased quickly in red pacu, *Piaractus brachypomus,* anaesthetised with MS-222 or clove oil. They hypothesized that, similar to what occurs in mammals; the sudden increase of glucose levels was due to catecholamine induced gluconeogenesis. However, a short-lived decrease in blood glucose concentration was observed in the rainbow trout, *Salmo gairdneri,* immediately after induction of MS-222 anaesthesia (Soivio et al., 1977).

Cortisol has been suggested to be one of the mediators causing an increase in plasma glucose levels as part of the physiology of the stress response (Barton *et al.*, 2000). It may be possible that the lack of change of glucose, lactate and ionic chloride concentration in response to clove oil, was due to the reduced levels of the cortisol response by clove oil.

Barton and Iwama (1991) proposed that an increase in the plasma cortisol concentration in fish is a first-order reaction associated with the stress response, often leading to an increase in glucose levels. Mildly sedating doses of anaesthetics such as MS222, used widely in aquaculture for handling fish, have been reported to cause elevations in plasma cortisol, similar to those normally associated with acute stress, while higher concentrations did not (Barton and Peter, 1982; Iwama et al., 1989).

In conclusion, the results suggest that clove oil was an effective anaesthetic, with 75 - 100 μl $L^{-1}$ inducing anaesthesia and recovery within the recommended time. Clove oil at high concentrations did not elicit stress responses. As a result, a dose of 50 μl $L^{-1}$ is suggested as a practical, minimum concentration of clove oil to handle Nile perch of size 0.5-12 kg $fish^{-1}$. A clove oil concentration of 5 - 10 μl $L^{-1}$ was adequate for sedating Nile perch without eliciting an increase in the cortisol level. Considering that an increase in blood plasma cortisol concentration has generally been associated with a reduction in gamete quality, testing its effect on blood chemistry in *L. niloticus* has practical applications for its aquaculture potential. *Lates. niloticus* broodstock is difficult to obtain and to keep in tanks as the fish are large and sensitive to environmental conditions and handling. Thus, they need to be sedated for artificial spawning. The findings suggest which clove oil concentrations can be safely used in this species for a given weight range, and that clove oil at the concentrations used herein may reduce that part of the stress response that results in elevated cortisol levels.

# CHAPTER SIX

## Cyto-histological examination of ovarian follicle maturation and follicular structural changes in Nile Perch, *Lates niloticus,* injected with a gonadotropin release hormone analogue and a dopamine antagonist (GnRHa + MET)

### Introduction

Nile perch, *L. niloticus,* is a prime food fish species for fisheries and a target species for aquaculture in Uganda. The species' total length at 50% sexual maturity has been estimated to be 59.4 cm in females and 57.8 cm in males (Chapter three of this thesis). As Nile perch populations in various water bodies have become exploited, the commercial Nile perch fishery has declined, due to ineffective fisheries management. As a result, the numbers of mature Nile Perch have decreased (LVFO, 2007; Njiru et al., 2008). There is a need to develop technologies such as induced spawning so as to initiate production of Nile perch in aquaculture. For this to happen, an understanding of processes such as ovarian follicle growth and oocyte maturation, which leads to the production of fertile eggs and how their development is controlled by gonadotropins, is needed.

*Ovarian follicle maturation (OFM)*

Control of reproduction under captive conditions is vital for aquaculture where the life cycle of the species is closed and for the captive breeding of exploited fish stocks. In many fishes, this is achieved by manipulating environmental variables such as photoperiod, temperature or the availability of spawning substrate (Mylonas et al., 2010). Secondary oocyte growth culminates in the formation of tertiary yolk vesicle oocyte formation, with high yolk content. In some species such as common carp, *Cyprinus carpio,* in temperate environments, oocytes mature early in

winter and do not undergo final oocyte maturation until the next spring (Sivakumaran et al., 2003). Thus, final oocyte maturation is triggered by environmental stimuli, in this case by changes in water temperature. These aspects of eco-biology of many fish species are not well understood. It has therefore been difficult to simulate conditions in captivity that moderate oocyte maturation and ovulation. In such species, hormonal manipulations may be employed to induce final oocyte maturation (Mylonas et al., 2010). Various sense organs perceive external stimuli and the information is transferred to the hypothalamus, where it triggers gonadotropin-releasing-hormone (GnRH) production.

In response to circulating GnRH, the pituitary gland synthesises and secretes the gonadotropin hormone GTH-I (follicle stimulating hormone), and GTH-II (luteinising hormone) in teleosts (Yoshizaki, et al., 2001, Mylonas et al., 2010). Luteinising hormone (GTH-II) stimulates the ovarian follicle cells to produce maturation inducing hormone (MIH). MIH, a steroid hormone, moderates the final stages of maturation leading to ovulation (Patiño et al., 2001, Patiño and Sullivan, 2002). Thus, GTH-II plays a key role in inducing ovarian follicle maturation (OFM) processes by binding to a specific receptor on the granulosa cells of the follicle and by stimulating a series of events that include the acquisition of oocyte maturation competence, production of maturation inducing hormone (MIH), and resumption of meiosis and cytoplasmic maturation (Mylonas et al., 1997, Patiño et al., 2001, Patiño and Sullivan 2002).

In order to efficiently induce OFM and spawning of both captive and wild brood fish, and also to produce viable eggs of good quality, hormonal induction is done when fish have completed the vitellogenic phase of oocyte growth before onset of oocyte atresia (Mylonas et al., 1997). It is vital therefore, to identify morphological parameters that can be used to monitor the progress of ovarian follicle maturation events. Furthermore, it is important to understand OFM and how the

process is regulated by hormonal influences, so as to be able to manipulate and time spawning under captive conditions.

Histology of pre-vitellogenic and vitellogenic oocytes has been described for *L. niloticus* in Chapter four, and by Basiita et al. (2011). This information provided the foundation for this research. No microscopic morphology of oocytes from Nile perch females undergoing OFM has been documented. The analysis of embryonic developmental stages provides the possibility to identify critical developmental stages and this information can be used as a tool for developmental definition in morpho-anatomic abnormalities (Estêvão et al., 2005) in fish culture conditions.

Histological descriptions of OFM and its comparison with macroscopic descriptions, such as gonad stages, are not available for the Nile perch. Such information is an important tool for timing of ovarian follicle maturation manipulation. Induction of OFM must be timed very well. For instance, early induction may be ineffective, potentially leading to atresia while late induction is likely to reduce fertility and offspring survival (Miranda et al., 1999; Palstra et al., 2005). Therefore, ovarian follicle and oocyte structural changes at the end of vitellogenesis (post-vitellogenesis) in Nile perch, subjected to GnRH via injection into the bloodstream, was documented in this study. In addition, OFM was examined in histological details in female *L. niloticus*, treated with gonadotropin-releasing hormone analogue (GnRHa), combined with an antagonist to the GnRH-inhibiting factor. This chapter describes the ovarian follicle maturation and egg structure in wild-caught *L. niloticus*, with the aim of understanding and providing information on artificial spawning of the species.

## Materials and methods

*Collection of broodstock*

Brood fish with a weight ranging from 3.6 to 18.3 kg, were caught, using gill nets (8-12 cm stretched mesh size) in combination with long line hooks (6" – 9" size), from Lake Victoria. The brood fish were kept in 100 $m^2$ circular tanks, supplied with raw lake water, at Interfish Fish Farm on the shores of Lake Victoria and the Kajjansi Aquaculture Research Development Centre. The time for the collection of fish was chosen based on temporal variations in the gonadosomatic index and the presence of oocytes in advanced stages of development, as determined in Chapter three of this thesis. The ripe females were captured from two locations, W*ayia* Bay, and an area between Buvu and Busi Islands of Lake Victoria (see Figure 2.1 in Chapter two of this thesis), in October 2010 and November 2010, when the moon was in the last and in the first quarter of the lunar phase, respectively. Water depth and temperature in these areas varied between 2.0-5.5 m and 23-25$^{o}$C, respectively. The dissolved oxygen ($O_2$) concentration ranged from 2.93 to 3.32 mg/L. Adult *L. niloticus* appears to migrate from deep waters to shallow waters in the sheltered bays or in rocky shallow waters during the last and the new lunar phases (own observation). Fishing activities of fishermen and the migration of fish between fishing grounds that are associated with water depth, are synchronized with the lunar phases.

*Broodstock selection and hormone application*

Prior to hormone administration, fish were tested for their readiness to spawn by sampling eggs from the genital pore, using a rubber cannula of 1.2 mm in diameter. The quality of the eggs was assessed qualitatively, based on colour (yellow to orange eggs), uniformity in size, lack of blood

and floating ability. The samples were treated with SERA's fluid (ethanol: formaldehyde: acetic acid 6:3:1 *v*/*v*) and observed under a light microscope.

*Hormone administration*

Fish were anaesthetised with clove oil 50 µL/L (eugenol) (Chapter five of this thesis), and weighed, prior to intramuscular injection of the ovulation-inducing agent between the dorsal fin and the lateral line. One inducing agent, traded as Dagin ([D-Arg6, Pro9-NEt]-sGnRH), combined with 20 mg/kg of powder, water-soluble dopamine receptor antagonist metoclopramidee (GnRH + MET) (Dagin purchased from Hebrew University, Israel and stored at 4$^{o}$C), was administered to ripe female Nile perch at a concentration of 10 µg kg$^{-1}$ body weight (Almendras, et al., 1988; Forniés et al., 2001). A two-stage hormone administration was used, i.e., a priming dose, as a tenth of the total dose and the remaining dose was given twelve hours later. Control fish were injected with physiological saline that was equivalent to the volume of the hormone solution administered. The induced fish, including the control fish, were kept individually in a tank covered with a dark tarpaulin material and aerated throughout the experimental time. Because of scarcity of broodstock, the fish were tested sequentially. The study lasted two months.

*Histology*

Weak and lethargic fish were euthanized with a high concentration of clove oil and the ovaries were removed and fixed in Bouin's solution for 48 hours. They were then dehydrated in a graded ethanol series (50%, 75%, 95% and 95%) for 15 minutes at each grade, followed by two 30-minute washes in 100% ethanol, and tissue samples were infiltrated and embedded in paraffin wax. Serial sections of 5 µm thick were stained, using Haematoxylin-Eosin (HE), and examined

under a light microscope. Images were captured using a digital camera (Olympus DP72 - Japan), attached to a microscope (Olympus BX50, Japan) and the images were digitally stored for analysis, using AnalySIS Imaging Software.

Thirteen female fish were used in the experiment. Ten fish were injected with the GnRH + MET hormone preparation and three with physiological saline. Fish were checked for ovulation after six hours by applying gentle pressure on the lower abdomen. Eggs were stripped and fertilised, using dry fertilisation and washed of excess milt obtained by stripping males. Obtaining eggs by stripping was rather difficult due to the large, strong muscles in the fish abdomen. Males were not induced since they released milt upon gentle pressure to the abdomen.

*Egg incubation*

Eggs were incubated in circular asbestos tanks that had been disinfected using 10% formalin, followed by sodium chloride, prior to adding water. Fertilization success was measured based on the number of eggs with evidence of cell division after five hours. In this experiment, fertilization success was estimated at 20%. The fertilized eggs were incubated in three tanks of 3 $m^3$ capacity, at a water temperature adjusted to an average of 26 ± 1.8$^oC$. Water was heated and mixed with cool water in a mixing tank (3000 L) and was maintained at 30 ± 1$^oC$. Water quality was monitored every two hours. The dissolved $O_2$ concentration ranged from 2.5 to 6.0 mg/l.

Egg development was observed once per hour by collecting a sample from the incubating tanks. The eggs were placed on a watch glass and observed under a light microscope (Zeiss) at x40 magnification, using the wet mounting technique. All eggs were examined; egg diameter and oil globules were measured and recorded every two hours. Egg images were captured using a digital

camera (Kodak Easy Share M763 7.2 mega pixels) and digitally stored. Egg size was measured using a stage micrometer, mounted on the microscope, at the magnifications stated above.

*Statistics*

This study was constrained by a scarcity of broodstock fish, due to severely declined Nile perch stock as a result of the fishery in Lakes Victoria, Kyoga, Albert, and Nabugabo. As a result, brood fish treatment could not be replicated in time. This is the reason that descriptive statistics was chosen to illustrate the results. A one sample t-test statistic was used to compare *L. niloticus* egg size with marine species with floating eggs that contained an oil globule.

**Results**

The mean diameter of oocytes before GnRH-Met injection was 550 ± 42 μm, n = 125. Post-vitellogenic oocytes were yellow to orange and they were opaque and granular (Fig. 6.1b). Histological examination of the gonads from the control fish did not show any egg structural changes (Fig. 6.1a). Among the control fish (n = 3) treated with saline solution, none showed signs of final oocyte maturation.

*Follicular oocyte maturation of GnRHa induced Nile perch*

Of the ten fish injected with GnRHa hormone, nine fish commenced ovarian follicle maturation, one fish ovulated and one fish had oocytes undergoing atresia. Of the nine fish that commenced oocyte maturation, eight died within 18 hours after handling. Final oocyte maturation began with lipid-droplet coalescence and was followed by germinal vesicle (GV) migration from the centre to the periphery of the oocyte (Fig. 6.2a). Only the largest oocytes (457 to 550 μm), with a high yolk globule content and large lipid droplets responded to the hormonal treatment. The GV and

yolk globules moved to the periphery of the oocyte as the centrally positioned oil globule expanded (Fig. 6.2b). In late oocyte maturation the germinal vesicle (GV) was seen juxtaposed with the oocyte plasma membrane while maintaining close contact with the enlarging oil globule and it was squashed between the two structures while remaining intact (Fig. 6.2c). The oil globule covered 20-33% of the egg size at the end of oocyte maturation. The large, central oil globule appeared as a round empty space in the centre of the maturing oocyte (Fig. 6.2c, d). Furthermore, the yolk globules did not liquefy during the process of final oocyte maturation, but enlarged and remained granular, occupying the peripheral areas of the oocyte. Throughout maturation, the oocyte diameter remained within the range of 457 to 550 µm.

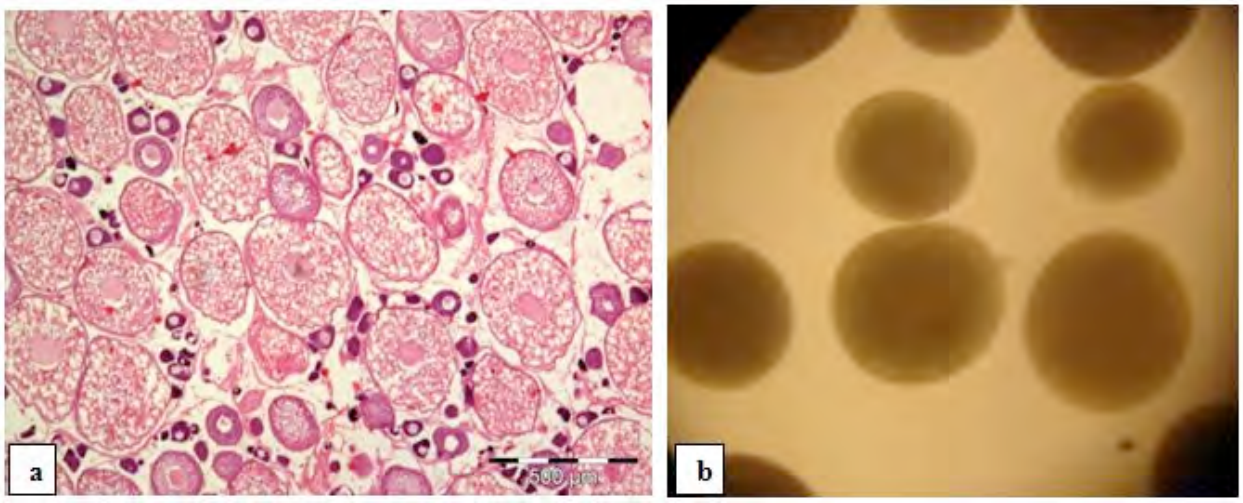


**Figure 6.1:** Histological section and light microscopy micrographs of post-vitellogenic oocytes in *L. niloticus,* induced with normal saline (control); a) histological section with all stages of oocyte development, b) vitellogenic oocytes from ovarian biopsy

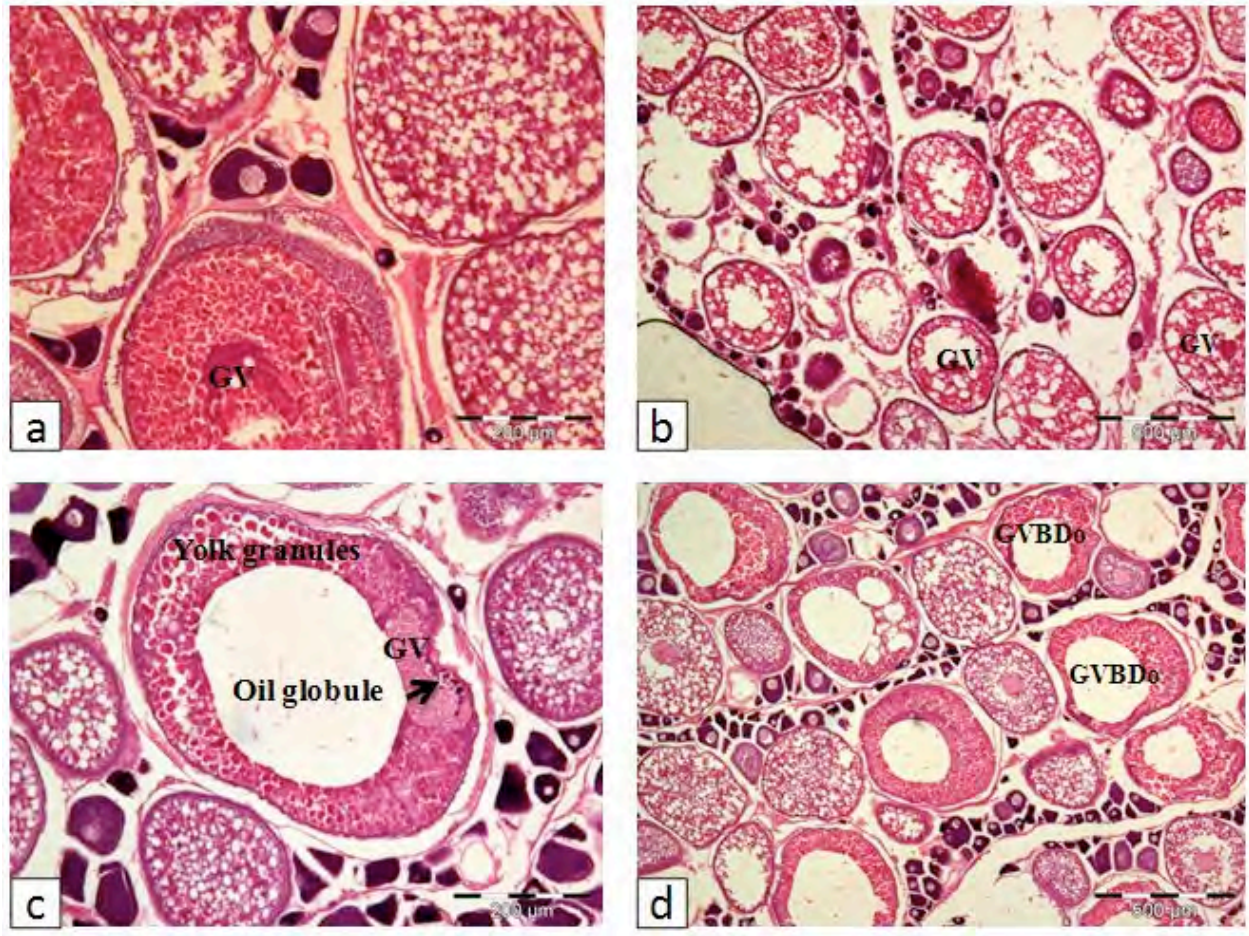


**Figure 6.2:** Histological sections of GnRHa-induced, wild-caught *L. niloticus* broodstock undergoing final oocyte maturation; a-b) early oocyte maturation (centre GV); c) oocyte with fully formed oil globule with periphery GV; d) oocytes undergoing germinal vesicle breakdown, GV= germinal vesicle, black arrow head = multiple nucleoli, GVBDo = germinal yolk vesicle breakdown oocyte

*Follicular layer changes following hormonal induction*

Structural changes in the oocyte follicular layers occurred in all fish induced with GnRHa + MET. No structural changes occurred in any of the follicular envelops of control fish. These changes included hypertrophy of the region between the theca cells and the basal lamina (Fig. 6.3a). The theca cells appeared like threaded beads and no changes in basal lamina structure occurred (Fig. 6.3b). The follicle cell layer was hypertrophied and this reaction was localized to one or two points on the maturing ovarian follicle (Fig.6.4a).

Following hormonal stimulation, only oocytes that had reached full vitellogenesis responded by entering into maturation. There were two batches of fully grown oocytes. One type did not respond to the hormonal GnRH-MET stimulation, while other showed structural changes. Among the hormone-responsive category, three subcategories could be distinguished. The first showed signs of lipid coalescence, followed by germinal vesicle migration to the periphery of the oocyte. The second category responded by their follicular layers undergoing both hyperplasia and hypertrophy but proceeded to enter maturational stages, and in the third category, oocytes did not enter maturation but progressed to atresia (Fig. 6.4). In some fish (n = 2), all oocytes underwent atresia, regardless of their stage of oocyte development (Fig. 6.5).

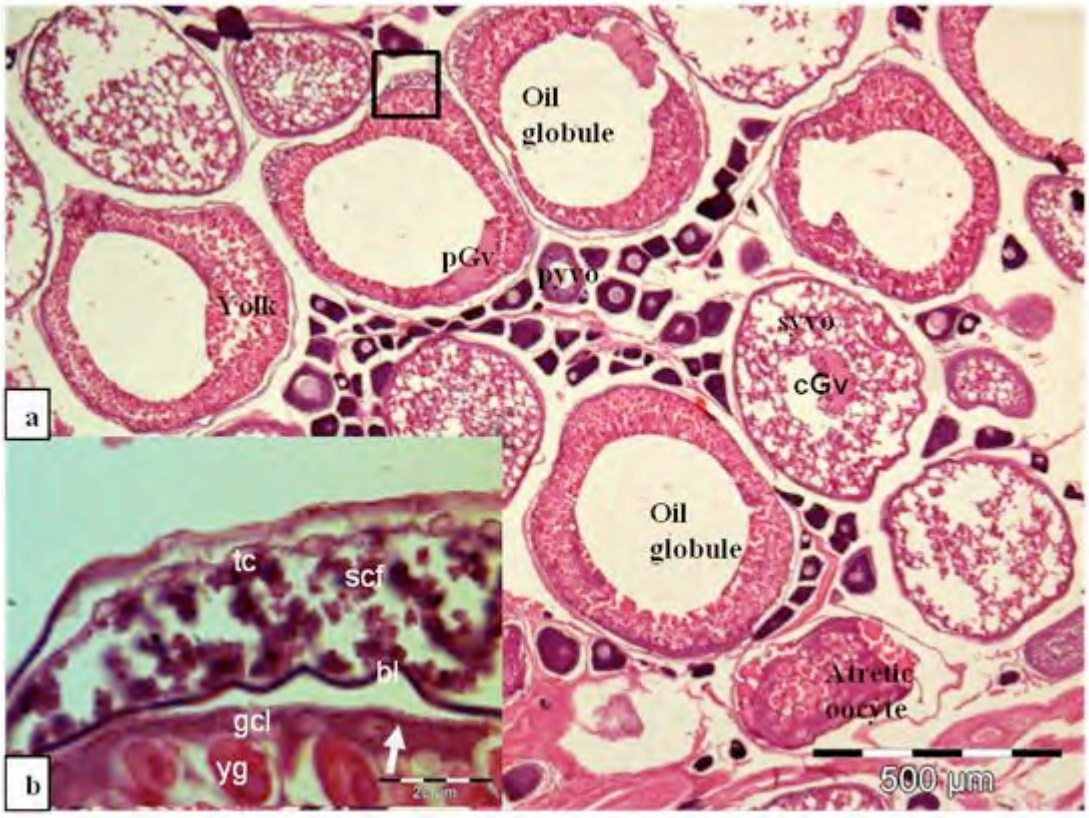


**Figure 6.3:** Histological micrographs showing follicular layer changes to GnRH injection a) ovary with maturing oocytes, b) hypertrophic collagen fibres and intact theca cells (appearing as threaded beads; pGv = periphery germinal vesicle, cGv = central germinal vesicle, syvo = secondary yolk vesicle oocyte, pyvo = primary yolk vesicle oocyte, tc = theca cells, scf = swollen collagen fibres, bl = basal lamina, arrow = granulosa cell layer, arrow head = zona radiata, and yolk granule. (The black square above indicates an area that was magnified to give figure 6.3b)

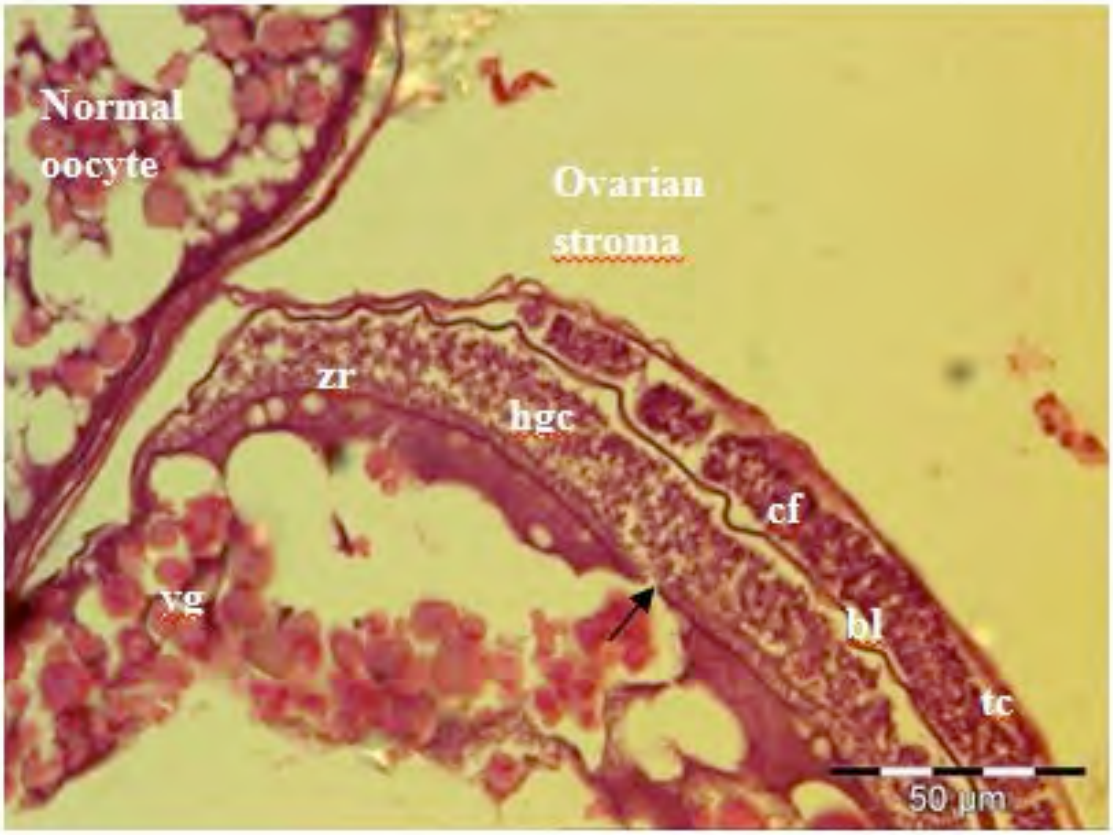


**Figure 6.4:** Histological section of post-vitellogenic oocytes; normal and hyperplasic and hypertrophic, follicular cell layer and collagen-filled space in induced wild caught *L. niloticus* brood fish. Arrow indicates fragmenting hypertrophic *zona radiata*; tcl = theca cell layer, bl = basal lamina, yg = yolk globule, scfl = swollen collagen fibre layer, hgcl = hypertrophic granulosa cells, and zr = *zona radiate*

*Atresia*

Several morphological changes were observed in the ovarian follicles. These changes included germinal vesicle membrane and follicular cell layer disintegration. Also numerous apoptotic bodies that stained purple with Haematoxylin and Eosin were observed in the theca-collagen

layer and some in the oocyte (Fig. 6.5a to b). In this type of oocytes, the yolk globules had fused and disintegrated and all layers of the follicle, including the *zona radiata,* had lost their integrity (Fig. 6.5a) The oocytes in these fish did not commence oocyte maturation, while the entire ovarian tissue was highly inflamed.

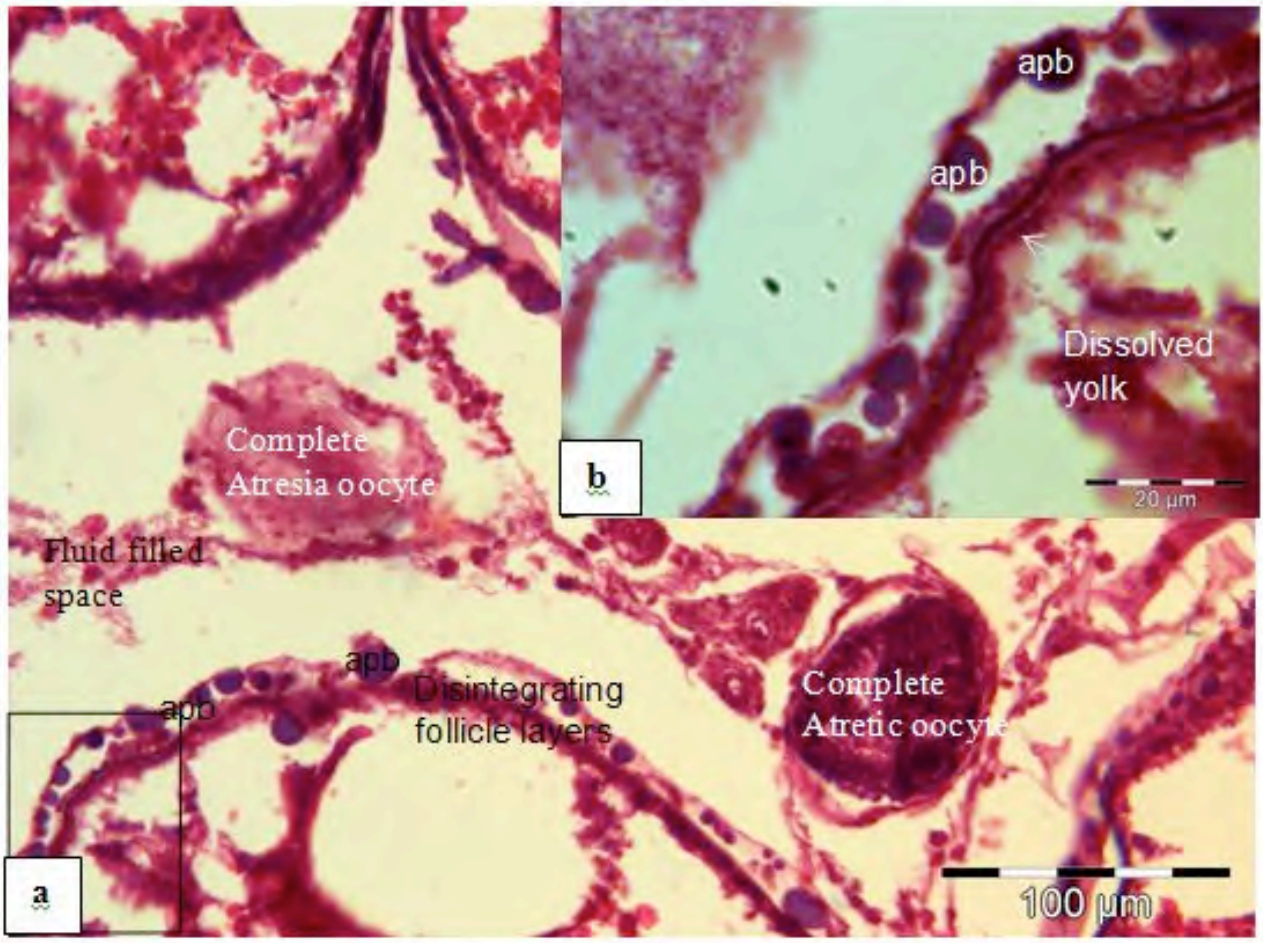


**Figure 6.5:** Total oocyte atresia in some fish induced with GnRHa-MET, b) disintegrating basal membrane and arrow = chorion, apb = apoptotic body

*Egg structure in Nile perch*

The pelagic eggs of *L. niloticus* were spherical, free floating, not attached to one another and transparent, with a uniform vitellus (Fig.6.6a). They contained one, centrally placed, lipid globule and the yolk was granular (6.6 b to d). The average egg size (ovulated egg diameter) was 550 ± 42 μm (n = 30) and increased significantly (t = 43.93, p = < 00001, df = 39) to an average of 726.30 ± 54.82 μm when yolk protein granules had dissolved (Fig. 6.6e to f). The oil globule size was 351 ± 23 μm (n = 40) and remained constant (p > 0.05) throughout the egg incubation period. When hydrated, Nile perch eggs and oil globule size were compared with marine species, *Diplodus puntazzo* (Klimgianni et al., 2011), *Dentex dentex* (Jug-Dujakoviće et al., 1995) and *Lates calcarifer* (Maneewongsa and Tattanon, 1982). *Lates niloticus* egg size was significantly smaller than all the three species, (egg diameter = 870 ± 30 μm), t = 18.89, df = 39, p < 0.001 (egg diameter = 920 ± 40 μm), t = 29.27, df = 39, p < 0.001 and (egg diameter = 800 ± 25 μm), t = 8.50, df = 39, p < 0.001, respectively. On the contrary, *Lates niloticus* eggs had a significant larger oil globule, occupying on average 48.90 ± 2.80% of the entire egg, than that found in all the three marine species (*D. puntazzo*, (oil globule = 240 ± 20μm), t = 46.96, df = 39, p < 0.001, *D. dentex*, (oil globule = 210 ± 30 μm), t = 61.99, df = 39 p < 0.001 and *L. calcarifer*, (oil globule = 20 μm), t =105.47, df = 39, p < 0.001).

Upon fertilization and activation in water, yolk globule coalescence began and was complete within six hours of egg incubation. At this point, the eggs were transparent and the oil globule was discernible as a sphere occupying the central region of the eggs (Fig. 6.6e to i). Embryo development commenced and stopped at the early gastrula stage at day six when it was followed by a mass mortality of fertilized eggs. Large masses of spores and threadlike filaments that formed the mycelia of a fungus were seen inside the infected eggs.

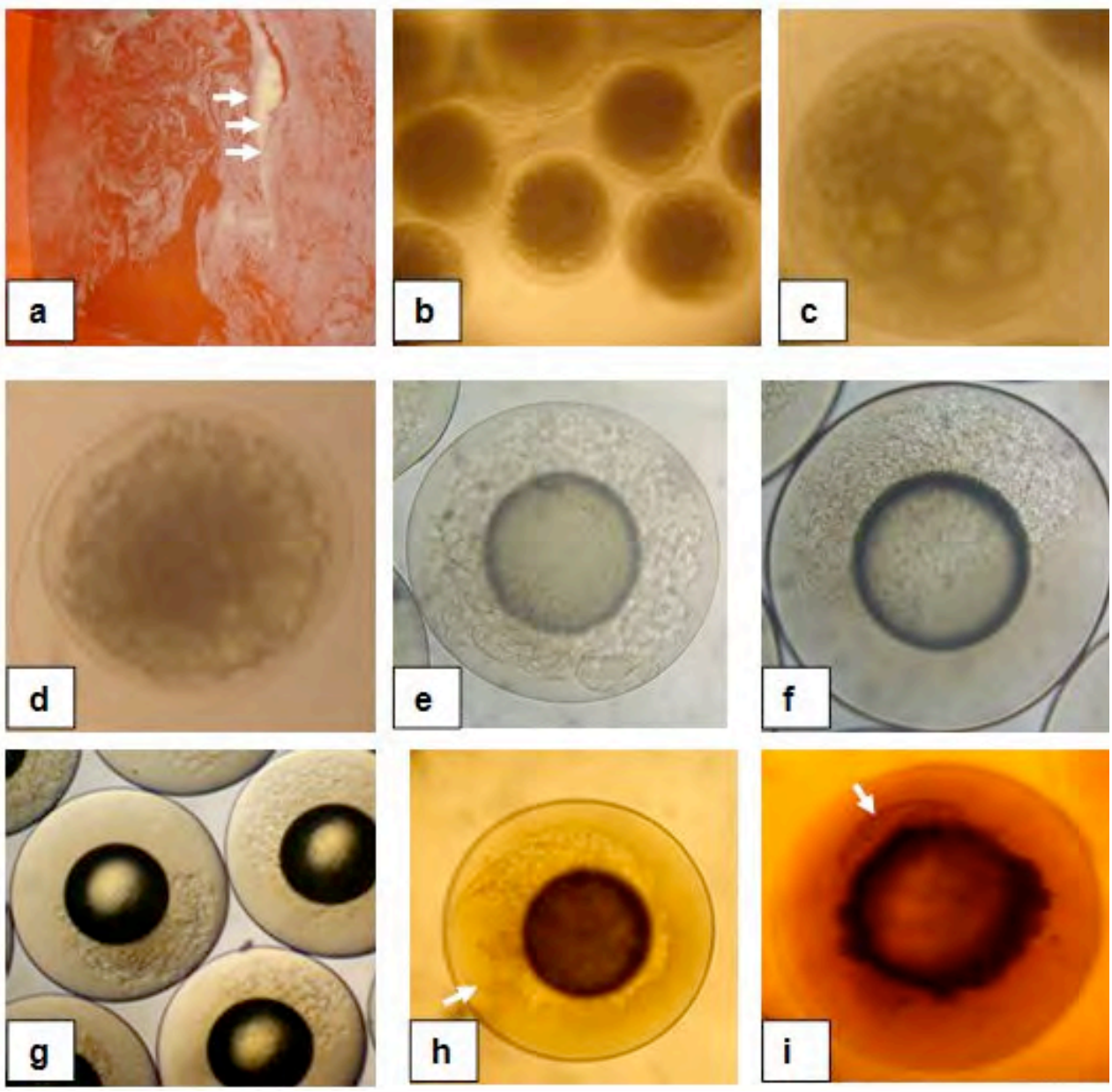


**Figure 6.6:** Light microscopy micrographs of ovulated eggs from GnRHa induced *L. niloticus* undergoing yolk granule proteolysis; a) *L. niloticus* eggs (arrows) floating in water in an incubation tank; b-f) yolk globule coalescence; e-f) eggs transparent and oil globule (centre) visible; g) hydrated oocytes; h) embryogenesis and i) early embryo, arrow

## Discussion

This study investigated the influence of GnRHa + MET on ovarian follicle maturation and atresia in *L. niloticus.* Fish with fully grown vitellogenic oocytes, injected with saline solution, did not undergo the final stages of follicle maturation that lead to ovulation. The results suggest that GnRHa + MET stimulated ovarian follicle maturation, cytoplasmic maturation and the resumption of meiosis in fully grown vitellogenic oocytes and that it caused hyperplasia and hypertrophy in localized regions of the follicular tissue in some fish.

### *Induced oocyte maturation*

The first sign of final oocyte maturation was the commencement of oil droplet coalescence and concomitant germinal vesicle migration to the periphery of the oocyte. Yolk globule dissolution did, however, not appear to take place and the yolk globules maintained their structure, size, and staining properties. Mylonas et al. (1997), similarly, did not observe any morphological changes in the yolk globules in captive-reared striped bass, *Morone saxatilis,* induced using GnRHa-implants. However, the authors reported that yolk coalescence occurred just before ovulation could take place. In contrast, Nile perch oocytes were ovulated with intact yolk globules as evidenced by opaque eggs that became transparent after fertilisation.

Yolk globule dissolution has been attributed to proteolysis, the hydrolysis of yolk proteins into peptides and amino acids by cleavage of their peptide bonds (Wallace and Selman, 1981; Craik and Harvey, 1987). Oocyte yolk has been implicated in oocyte hydration in marine species (Craik and Harvey, 1987; Fabra et al., 2006) and contributes to the buoyancy of the marine fish eggs (Craik and Harvey, 1987; Fabra, et al., 2006; Cerdá, 2009; Lubzens et al., 2010; Skoblina, 2010). Proteolysis of the yolk granules began after fertilisation. This was supported by the gradual clearance of ovulated Nile perch eggs in which fertilisation was attempted and eggs

incubated. Yolk globule hydrolysis has been reported to increase the osmotic potential of the marine floating eggs, hence aiding in the uptake of water, resulting into reduced density of the eggs, in relation to sea water (Craik and Harvey 1987; Fabra et al., 2006). I therefore suggest that a delay in oocyte proteolysis in Nile perch might be a reproductive strategy to control excess egg water uptake whereby free amino acids (FAA) in the egg are removed by the developing embryo, as they are formed, thus avoiding elevated osmotic potential that would result from FAA accumulation (Craik and Harvey, 1987; Cerdá, 2009; Mylonas et al., 2010). I further suggest that this strategy maintains a low osmotic potential, hence preventing eggs from excessive hydration that would lead to increased internal pressure, due to water influx, which might be detrimental to the developing Nile perch embryo.

As only one fish survived beyond 18 hours, behaviour was not recorded. The high mortality observed in this study cannot be attributed to either hormonal or anaesthetic induction effects since most if not all fish exhibited signs of barotrauma (refer to chapter two of this thesis) as a result of rapid elevation from deep high pressure waters to the low pressure surface waters. The inflated swim bladder observed in most specimens implies that Nile perch is a physoclistous fish with a closed swim bladder.

Spawning occurs only when oocytes have been ovulated (Jalabert, 1978; Mylonas et al., 1997; Mylonas et al., 2010). For ovulation to occur, oocytes must complete oocyte maturation. Gonadotropin releasing hormones have been used to induce ovulation and spawning in many fish species (Barbaro et al., 2002). In Baramundi, *L. calcarifer*, GnRHa was used to induce spawning, 32 hours after induction (Almendras et al., 1988). Similarly Rutaisire and Booth (2004) used a combination of GnRHa with a water soluble dopamine receptor antagonist metoclopramide (GnRHa + MET) to induce ovulation and spawning in *Labeo victorianus*. More studies are

needed using a sustained hormone delivery system on acclimatised Nile perch broodstock, kept in ponds, once they have recovered from capture and barotrauma-related stresses.

*Follicular cell layer changes*

The reasons for localized hypertrophy of the collagen-filled region between theca cells and the basal lamina could not be determined in this study. In rainbow trout, *Oncorhynchus mykiss,* an increase in oocyte size due to hydration, facilitated the oocyte ovulation process by mechanically increasing intra-follicular pressure (Lubzens et al., 2010). In this case, it has been suggested that the oocyte exerts considerable pressure on the follicular cell layers, resulting in rupture in localized areas that were probably weakened by proteolytic enzyme action (Jalabert, 1978; Lubzens et al., 2010). Nile perch oocytes did not undergo hydration before ovulation as was reported for *L. calcarifer* (Maneewongsa and Tattanon, 1982), *D. dentex* (Jug-Dujakoviće et al., 1995), and *D. puntazzo*, (Klimgianni et al., 2011), thus there was no substantial oocyte volume change. The follicular layer can therefore not rupture mechanically. It is likely that the observed hyperplasia, combined with hypertrophy of the follicle layer at some localized points, might facilitate oocyte ovulation since the oocytes did not hydrate to cause follicular internal pressure build up, as has been reported in rainbow trout (Lubzens et al., 2010). It is hypothesised that the observed follicle structural change might be a mechanism of chemical (proteolytic enzyme) action or a cell death, which facilitates the oocyte to rupture at the weakened points in the follicular cell layers. More research is needed in order to understand the cause and mechanisms of follicle rupture during ovulation in this species.

*Follicular atresia*

Follicular atresia is a natural process, occurring at various stages of development in teleosts and other vertebrates (Miranda et al., 1999). Several factors have been reported to induce follicular atresia. They include temperature change, low steroid hormone and high testosterone levels, handling stress, starvation and confinement (Nagahama, 1983; Miranda et al., 1999).

The ovaries of *L. niloticus*, which did not respond to hormone induction, showed atretic oocyte follicles in all stages of development. A similar response was observed in captive, hormonally induced *Leporhinus reinhardti* and *Astyanas bimaculatus lacustris* broodfish that did not respond to hormone treatment (Miranda et al., 1999). In the histological sections of *L. niloticus* ovaries induced with GnRHa-MET, there were apoptotic bodies in the theca and the granulosa layers, and there were a few such structures in those oocytes showing degeneration of the yolk globules, basal lamina, granulosa and thecal cell layers and dissolution of the germinal vesicle. The mechanisms of follicular atresia could not be described in this species. Future studies involving histochemistry should be conducted in order to understand the process of atresia in this species and the factors influencing it.

*Nile perch egg structure*

Nile perch, *L. niloticus* spawned buoyant eggs that floated on the water surface. Unlike in some other members of the order, such as sharpsnout sea bream, *Diplodus puntazzo* (Klimgianni et al., 2011), *L. niloticus* produces eggs that are free and floating on the water surface (pelagic eggs). The floating behaviour was used as a measure of egg quality in this study. Eggs that did not float were considered unfertilisable. Preliminary findings suggest that morphological characteristics of the eggs of this species were closely related to the marine species *L. calcarifer* (Maneewongsa

and Tattanon, 1982) sharpsnout sea bream *D. puntazo* (Klimgianni et al., 2011), as they had a large oil globule that allowed the eggs to float.

The egg diameter and oil globule diameter have been compared to the marine *centropomidae* and *sparidae* groups (Table 1). The diameter of *L. niloticus* was smaller than that of *L. calcarifer* (Maneewongsa and Tattanon, 1982), *Dentex dentex* (Jug-Dujakoviće et al., 1995) and, *D. puntazzo* (Klimgianni et al., 2011). However, the oil globule in *L. niloticus* was the largest of these species. This is because freshwater pelagic eggs require the oil globule to achieve buoyancy. In marine species, buoyancy is achieved by having large volumes of an aqueous solution that make up to 92% of the egg weight. Floating marine eggs have been reported to have lipids that make up less than 10% of the egg content (Craik and Harvey, 1987).

**Table 6.1:** The egg diameter and oil globule diameter, expressed as a percentage of egg diameter of *L. niloticus*, compared with some marine species with a similar egg structure

| Species | Egg diameter (mm) | Oil globule as percentage of egg diameter (%) | Source |
|---|---|---|---|
| *Diplodus puntazzo* | 0.85-0.89 | 25.88 - 28.09 | Klimgianni et al. (2011) |
| *Dentex dentex* | 0.94 - 0.98 | 20.21 - 25.51 | Jug-Dujakoviće et al. (1995) |
| *Lates calcarifer* | 0.8 | 25 | Maneewongsa and Tattanon (1982) |
| *Lates niloticus* | 0.55 - 0.84 | 56.36 - 45.24 | This study |

This study made a contribution to the understanding of egg histology and size in this species. Such information provides a foundation for controlled egg production and the establishment of an egg rearing protocol in a hatchery. It also provides a basis for the establishment of egg quality standards that are important in hatchery operations. GnRHa + MET induced oocyte maturation and ovulation in *L. niloticus*. Egg structure and type is similar to that found in marine species, although in Nile perch, oocyte hydration appeared to occur during early embryo development, which was similar to that reported for *L. calcarifer.*

# CHAPTER SEVEN

## General discussion and management considerations

Fish stocks have diminished, leading to a lower catch per unit effort, and resulting in a stagnation in total fish production at around 220 000 t (Nyombi and Bolwig, 2004). Several indicators point to excessive fishing and the use of unsustainable fishing methods over the last two decades. This has resulted in the exploitation of the resource, above the maximum sustainable yield, due, in part, to ineffective national and regional regulation. One of the strategic goals for the Lake Victoria Management Action Plan is to harmonise and strengthen the institutional environment for fisheries development, research and management. Studies on the reproductive biology of the Nile perch have been based on data from studies that did not cover a twelve-month cycle. Lake-wide survey methods usually cover one to four sampling activities in a year, depending on resource availability; hence such studies did not include monthly variations in reproductive variables. Some management strategies of the Nile perch fishery have been based on assumptions rather than on data describing the reproductive biology of the species (Wilson Mwanja, personal communication). For example, gear size limit for the Nile perch fishery was set based on the assumption that Nile perch change sex from male to female and that the male population will always contribute to the female population through sex change, from there the small mesh size limit (Mwanja W., personal communication). Histological studies by Basiita et al. (2011), combined with the data generated in Chapter four of this thesis, suggest that sex change does not occur in *L. niloticus*. This therefore calls for the review of the Nile perch fisheries regulations in order to avoid the consequences of an overexploited or a collapsed fisheries resource.

Results from this thesis provide information that can be used for *L. niloticus* fisheries management and to evaluate the species' potential for aquaculture and so to have a sustainable Nile perch supply from Lake Victoria and similar ecosystems where commercial *L. niloticus* fisheries exist. The research was designed to study the reproductive biology of *L. niloticus* (Chapter three). Based on the results from this research, the flow chart in Figure 7.1 suggests a research approach into the reproductive biology and management strategies of *L. niloticus* that can lead to increased fisheries production. Technical aspects of the proposed strategy, together with their scientific and management application will be discussed.

## Reproductive biology

### *Factors influencing reproduction*

Most of the reproductive variables examined in this thesis (Chapter three) were not influenced by rainfall. A positive relationship was observed between the monthly rainfall average and the frequency of atretic oocytes as the percentage of atretic oocytes was highest in April. Rainfall may thus induce the resorption of unspent yolk in oocytes. Rainfall average, the month before sampling, correlated to the gonadosomatic index. This suggests that rainfall contributed to the environmental factors that moderated vitellogenesis and resorption of unspawned vitellogenic oocytes, so that vitellogenesis of new oocytes might occur during the rainy season.

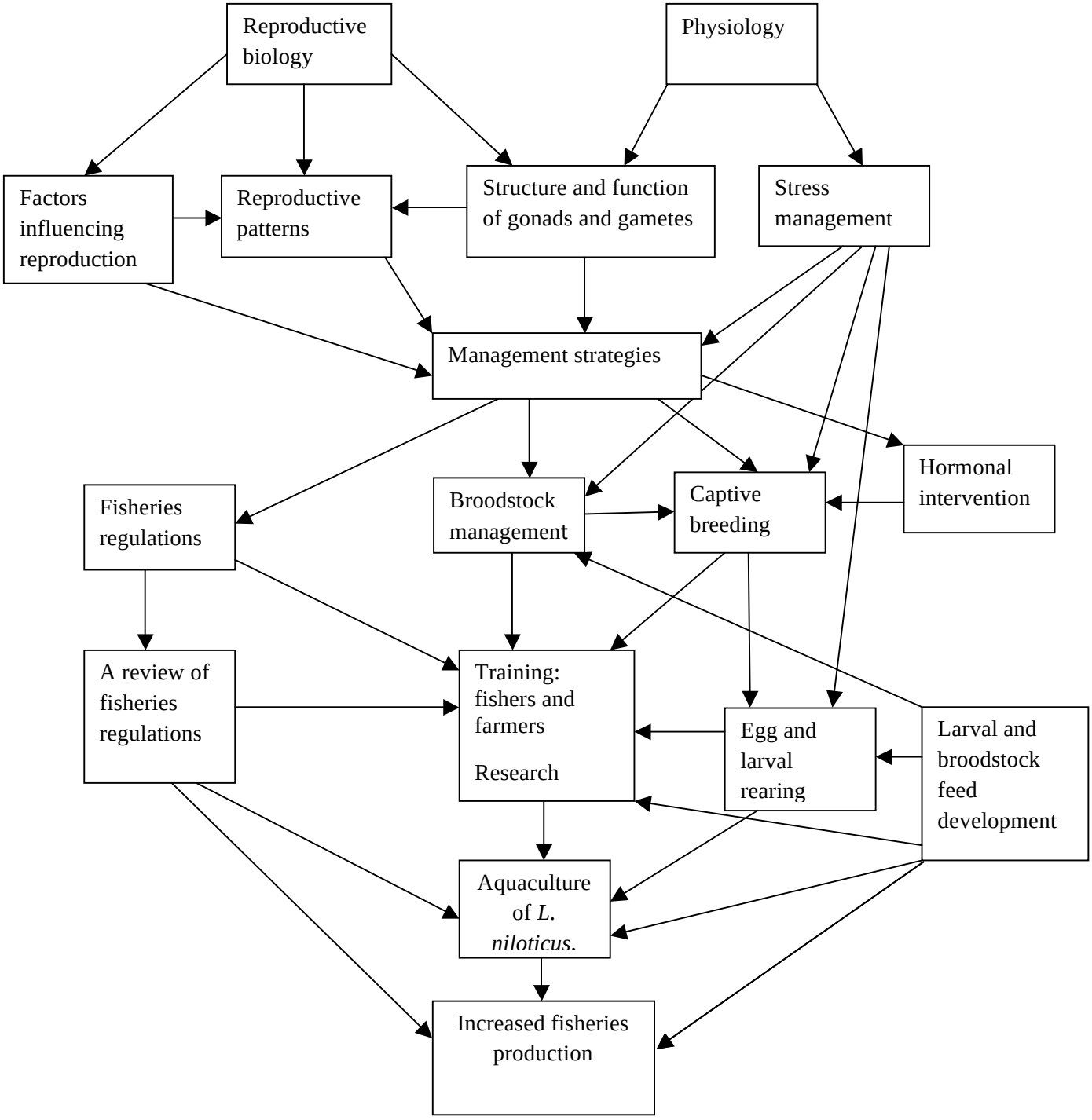


**Figure 7.1:** Flow chart of the proposed management strategies geared towards sustainable *Lates niloticus* fisheries production

Neither temperature, nor wind speed influenced most reproductive variables, except for a negative relationship between the frequency of tertiary oocytes and both temperature and wind speed. Wootton (1998) suggested that, in low latitudes, the cues used by fish in timing their reproductive development are not clear and he hypothesised that change in food abundance and water chemistry may play an important role in the timing of reproduction in a range of fishes.

***Reproductive patterns***

This thesis provides information on the reproductive biology and factors influencing reproductive patterns and size at sexual maturity in *L. niloticus* from Lake Victoria, Uganda. *Lates niloticus* in Lake Victoria does not spawn throughout the year, as has been reported in many fishes inhabiting the tropical equatorial aquatic environment (Munro, 1990). Nile perch begin spawning in November and end in February. For example, the lowest GSI data occurred in March. However, the low GSI–values observed for August and September could only be explained using ovarian histology. From this, I hypothesise that *L. niloticus* spawns several times during the same spawning season, and can therefore be considered a protracted unimodal batch spawner.

The breeding time was inferred from the frequency of advanced tertiary oocytes, the presence of both post ovulatory follicles (POFs) and atretic oocytes (Chapter three). However, the absence of POFs from April to November suggests a cessation of spawning during the months when rainfall was high (Chapter three). The high frequency of primary yolk vesicle oocytes, atretic oocytes and absence of post-ovulatory follicles from April to November (Chapter three, Fig. 3.5) provides a basis for the hypothesis that increased monthly average rainfall acted as a cue to stop spawning and triggered the onset of another phase of oocyte secondary growth. This suggests

that secondary gametogenesis in Nile perch occurs during the rainy season and spawning takes place at the end of rainy season. Thus, there appears to be a difference in seasonality between Lake Victoria and Lake Albert, where Nile perch spawn throughout the year (Hamblyn, 1962), possibly attributed to differences between these environments. Wootton (1998) suggested that the timing of reproduction in fish is flexible and that fish breed within a range of environmental conditions such as prey availability for both larvae and broodfish. The new habitat in Lake Victoria may have resulted in the species altering the spawning pattern so as to maximize offspring survival. Some fish species i.e. American shad, *Alosa sapidissima*, an introduced species, and sockeye salmon, *Oncorhynchus nerka*, a native species, which migrate up the Columbia River (United States) in late spring and early summer to spawn, have demonstrated this plasticity. They have adjusted the dates by which 50% of the spawning adults migrate past the dam by 38 days, while the native species has adjusted to 6 days earlier than it was six decades ago (Quinn & Adams, 1996). This change has been reported by Quinn and Adams (1996) to correlate with this shift in thermal and flow regimes of the river and the dam. This suggests that the two species have evolved migratory patterns that allow a greater behavioural response to environmental fluctuations.

In the equatorial region, seasonal changes are well entrenched in the reproductive cycles of most fishes. Some riverine species have spawning seasons related to flooding, which results in the availability of spawning sites, while in lacustrine potamodromous species, in natural lakes and in manmade lakes, migration is from the river mouth, upstream and they may start the migration months before the arrival of floods (Munro, 1990). Spawning site may not be a factor for those species that breed in the lake, but rather temperature, mixing, wind speed, atmospheric pressure,

or lunar cycles may become important factors in influencing spawning. This may not be the case with a predator occupying the highest trophic level, as is the case with *L. niloticus,* but instead timing of predator energy requirements and resource availability (Cushing 1990; Durant et al., 2005; 2007) might play an important role in the timing of spawning in this species.

For marine ecosystems, it has been suggested that the inter-annual variability in fish recruitment is a function of the timing of the production of food for the larvae (Cushing, 1990). Cushing (1990) put forward a match/mismatch hypothesis which predicts that predators' recruitment will be high if the prey availability temporally matches that period in which predators' offspring have the highest energy demand, whilst a mismatch will lead to poor recruitment. Durant et al. (2005) found that cod recruitment was affected by both the timing and the total annual abundance of zooplankton. This has also been found in a bird-of-prey population of the Atlantic puffin nestling that depends on young herring that drift with the costal current from the spawning grounds (Durant et al., 2003).

The deviation of *L. niloticus* from a rain-dependent spawning strategy, as has been shown in other tropical equatorial species found in the same lake system, can be explained by the fact that Nile perch is a top predator, occupying the highest trophic level. *Lates. niloticus* produces small eggs (Chapter six). Nile perch has been reported to grow fast, attaining a weight of over one kilogram in one year (Hughes, 1992). The fast growth rate may confer an advantage on the Nile perch larvae, such that by the time the other species, such as *haplochromines*, their prey items, spawn and hatch, the Nile perch larvae are large enough to feed on cichlid juveniles. *Haplochromis spp.* breeding activity is mostly seasonal with spawning occurring at the end of the rainy season (Goldschmidt and Witte, 1990). Data on haplochromine breeding revealed that

brooding females are frequently collected between June and October (Goldschmidt and Witte, 1990), thus providing ample time for the Nile perch (that breed between November and February) larvae to grow to a large size before many haplochromine species begin to spawn. The timing of breeding in the Nile perch might be induced by prey or food availability for both broodstock and larvae. To test this hypothesis, studies are required to quantify prey abundance and correlate it to breeding activity of *L. niloticus*.

***Size at sexual maturity***

The reduction of size at sexual maturity of *L. niloticus* in Lake Victoria is of concern to fisheries managers (Fig. 3.10). The estimated size at sexual maturity of female *L. niloticus* in Lake Victoria was lower than estimated in the 1990s, while values of males remained stable. The decrease in female size at sexual maturity, between 1990 and 2007 (Chapter three), coincides with the time when Nile perch was targeted by fisheries of Lakes Victoria and Kyoga (Lake Victoria Fisheries Organisation, LVFO, 2007). This suggests that the Lake Victoria Nile perch population has been fished unsustainably. There is a need to review the Nile perch fisheries management strategy and educate both fishermen and policy makers on the consequences of removing large females from the Nile perch population.

Life-history theory predicts that a high adult mortality rate leads to early maturity and smaller size at sexual maturity (Reznick and Ghalambor, 2005). This suggests that large animals or late maturing specimens have been removed through fishing. Early sexual maturity and reduced body size at sexual maturity have been reported in many commercially exploited fish populations (Reznick and Ghalambor, 2005). Mortality due to commercial fishing is higher in old and large

fish than in small fish (Rochet, 1998; Reznick and Ghalambor, 2005). The change in population structure of fish species that are subject to commercial exploitation tend to show patterns such as earlier maturity at a smaller size, within decades (Reznick and Ghalambor, 2005). Changes in size at maturity over time in *L. niloticus* reported in Chapter three support this argument. Furthermore, fishing mortality has been reported to reduce fish stock biomass, resulting in relieving the target fish population from intra-specific competition (Law, 2000). This can influence life history and so enable faster growth and earlier sexual maturation if maturation is size-dependent. However, Reznick and Yang (1993) warned that plasticity in life-history traits tends to take on different values depending on the environmental condition. For instance, Horwood et al. (2000) reported regulation of the plankton, while Bromley (1989) observed that apparent density-dependent growth can be area-specific, that is, some areas having larger numbers of smaller fish than others due to temperature, inter-specific competition and size-dependent migrations.

**Structure and function of gonads and gametes**

Histological examination of the gonads provides a platform for validation of field macroscopic observations that may otherwise be considered inconclusive, owing to the subjective nature of assigning gonad stages, using macroscopic assignment. The results in Chapter three were augmented by the histological data from Chapter four. Spermatogenesis in *L. niloticus* is cystic and sperm development is through asynchronous activation of the germ cells. Type II spermiogenesis, which does not involve flagella rotation, occurred in Nile perch. Nile perch produces spermatozoa that are simple, uniflagellate aquasperm, without an acrosome.

Fish caught in May had peculiar characteristics in that the milt did not readily dissolve in water. The testes from these individuals exhibited some form of cell fragmentation and nuclear disolution, with ruptured spermatozoa (Chapter four, Fig. 4.9). These anomalies were common in sperm from spent *L. niloticus.* These features can therefore be used in sperm quality assurance during artificial fertilisation. It is also reported that Sertoli cells play multiple roles, depending on the stage of reproduction. Chapter four has demonstrated that Sertoli cells work to eliminate unspawned sperm cells by phagocytosis. It is not clear from this study what factors trigger these cells to change roles.

**Stress management in broodstock**

Clove oil was an effective anaesthetic for the handling of Nile perch, *L. niloticus*, as Nile perch is a large fish that inhabits most of the lake niches, ranging from 3.5 m water depth to a depth of more than 15 m. It is highly susceptible to environmental, capture and handling stresses. Nile perch caught in waters deeper than 10 m, can suffer from barotrauma which results in compromised brood fish that rarely survive beyond twelve hours in captivity (own observations).

Since Nile perch is a new candidate species with a potential for aquaculture and considering that the fish are very are susceptible to stress when being handled, it was prudent that studies into stress management be carried out in order to establish suitable doses of a chosen anaesthetic. Clove oil, a plant extract, was chosen due to its availability on the market, its low cost and a wide safety margin for both fish and humans. Clove oil was effective as an anaesthetic at low concentrations (Chapter five) within the recommended time of induction (4 minutes) and recovery (12 minutes).

Stress in fish evokes a variety of responses that may be adaptive or maladaptive. The overall effect of stress may be a change in biological condition, beyond the normal resting state, that challenges homeostasis and, therefore presents a threat to the health of the fish. These stress-induced changes are grouped as primary and secondary and they include metabolic, haematological, hydromineral and structural and tertiary responses, which involves whole animal responses such as reduced growth. An increase in blood cortisol concentration is a primary response to stress. In *L. niloticus,* blood cortisol concentration was negatively correlated to clove oil concentration (Chapter five), suggesting that clove oil reduced this aspect of the stress response in Nile perch. The blood glucose level increased with increasing clove oil concentration. Maricchiolo and Genovese (2011) reported an increase in blood plasma glucose concentration in Black spotted sea bream *Pagellus bogaraveo* and amberjack *Seriola dumeralii,* following treatment with clove oil in the first 15 minutes of exposure. Also Wagner et al. (2002), using clove oil and MSS 222 successfully, blocked cortisol secretion, but increased glucose secretion in rainbow trout. Martínes-Porchas et al. (2009) suggested that the efficacy of an anaesthetic in reducing glucose increase might be species specific.

The combined effect of the two main effects “clove oil concentration” and “duration of exposure” and their interaction on the blood plasma level of cortisol, glucose, lactate and chloride concentrations, using fish weight as a covariate, suggested no effect of clove oil concentration on any of these variables. This suggests that clove oil did not elicit a physiological stress response in the Nile perch. This is an important factor in the choice of a suitable anaesthetic agent. Results by Keene et al. (1998), Prince and Powell (2000), Sladky et al. (2001), Iversen et al. (2003), Roubach et al. (2006) and Yamanaka et al. (2011), and data generated in

Chapter five of this thesis suggests that clove oil is effective in inducing anaesthesia at concentrations of 50-70 μml/L, and sedation was achieved at concentrations of 2.5 - 5 μml/l. Stress in fish can have a profound effect on their reproductive capacity by affecting seasonal patterns, or circulating levels of reproductive hormones (Pankhurst and van der Kraak, 1997). Chronically elevated cortisol levels have been reported to suppress plasma testosterone, oestradiol and gonadotropin and pituitary levels of gonadotropins in vitro (Carragher and Sumpter, 1990). Therefore, the concentrations of clove oil that suppressed cortisol metabolism in Chapter five of the thesis provide future prospects in the management of Nile perch broodstock in captivity.

**Hormonal intervention**

Global demand for food fish has steadily increased in the last three decades**.** At the same time, wild fish population sizes have declined, due to environmental degradation, poor fisheries management, or a combination of both factors. The Nile perch population in Lake Victoria is no exception. For instance, Lake Victoria Fisheries Organisation (www.LVFO.org, downloaded in February, 2012) reports a decrease in the Nile perch contribution to mean standing stock, from 1.29 million t in 1999/2001 to 0.82 million t in 2005/2006, contributing 59% in the earlier years, but only 39% of the total standing crop in 2005/2006. As a result, efforts to generate knowledge and develop technologies for hormonally induced spawning were made in this research. The majority of commercially farmed fish species do not undergo final oocyte maturation, ovulation and subsequent spawning under captive conditions, but require hormonal intervention if they are to spawn in captivity (Brooks et al., 1997). This is mainly due to a lack of proximate cues such as environmental cues, spawning substrate, availability of prey for larvae and juveniles, or

temperature (Munro, 1990). Although environmental factors have been reported as one of the important considerations for inducing fish to spawn, the stage of tertiary oocyte development is crucial for the successful induction of spawning. Based on observations made in Chapter six, it is hypothesised that the frequency of the largest tertiary oocytes is important for the successful hormonal spawning induction. Some fish exposed to GnRH + MET, exhibited atresia of the oocytes at all stages of development. Several factors have been implicated in causing follicular atresia in teleost fish. Guraya (1986) pointed out administration of steroid hormones, temperature change, hypophysectomy, starvation and confinement as some of the factors that may cause follicular atresia in fish. Nevertheless, the stress response of broodstock has been reported as an important factor in successful oocyte maturation induction and the subsequent production of viable eggs (Pankhurst and van der Kraak, 1997).

**Management of populations of *L. niloticus***

Information generated on the reproductive biology (Chapter three) and gamete structure as is presented in Chapters four and six, have provided information for the suggested management plans of wild *L. niloticus* populations. Moreover it provides information on timing of induced spawning that is useful for gamete quality control during artificial breeding. Furthermore, data from the clove oil efficacy studies can be provided to researchers and farmers to aid them in ameliorating stress-related mortalities in this species. Increased propagation of *L. niloticus* under captive conditions should be conducted simultaneously with education of the fishing communities about the potential of *L. niloticus* as an aquaculture species, while addressing the environmental concerns associated with introducing the Nile perch to new environments were

the species has not yet been introduced. Cultivation of Nile perch should be limited to regions or areas were it already exists.

## Conclusion

In summary, this thesis suggests a research approach that provides a basis for aquaculture of a new species from the equatorial region by first studying reproductive biology patterns and linking the information to gonad and gamete structure so that spawning times could be estimated. It also provides insights into the reproductive biology, effects of environmental factors on reproduction, and the influence of hormonal intervention on oocyte development, by showing when and at what stage of oocyte development hormones should be applied. Finally, clove oil can be used to anaesthetise and sedate Nile perch brood fish to reduce stress related to handling.

# REFERENCES


Almeida, F. F. L., Kristoffersen, C., Taranger, G. L. and Schulz, R. W. (2008). Spermatogenesis in Atlantic cod, (*Gadus morhua*): A Novel Model of Cystic Germ Cell Development. *Biology of Reproduction, 78*, 27-34.

Afzelius, B. A. (1978). Fine structure of the garfish spermatozoon. *Journal of Ultrastructure Research, 64*, 309-314.

Allendorf, F. W. and Hard, J. J. (2009). Human-induced evolution caused by unnatural selection through harvest of wild animals. *Proceedings of the National Academy of Sciences of the United States of America, 106*, pp. 9987-9994. Seattle: www.pnas.org/cgi/.

Almendras, J. M., Duenas, C., Nacario, J., Sherwood, N. and Crim, L. W. (1988). Sustained hormone release. Use of gonadotropin releasing hormone agonists to induce multiple *spawning* in sea bass, *Lates calcarifer*. *Aquaculture*, *74*, 97-111.

Aruho, C. (2009). *The reproductive biology of Bagrus docmak in the Lake Victoria, Uganda.* Makerere University - Kampala.

Balirwa, J. S. (2007). Ecological, environmental and socioeconomic aspects of the Lake Victoria's introduced Nile perch fishery in relation to the native fisheries and the species culture potential: lessons to learn. *African Journal of Ecology, 45*, 120-129.

Barbaro, A., Francescon, A., Bertotto, D., Bozzato, G., Di Maria, I., Patarnello, P., Furlan, F. and Colombo, L. (2002). More effective induction of spawning with long-acting GnRH agonist in the shi drum *Umbrina cirrosa* L. (Sciaenidae, Teleostei), a valuable candidate for Mediterranean mariculture. *Journal of Applied Ichthyology, 18*, 192-199.

Barrel, C. D., Dorit, R., Greenwood, P. H., Fryer, G., Hughes, N. and Jackson, P. B. (1985). Destruction of fisheries in Africa's lakes. *Nature, 315*, 19-20.

Barton, B. A. and Peter, R. E., (1982). Plasma cortisol stress response in fingerling rainbow trout, S*almo gairdneri* Richardson, to various transport conditions, anaesthesia, and cold shock. *Journal of Fish Biology, 20*, 39-51.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrated Comperative Biology, 42*, 517-525.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases, 1* 3-26.

Barton, B. A., Bollig, H., Hauskins, B. L. and Jansen, C. R. (2000). Juvenile pallid, (*Scaphirhynchus albus)* and hybrid pallidxshovelnose (*S. albus* x *platorynchus)* sturgeons exhibit low physiological responses to acute handling and severe confinement. *Comparative Biochemistry and Physiology - Part A*: *Molecular & Integrative Physiology 126*, 125-134.

Basiita, R. K., Aruho, C., Kahwa, D., Nyatia, E., Bugenyi, F. W. and Rutaisire, J. (2011). Differentiated gonochorism in Nile perch *Lates niloticus* from Lake Victoria, Uganda. *African Journal of Aquatic Sciences, 36 (1),* 89-96.

Bazzoli, N. and Godinho, H. P. (1995). Comparative morphology of the yolk nucleus (Balbiani body) in fresh water neotropical teleost fish. *Reviews of Brazilian Biology, 55*, 207-214.

Begovac, P. C. and Wallace, R. A. (1989). Major vitelline envelope proteins in pipefish oocytes originate within the follicle and are associated with the Z3 layer. *Journal of Experimental Zoology, 251*, 56-73.

Billard, R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. *Aquaculture, 14 (3),* 187-198.

Billard, R. (1984). Ultrastructural changes in the spermatogonia and spermatocytes of *Poecilia reticulata* during spermatogenesis. *Cell and Tissue Research, 237*, 219-226.

Bloom, W. and Fawcett, D. W. (1994). *A textbook of histology* (12 ed.). pp. 988. New York: Chapman and Hall, New York.

Booth, A. J. (1997). On the life history of the lesser gurnard (Scorpaeniformes: Triglidae) inhabiting the Agulhas Bank, South Africa. *Journal of Fish Biology, 51 (6)* 1155-1173.

Booth, A. J. and Weyl, O. L. F. (2000). Histological validation of gonadal macroscopic staging criteria for *Labeo cylindricus* (Pisces: Cyprinidae). *African Zoology, 35 (2),* 223-231.

Bromage, N. (1995). Broodstock management and seed quality. In N. R. Bromage and R. J. Roberts, *Broodstock management and egg and larval quality* (pp. 1-24). London, United Kingdom: Blackwell Science, London, UK.

Bromley, P. J. (1989). Evidence for density-dependent growth in North Sea gadoids. *Journal of Fish Biology, 35 (A),* 117-123.

Brooks, S., Tyler, C. R. and Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries, 7,* 387-416.

Brown, M. L. and Murphy, B. R. (2004). Seasonal dynamics of direct and indirect condition indices in relation to energy allocation in largemouth bass *Micropterus salmoides* (Lacèpede). *Ecology of Freshwater Fish, 13 (1),* 23-36.

Brown-Peterson, N. J., Wyanski, D. M., Saborido-Rey, F., Macewicz, B. J. and Lowerre-Barbieri, S. K. (2011). A Standardized Terminology for Describing Reproductive Development in Fishes. *Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science, 3 (1),* 52-70.

Bruslé, S. (1980). Fine structure of early previtellogenic oocytes in *Mugil (Liza) auratus* Risso, 1810 (Teleostei Mugilidae). *Cell and Tissue Research, 207 (1),* 123-134.

Campton, D. and Gall, G. A. E. (1988). Responses to selection for body size and age at sexual maturity in the mosquitofish, *Gambusia affinis*. *Aquaculture, 68 (3),* 221-241.

Carragher, J. F. and Sumpter, J. P. (1990). The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *General and Comparative Endocrinology, 77 (3),* 403-407.

Cerdà, J. (2009). Molecular pathways during marine fish egg hydration: the role of aquaporins. *Journal of Fish Biology, 75 (9),* 2175-2196.

Chung, E. -Y. (2008). Ultrastructure of germ cells, the Leydig cells, and Sertoli cells during spermatogenesis in *Boleophthalmus pectinirostris* (Teleostei, Perciformes, Gobiidae). *Tissue and Cell, 40 (3),* 195-205.

Chung, E. -Y., Yang, Y. -C., Kang, H. -W., Choi, K. -H., Jun, J. -C. and Lee, K. -Y. (2010). Ultrastructure of Germ Cells and the Functions of Leydig Cells and Sertoli cells Associated with Spermatogenesis in *Pampus argenteus* (Teleostei: Perciformes: Stromateidae). *Zoological Studies, 49 (1),* 39-50.

CIA. (2011). Central Intelligence Agency, The World Factbook. https://www.cia.gov/library/-publications/the-world-factbook.

Clearwater, S. J. and Pankhurst, N. W. (1994). Reproductive biology and endocrinology of female red gurnard, *Chelidonichthys kumu* (Lesson and Garnot) (family Triglidae), from the Hauraki Gulf, New Zealand. *Australian Journal of Marine and Freshwater Research, 45 (2),* 131-139.

Costas, B., Aragao, C., Mancera, J. M., Dinis, M. T. and Conceiçao, L. E. C. 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. Aquaculture Research, **39**: 1-9. DOI: 10.1111/j.1365-2109.2007.01845.x

Coward, K., Bromage, N. R., Hibbitt, O. and Parrington, J. (2002). Gamete physiology, fertilization and egg activation in teleost fish. *Reviews in Fish Biology and Fisheries, 12 (1)*, 33-58.

Craik, J. C. A. and Harvey, S. M. (1987). The causes of buoyancy in eggs of marine teleosts. *Journal of the Marine Biological Association of the United Kingdom*, *67*, 169-182.

Cushing, D. H. (1990). Plankton production and year-class strength in fish populations: an update of the match/mismatch hypothesis. *Advances in Marine Biology*, *26*, 249-293.

Dufour, S., Sebert, M. E., Weltzien, F. A., Rousseau, K. and Pasqualini, C. (2010). Neuroendocrine control by dopamine of teleost reproduction. *Journal of Fish Biology, 76 (1),* 129-160.

Durant, J. M., Anker-Nilssen, T. and Stenseth, N. C. (2003). Trophic interactions under climate fluctuations: the Atlantic puffin as an example. *Proccedings of the Royal Society, B: Biological Sciences, 270,* pp. 1461-1466.

Durant, J. M., Hjermann, D. Ø., Anker-Nilssen, T., Beaugrand, G., Mysterud, A., Pettorelli, N. and Stenseth, N. C. (2005). Timing and abundance as key mechanisms affecting trophic interactions in variable environments. *Ecology Letters, 8 (9)*, 952-958.

Durant, J. M., Hjermann, D. Ø., Ottersen, G. and Stenseth, N. C. (2007). Climate and the match or mismatch between predator requirements and resource availability. *Climate Research, 33*, 271-283.

Eddy, E. M. (1974). Fine structural observations on the form and distribution of nuage in germ cells of the rat. *The Anatomical Record, 178 (4)*, 731-757.

Eddy, E. M. (1975). Germ plasm and the differentiation of the germ cell line. *International Reviews in Cytology, 43*, 229-280.

Eddy, E. M., Toshimori, K. and O'Brien, D. A. (2003). Fibrous sheath of the mammalian spermatozoa. *Microscopy Research and Technique, Special Issue: The Biology of Spermatozoa Maturation, 61 (1)*, 103-115.

Estêvão, M. D., Redruello, B., Canario, A. V. M. and Power, D. M. (2005). Ontogeny of osteonectin expression in embryos and larvae of sea bream (*Sparus auratus*). *General and Comparative Endocrinology*, *142 (1) and (2)*, 155-162.

Extavour, C. G. and Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development*, *130 (24)*, 5869-5884.

Extavour, C. and García-Bellido, A. (2001). Germ cell selection in genetic mosaics in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, *98 (20)*, 11841-11846.

Fabra, M., Raldua, D., Bozzo, M. G., Deen, P. M. T., Lubzens, E. and Cerdá, J. (2006). Yolk proteolysis and aquaporin-1o play essential roles to regulate fish oocyte hydration during meiosis resumption. *Developmental Biology*, *295 (1)*, 250-262.

FAO. (2010). *The State of World Fisheries and Aquaculture 2010.* Rome: Food and Agriculture Organisation of the United Nations, 218 pp, ISBN 978-92-5-106675-1, Rome.

FDA. (2002). *Guidance for the industry. Status of clove oil and eugenol for anaesthesia of fish.* Food and Drug Administration, 150, 1-3, Food and Drug Administration, Rockville, USA.

Flik, G., Klaren, P. H. M., Van den Burg, E. H., Metz, J. R. and Huising, M. O. (2006). CRF and stress in fish. *General and Comparative Endocrinology*, *146 (1)*, 36-44.

Forchhammer, M. C., Post, E. and Stenseth, N. C. (1998). Breeding phenology and climate. *Nature*, *391*, 29-30.

Fordham, S. E. and Trippel, E. A. (1999). Feeding behaviour of cod (*Gadus morhua*) in relation to spawning. *Journal of Applied Ichthyology*, *15 (1)*, 1-9.

Forniés, M. A., Mañanòs, E., Carrillo, M., Rocha, A., Laureau, S., Mylonas, C. C., Zohar, Y. and Zanuy, S. (2001). Spawning induction of individual European sea bass females (*Dicentrarchus labrax*) using different GnRHa-delivery systems. *Aquaculture, 202 (3) and (4),* 221-234.

García-López, A., de Jonge, H., Nóbrega, R. H., de Waal, P. P., van Dijk, W., Hemrika, W., Taranger, G. L., Bogerd, J. and Schulz, R. W. (2010). Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. *Endocrinology, 151 (5),* 2349-2360.

Gee, J. M. (1964). *Nile perch investigation,* Annual Report, East African Freshwater Fisheries Research Organisation (E.A.F.R.O.), Jinja - Uganda p.76.

Genten, F., Terwinghe, F. and Danguy, A. (2009). *Atlas of fish histology.* 223 pp., ISBN13: 9781578085446, New Hampshire: Science Publishers.

Goldschmidt, T. and Witte, F. (1990). Reproductive strategies of zooplanktivorous haplochromine cichlids (Pisces) from Lake Victoria before the Nile perch boom. *OIKOS, 58 (3),* 356-368.

Goldschmidt, T., Witte, F. and Wanink, J. (1993). Cascading Effects of the Introduced Nile Perch on the Detritvorous/Phytoplanktivorous Species in the Sublittoral Areas of Lake Victoria. *Conservation Biology, 7 (3),* 686-700.

Gresik, E. W., Quirk, G. B. and Hamilton, G. B. (1973). A fine structural and histochemical study of the Leydig cell in the testis of the teleost, *Oryazias latipes* (Cyprinodontiformes). *General and Comparative Endocrinology, 20,* 86-99.

Grier, H. J. (2000). Ovarian germinal epithelium and folliculogenesis in the common snook, *Centropomus undecimalis* (Teleostei: Centropomidae). *Journal of Morphology, 243,* 265-281.

Grier, H. J. (2002). The germinal epithelium: Its dual role in establishing male reproductive classes and understanding the basis for indeterminate egg production in female fishes. In: *Proceedings of the 53rd Annual Gulf and Caribbean Fisheries Institute*, 537-552.

Grier, H. J., Linton, J. R., Leatherland, J. F and De Vlaming, V. L. (1980). Structural evidence for two different testicular types in teleost fishes. *American Journal of Anatomy*, *159 (3)*, 331-345.

Grier, H. J., van den Hurk, R. and Billard, R. (1989). Cytological identification of cell types in the testis of *Esox lucius* and *E. niger*. *Cell and Tissue Research*, *257 (3)*, 491-496.

Grier, H. J. (1981). Cellular Organization of the Testis and Spermatogenesis in Fishes. *American Zoologist*, *21 (2)*, 345-357.

Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J. and Jalabert, B. (1994). Reproductive cycle and sex inversion of the seabass, *Lates calcarifer*, reared in sea cages in French Polynesia: histological and morphometric description. *Environmental Biology of Fishes*, *39 (3)*, 231-247.

Guimarães, A. C. D. and Quagio-Grassiotto, I. (2001). Ultrastructural aspects of oogenesis and oocyte primary growth in *Serrasalmus spilopleura* (Teleostei, Characiformes, Characidae). *Tissue and Cell*, *33* (3), 241-248.

Guo, G. -Q. and Zheng, G. -C. (2004). Hypotheses for the functions of intercellular bridges in male germ cell development and its cellular mechanisms. *Journal of Theoretical Biology*, *229 (1)*, 139-146.

Guraya, S. S. (1994). Gonadal development and production of gametes in fish. *Proceedings of Indian National Science Academy*, *B60*, 15-32.

Guraya, S. S. (1986). The cell and molecular biology of fish oogenesis (Monographs in Developmental Biology) (pages 223) Publisher Karger, ISBN-13: 978-3805542531

Gusmão-Pompiani, P. , Foresti, F. and Quagio-Grassiotto, I. (1999). The ultrastructure of spermiogenesis in *Plagioscion squamosissimus* (Teleostei, Perciformes, Sciaenidae). *Tissue and Cell, 31*, 627-633.

Gusmão-Pompiani, P., Oliveira, C. and Quagio-Grassiotto, I. (2005). Spermatozoa ultrastructure in Sciaenidae and Polynemidae (Teleostei: Perciformes) with some consideration on Percoidei spermatozoa ultrastructure. *Tissue and Cell, 37 (3),* 177-191.

Gwo, J. -C., Chiu, J. -Y., Lin, C. -Y. and Yu, S. -L. (2005). Spermatozoal ultrastructure of four Sparidae fishes: *Acanthopagrus berda*, *Acanthopagrus australis, Lagodon rhomboids* and *Archosargus probatocephus*. *Tissue and Cell, 37 (2),* 109-115.

Haqlund, K., Nezis, I. P and Stenmark, H. (2011). Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development. *Communicative and Integrative Biology, 4 (1),* 1-9.

Hamaguchi, S. (1987). The structure of the germinal dense bodies (nuages) during differentiation of the male germ line of the teleost, *Oryzias latipes*. *Cell and Tissue Research, 248 (2),* 375-380.

Hamblyn, E. L. (1962). *Nile perch investigation.* East Africa Freshwater Fisheries Research Organisation, Jinja, 76 pp.

Hammerle, M., Heimur, T., Maggard, K., Paik, J. and Valdivia, S. (2010). The Fishing Cluster in Uganda: Final Report for Microeconmics of Competitiveness. *HARVARD Bussiness School: Harvard Kennedy School of Government,* 31 pp.

Hecht, N. B. (1995). The making of a spermatozoon: A molecular perspective. *Developmental Genetics, 16 (2),* 95-103.

Hopson, A. J. (1972). *A study of the Nile perch (Lates niloticus (L.) Pisces: Centropomidae in Lake Chad.* Overseas Research Publication, *19,* 1-93.

Horwood, J, Cushing, D. and Wyatt, T. (2000). Planktonic determination of variability and sustainability of fisheries. *Journal of Plankton Research, 22 (7),* 1419-1422.

Hughes, N. F. (1992). Growth and reproduction of the Nile perch, *Lates niloticus*, an introduced predator, in Nyanza Gulf, Lake Victoria, East Africa. *Environmental Biology of Fishes, 33 (3),* 299-305.

Hunter, J. R. and Goldberg, S. R. (1980). Spawning incidence and batch fecundity in Northern anchovy, *Engraulis mordax*. *Fishery Bulletin, 77,* 641-652.

Hunter, J. R. and Leong, R. (1981). The spawning energetics of female northern anchovy, *Engraulis mordax*. *Fishery Bulletin U.S., 79,* 215-230.

Hunter, J. R. and Macewicz, B. J. (1985). Rates of atresia in the ovary of captive and wild Northern anchovy, *Engraulis mordax*. *Fishery Bulletin 83 (2),*119-136.

Hussein, M. M. A., Wada, S., Hatai, K. and Yamamoto, A. (2000). Antimycotic Activity of Eugenol against Selected Water Molds. *Journal of Aquatic Animal Health, 12 (3),* 224-229.

Inaba, K. (2007). Molecular Basis of Sperm Flagellar Axonemes: Structural and Evolutionary Aspects. *Annals of the New York Academy of Sciences*, *1101*, *Reproductive Biomechanics,* 506-526.

Isaacs, G. (1983). Permanent local anaesthesia and anhidrosis after clove oil spillage. *Lancet, 321,* 882-883.

Isumbisho, M., Sarmento, H., Kaningini, B., Micha, J. -C. and Descy, J. -P. (2006). Zooplankton of Lake Kivu, East Africa, half a century after the Tanganyika sardine introduction. *Journal of Plankton Research, 28 (11),* 971-989.

Iversen, M., Finstad, B., McKinley, R. S. and Eliassen, R. A. (2003). The efficacy of metomidate, clove oil, Aqui-S$^{Tm}$ and Benzoak® as anaesthetics in Atlantic salmon (*Salmo salar L.*) smolts, and their potential stress-reducing capacity. *Aquaculture, 221 (1 to 4),* 549-566.

Iwama, G. K., McGeer, J. C. and Pawluk, M. P. (1989). The effects of five fish anaesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Canadian Journal of Zoology, 67 (8),* 2065-2073.

Iwama, G. K., Afonso, L. O. B., Todgham, A., Ackerman, P. and Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? *The Journal of Experimental Biology, 207*, 15-19.

Iwao, Y. (2000). Mechanisms of Egg Activation and Polyspermy Block in Amphibians and Comparative Aspects with Fertilization in Other Vertebrates. *Zoological Science, 17 (6),* 699-709.

Jaffe, L. A. (1980). Electrical polyspermy block in sea urchins: nicotine and low sodium experiments. *Development, Growth and Differentiation, 22 (3),* 503-507.

Jalabert, B. (1978). Production of fertilizable oocytes from follicles of rainbow trout (*Salmo gairdnerii*), following *in vitro* maturation and ovulation. *Annales de Biologie Animale Biochimie Biophysique, 18 (2B),* 461-470.

Jamieson, B. G. (1991). *Fish evolution and systematics: Evidence from spermatozoa.* Cambridge University Press, Cambridge, 319 pp.

Jamieson, B. G., & Leung, K. P. (1991). Introduction to fish spermatozoa and the micropyle p 57-72 . In B. G. M. Jamieson, *Fish evolution and systematics:Evidence from spermatozoa* (p. 319). Cambridge, Great Britain: Cambridge University Press.

Johnson, A. D., Drum, M., Bachvarova, R. F., Masi, T., White, M. E. and Crother, B. I. (2003). Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. *Evolution & Development, 5 (4),* 414-431.

Jørgensen, T. (1990). Long-term changes in age at sexual maturity of Northeast Arctic cod (*Gadus morhua* L.). *ICES Journal of Marine Science, 46 (3),* 235-248

Jug-Dujaković, J., Dulčić, J. and Katavić, I. (1995). Embryonic and yolk-sac larval development of the sparid *Dentex (Dentex) dentex* (Linnaeus, 1758). *Fisheries Research, 24 (2)*, 91-97.

Kaelin, A. J., & Cowx, I. G. (2002). Outline of the path forward in Uganda's fisheries sector. *The Presidential Conference on Export Competitiveness* Kampala - Uganda, 15 pp.

Kaiser, H., Brill, G., Cahill, J., Collett, P., Czypionka, K., Green, A., Orr, K., Pattrick, P., Scheepers, R., Stonier, T., Whitehead, M. A. and Yearsley, R. (2006). Testing clove oil as an anaesthetic for long-distance transport of live fish: the case of the Lake Victoria cichlid *Haplochromis obliquidens*. *Journal of Applied Ichthyology*, *22 (6)*, 510-514.

Karapinar, M. and Aktuğ, S. E. (1987). Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. *International Journal of Food Microbiology*, *4 (2)*, 161-166.

Kaufman, L. and Ochumba, P. (1993). Evolutionary and Conservation Biology of Cichlid Fishes as Revealed by Faunal Remnants in Northern Lake Victoria. *Conservation Biology*, *7 (3)*, 719-730.

Keene, J. L., Noakes, D. L. G., Moccia, R. D. and Soto, C. G. (1998). The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, *29 (2)*, 89-101.

Kerr, J. B. and Dixon, K. E. (1974). An ultrastructural study of germ plasm in spermatogenesis of *Xenopus laevis*. *Journal of Embryology and Experimental Morphology*, *32*, 573-592.

Kiørboe, T. and MacKenzie, B. (1995). Turbulence-enhanced prey encounter rates in larval fish: effects of spatial scale, larval behaviour and size. *Journal of Plankton Research*, *17 (12)*, 2319-2331.

Kitchell, J. F., Schindler, D. E., Ogutu-Ohwayo, R. and Reinthal, P. N. (1997). The Nile perch in Lake Victoria: Interactions between predation and fisheries. *Ecological Applications*, *7 (2)*, 653-664.

Kjørsvik, E., Mangor-Jensen, A. and Holmefjord, I. (1990). Egg Quality in Fishes. *Advances in Marine Biology, 26*, 71-105.

Klimgianni, A., Kalanji, M., Pyrenis, G., Zoullioti, A. and Trakos, G. (2011). Ontogeny of embryonic and yolk sac larval stage of the sparid sharpsnout Sea bream, *Diplodus puntazzo*, Cetti 1777. *Academic Journals Inc., 6 (1)*, 62-73.

Kloc, M. and Etkin, L. D. (1995). Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus oocytes*. *Development, 121*, 287-297.

Kloc, M., Bilinski, S., Dougherty, M. T., Brey, E. M. and Etkin, L. D. (2004). Formation, architecture and polarity of female germline cyst in *Xenopus*. *Developmental Biology, 266 (1)*, 43-61.

Koya, Y., Itazu, T. and Inoue, M. (1998). Annual reproductive cycle based on histological changes in the ovary of the female mosquitofish,*Gambusia affinis*, in central Japan. *Ichthyological Research, 45 (3)*, 241-248.

Lake, J. S. (1967). Rearing experiments with five species of Australian freshwater fishes. I. Inducement to spawning. *Australian Journal of Marine and Freshwater Research, 18 (2)*, 137-153.

Lambert, T. C. and Ware, D. M. (1984). Reproductive Strategies of Demersal and Pelagic Spawning Fish. *Canadian Journal of Fisheries and Aquatic Science, 41 (11)*, 1565-1569.

Law, R. (2000). Fishing, selection, and phenotypic evolution. *ICES Journal of Marine Science, 57 (3)*, 659-668.

Lloret, J., Demestre, M. and Sánchez-Pardo, J. (2008). Lipid (energy) reserves of European hake (*Merluccius merluccius*) in the north-western Mediterranean. *Vie Et Milieu: Life and environment, 58 (1)*, 75-85.

Lo Nostro, F. L., Grier, H., Meijide, F. J. and Guerrero, G. A. (2003). Ultrastructure of the testis in *Synbranchus marmoratus* (Teleostei, Synbranchidae): the germinal compartment. *Tissue and Cell, 35 (2),* 121-132.

Lubzens, E., Young, G., Bobe, J. and Cerdà, J. (2010). Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology, 165 (3),* 367-389.

LVFO (2007). *Report of the fish factory sampling excercise: length at sexual maturity of Nile perch in Lake Victoria. Implementation of a fisheries management plan.* Jinja, Uganda: Lake Victoria Fisheries Reseach Organisation.

LVFO (2013) Status of fish stocks in Lake Victoria, Lake Victoria Fisheries Organisation website http://www.lvfo.org accessed on 25th March 2013.

MAAIF. (2004). *National Fisheries Policy for Uganda.* Entebbe: Ministry of Agriculture Animal Industry and Fisheries Entebbe- Uganda.

McDonald, G. and Milligan, L. (1997). Ionic, osmotic and acid-base regulation in stress. In: *Fish Stress and Health in Aquaculture* (eds G. W. Iwama, A. D. Pickering, J. P. Sumpter and C. B. Shreck), pp. 119-142. University Press, Cambridge.

MacFarlane, R. B., Norton, E. C. and Bowers, M. J. (1993). Lipid Dynamics in Relation to the Annual Reproductive Cycle in Yellowtail Rockfish (*Sebastes flavidus). Canadian Journal of Fisheries and Aquatic Sciences, 50 (2),* 391-401.

Maneewongsa, S. and Tattanon, T. (1982). *Nature of eggs, larvae and juveniles of the sea bass.* Manila, Philippines: South China Sea Fisheries Development and Coordinating Programme.

Maricchiolo, G. and Genovese, L. (2011). Some Contributions to Knowledge of Stress Response in Innovative Species with Particular Focus on the Use of the Anaesthetics. *The Open Marine Biology Journal, 5,* 24-33.

Marking, L. L. and Meyer, F. P. (1985). Are Better Anaesthetics Needed in Fisheries? *Fisheries, 10 (6),* 2-5.

Martínez-Porchas, M., Martínez-Córdova, L. R. and Ramos-Enriquez, R. (2009). Cortisol and glucose: Reliable indicators of fish stress. *Pan-American Journal of Aquatic Sciences, 4 (2),* 158-178.

Matsuishi, T., Muhoozi, L., Mkumbo, O., Budeba, Y., Njiru, M., Asila, A., Othina, A. and Cowx, G. (2006). Are the exploitation pressures on the Nile perch fisheries resources of Lake Victoria a cause for concern? *Fisheries Management and Ecology, 13 (1),* 53-71.

Mattei, X. (1991). Spermatozoon ultrastructure and its systematic implications in fishes. *Canadian Journal of Zoology, 69 (12),* 3038-3055.

Mattei, X. (1988). The flagellar apparatus of spermatoza in fish. Ultrustructure and evolution. *Biology of the Cell, 63 (2),* 151-158.

Mattei, X., Siau, Y., Thiaw, O. T. and Thiam, D. (1993). Peculiarities in the organization of testis of *Ophidian sp*. (Pisces Teleostei). Evidence for two types of spermatogenesis in teleost fish. *Journal of Fish Biology, 43 (6),* 931-937.

Mbatia, B., Adlercreutz, P., Mulaa, F. and Mattiasson, B. (2010). Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil. *European Journal of Lipid Science and Technology, Special Issue: Focus Issue Euro Fed Lipid Highlights 112 (9),* 977-984.

Meinhardt, A., Wilhelm, B. and Seitz, J. (1999). Mini symposium. New aspects of spermatogenesis: Expression of mitochodrial marker proteins during spermatogenesis. *Human Reproduction Update, 5 (2),* 108-119.

Migaud, H., Davie, A. and Taylor, J. F. (2010). Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. *Journal of Fish Biology, 76 (1),* 27-68.

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M. and O-Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the Natural Academy of Sciences of the United States of America, 101*, 16501-16506.

Miranda, A. C. L., Bazzoli, N., Rizzo, E. and Sato, Y. (1999). Ovarian follicular atresia in two teleost species: a histological and ultrastructural study. *Tissue and Cell, 31 (5)*, 480-488.

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries, 9 (3)*, 211-268.

Munro, A. D. (1990). General Introduction. In A. D. Munro, A. P. Scott and T. J. Lam, *Reproductive seasonality in teleosts: Environmental influences* (1st ed., pp. 1-12). CRC Press, Inc., ISBN: 0849368758.

Murua, H. and Saborido-Rey, F. (2003). Female Reproductive Strategies of Marine Fish Species of the North Atlantic. *Journal of Northwest Atlantic Fishery Science, 33*, 23-31.

Mylonas, C. C., Fostier, A. and Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *General and Comparative Endocrinology, 165 (3)*, 516-534.

Mylonas, C. C., Woods III, L. C. and Zohar, Y. (1997). Cyto-histological examination of post-vitellogenesis and final oocyte maturation in captive-reared striped bass. *Journal of Fish Biology, 50 (1)*, 34-49.

Nagahama, Y. (1983). The functional morphology of teleost gonads. In W. S. Hoar, D. J. Randall, and E. M. Donaldson, *Fish Physiology*, London: Academic Press, pp. 233-275.

Nakamura, S., Kobayashi, K., Nishimura, T. and Tanaka, M. (2011). Ovarian Germline Stem Cells in the Teleost Fish, Medaka (*Oryzias latipes*). *International Journal of Biological Sciences, 7 (4)*, 403-409.

NEMA. (2008). *State of environment report for Uganda 2008.* Kampala: National Environment Management Authority Uganda.

Nicotra, A. and Mura, G. (1985). Mitochondrial behavior during spermiogenesis of *Branchipus visnyai* (Crustacea, Anostraca). *Journal of Crustacean Biology, 5 (4),* 683-685.

Njiru, M., Kazungu, J., Ngugi, C. C., Gichuki, J. and Muhoozi, L. (2008). An overview of the current status of Lake Victoria fishery: Opportunities, challenges and management strategies. *Lakes & Reservoirs: Research & Management, 13 (1),* 1-12.

Njiru, M., Mkumbo, O. C. and van der Knaap, M. (2010). Some possible factors leading to decline in fish species in Lake Victoria. *Aquatic Ecosystem Health & Management, 13 (1),* 3-10.

Nóbrega, R. H. and Quagio-Grassiotto, I. (2007). Morphofunctional changes in Leydig cells throughout the continuous spermatogenesis of the freshwater teleost fish, *Serrasalmus spilopleura* (Characiformes, Characidae): an ultrastructural and enzyme study. *Cell and Tissue Research, 329 (2),* 339-349.

Nyombi, K. and Bolwig, S. (2004). *A Qualitative Evaluation of Alternative Development Strategies for Ugandan Fisheries. A Contribution to the Strategic Criteria for Rural Investments in Productivity (SCRIP) Program of the USAID Uganda Mission; The International Food Policy Research Institute, Washington DC.*

Odongkara, K., Abila, R. and Luomba, J. (2009). The Contribution of Lake Victoria Fisheries to National Economies. *African Journal of Tropical Hydrobiology and Fisheries, 12 (1),* 47-51.

Ogutu-Ohwayo, R. (2004). Management of the Nile perch, *Lates niloticus* fishery in Lake Victoria in light of the changes in its life history characteristics. *African Journal of Ecology, 42 (4),* 306-314.

Ogutu-Ohwayo, R. (1988). Reproductive potential of the Nile perch, *Lates niloticus* L. and the establishment of the species in Lakes Kyoga and Victoria (East Africa). *Hydrobiologia, 162 (3),* 193-200.

Ogutu-Ohwayo, R. (1990). The decline of the native fishes of lakes Victoria and Kyoga (East Africa) and the impact of introduced species, especially the Nile perch, *Lates niloticus*, and the Nile tilapia, *Oreochromis niloticus*. Environmental Biology of Fishes, 27 (2),* 81-96.

Okedi, J. (1970). *Further observations on the ecology of the Nile perch (Lates niloticus. Linne) in Lake Victoria and Kyoga* p.78, Jinja - Uganda: EAFFRO.

Olsen, E. M., Heino, M., Lilly, G. R., Morgan, M. J., Brattey, J., Ernande, B. and Dieckmann, U. (2004). Maturation trends indicative of rapid evolution preceded the collapse of northern cod. *Nature, 428,* 932-935.

Otero, O. and Gayet, M. (2001). Palaeoichthyofaunas from the Lower Oligocene and Miocene of the Arabian Plate: palaeoecological and palaeobiogeographical implications. *Palaeogeography, Palaeoclimatology, Palaeoecology, 165 (1to 2),* 141-169.

Otero, O., Pinton, A., Mackaye, H. T., Likius, A., Vignaud, P. and Brunet, M. (2010). The early/late Pliocene ichthyofauna from Koro-Toro, Eastern Djurab, Chad. *Geobios, 43 (2),* 241-251.

Palić, D., Herolt, D. M., Andreasen, C. B., Menzel, B. W. and Roth, J. A. (2006). Anesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque 1820). *Aquaculture, 254 (1to 4),* 675-685.

Palstra, A. P., Cohen, E. G. H., Niemantsverdriet, P. R. W., van Ginneken, V. J. T. and van den Thillart, G. E. E. J. M. (2005). Artificial maturation and reproduction of European silver eel: Develeopment of oocytes during final maturation. *Aquaculture, 249 (1 to 4),* 533-547.

Pankhurst, N. W. and King, H. R. (2010). Temperature and salmonid reproduction: implications for aquaculture. *Journal of Fish Biology, 76 (1),* 69-85.

Pankhurst, N. W. and van der Kraak, G. (1997). Effects of stress on reproduction and growth in fish. In G. K. Iwama, A. D. Pickering, J. P. Sumpter and C. B. Schreck (Eds.), *Fish stress and health in aquaculture* (pp. 73-93). Cambridge: Cambridge University Press.

Parenti, L. R. and Grier, H. J. (2004). Evolution and Phylogeny of Gonad Morphology in Bony Fishes. *Integrative & Comparative Biology 44 (5),* 333-348.

Patiño, R. and Sullivan, C. V. (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry, 26 (1),* 57-70.

Patiño, R., Yoshizaki, G., Thomas, P. and Kagawa, H. (2001). Gonadotropic control of ovarian follicle maturation: the two-stage concept and its mechanisms. *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology 129,* 427-439.

Pepling, M. E. (2006). From primordial germ cell to primordial follicle: mammalian female germ cell development. *Genesis, 44 (12),* 622-632.

Perazzolo, L. M., Coward, K., Davail, B., Normand, E., Tyler, C. R., Pakdel, F., Schneider, W. J. and Le Menn, F. (1999). Expression and Localization of Messenger Ribonucleic Acid for the Vitellogenin Receptor in Ovarian Follicles Throughout Oogenesis in the Rainbow Trout, *Onchorhynchus mykiss*. *Biology of Reproduction, 60 (5),* 1057-1068.

Peter, R. E. and Yu, K. L. (1997). Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. *Reviews in Fish Biology and Fisheries, 7 (2),* 173-197.

Pfisterer, U., Kirkeby, A., Torper, O., Wood, J., Nelander, J., Dufour, A., Björklund, A., Lindvall, O., Jakobsson, J. and Parmar, M. (2011). Direct conversion of human fibroblasts to dopaminergic neurons. *Proceedings of the National Academy of Science, 108 (25),* 10343-10348.

Phillippart, J. and Ruet, J. (1982). Ecology and distribution of tilapias. In R. S. Pullin, & R. H. Lowe-McConnell (Ed.), *International Center for Living Aquatic Resources Management, 7*, pp. 15-59. Manila, Philippines.

Pinto, E., Vale-Silva, L., Cavaleiro, C. and Salgueiro, L. (2009). Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species. *Journal of Medical Microbiology, 58 (11),* 1454-1462.

Pirhonen, J. and Schreck, C. B. (2003). Effects of anaesthesia with MS-222, clove oil, and $CO_2$ on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). *Aquaculture 220 (1 to 4),* 507-514.

Prince, A. and Powell, C. (2000). Clove Oil as an Anesthetic for Invasive Field Procedures on Adult Rainbow Trout. *North American Journal of Fisheries Management 20 (4),* 1029-1032.

Psenicka, M., Hadi Alavi, S. M., Rodina, M., Gela, D., Nebesarova, J. and Linhart, O. (2007). Morphology and ultrastructure of Siberian sturgeon (*Acipenser baerii*) spermatozoa using scanning and transmission electron microscopy. *Biology of the Cell, 99 (2),* 103-115.

Psenicka, M., Vancova, M., Koubek, P., Tesitel, J. and Linhart, O. (2009). Fine structure and morphology of sterlet (*Acipenser ruthenus* L. 1758) spermatozoa and acrosin localization. *Animal Reproduction Science, 111 (1),* 3-16.

Quagio-Grassiotto, I., Antoneli, F. N. and Oliveira, C. (2003). Spermiogenesis and sperm ultrastructure in *Cichla intermedia* with some considerations about Labroidei spermatozoa (Teleostei, Perciformes, Cichlidae). *Tissue and Cell, 35 (6),* 441-446.

Quinn, T. P. and Adams, D. J. (1996). Environmental changes affecting the migratory timing of American shad and sockeye salmon. *Ecology, 77 (4),* 1151-1162.

Rainuzzo, J. R., Reitan, K. I. and Olsen, Y. (1997). The significance of lipids at early stages of marine fish: a review. *Aquaculture, 115 (1 to 4),* 103-115.

Reid, S. G., Bernier, N. J. and Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comparative Biochemistry and Physiology, Part C: Pharmacology, Toxicology and Endocrinology 120 (1),* 1-27.

Reznick, D. N. and Ghalambor, C. K. (2005). Can commercial fishing cause evolution? Answers from guppies (*Poecilia reticulata*). *Canadian Journal of Fisheries and Aquatic Sciences, 62 (4),* 791-801.

Reznick, D. and Yang, A.P. (1993). The influence of fluctuating resources on life history: Patterns of allocation and plasticity in female guppies. *Ecology, 74 (7),* 2011-2019.

Ribbink, A. J. (1987). African lakes and their fishes: conservation scenarios and suggestions*. *Environmental Biology of Fishes, 19 (1),* 3-26.

Rochet, M. -J. (1998). Short-term effects of fishing on life history traits of fishes. *ICES Journal of Marine Science, 55 (3),* 371-391.

Roubach, R., Gomes, L. C., Fonseca, F. A. L. and Val, A. L. (2005). Eugenol as an efficacious anaesthetic for tambaqui, *Colossoma macropomum* (Cuvier). *Aquaculture Research, 36 (11),* 1056-1061.

Rutaisire, J. (2003). The reproductive biology and artificial breeding of ningu, *Labeo victorianus* (Pisces: Cyprinidae). *PhD Thesis,* Rhodes University, Grahamstown, South Africa, 183 pp.

Rutaisire, J. and Booth, A. J. (2004). A histological description of ovarian recrudescence in two *Labeo victorianus* populations. *African Journal of Aquatic Science, 29 (2),* 221-228.

Rutaisire, J. and Booth, A. J. (2004). Induced Ovulation, Spawning, Egg Incubation, and Hatching of the Cyprinid Fish *Labeo victorianus* in Captivity. *Journal of the World Aquaculture Society, 35 (3),* 383-391.

Saidapur, S. K. (1978). Follicular Atresia in the Ovaries of Nonmammalian Vertebrates. *International Review of Cytology, 54,* 225-244.

Santos, R. N. dos., Amadio, S. and Ferreira, E. J. G. (2010). Patterns of energy allocation to reproduction in three Amazonian fish species. *Neotropical Ichthyology*. *8 (1),* 155-162.

Sargent, J. R. (1995). Origins and functions of egg lipids: Nutritional implications. In J. R. Bromage and R. J. Roberts, *Broodstock management and egg and larval quality* (pp. 353-372). Wiley-Blackwell, ISBN: 10: 0632035919.

Sivakumaran, K. P., Brown, P., Stoessel, D. and Giles, A. (2003). Maturation and reproductive biology of female wild carp, *Cyprinus carpio*, in Victoria, Australia. *Environmental Biology of Fishes*, *68 (3),* 321-332.

Skoblina, M. N. (2010). Hydration of Oocytes in Teleost Fishes. *Russian Journal of Developmental Biology, 41 (1),* 1-12.

Sladky, K. K., Swanson, C. R., Stoskopf, M. K., Loomis, M. R. and Lewbart, G. A. (2001). Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetics in red pacu (*Piaractus branhypomus*). *American Journal of Veterinary Research, 62 (3),* 337-342.

Smith, J. M., Cridge, A. G. and Dearden, P. K. (2010). Germ cell specification and ovary structure in the rotifer *Brachionus plicatilis*. *EvoDevo Journal*, *1 (5),* 1-10.

Söderström, K.-O. (1981). The Relationship Between the Nuage and the Chromatoid Body During Spermatogenesis in the Rat. *Cell and Tissue Research, 215 (2),* 425-430.

Soivio, A., Nyholm, K. and Huhti, M. (1977). Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout, *Salmo gairdneri*. *Journal of Fish Biology, 10 (1),* 91-101.

Son, H. M., Park, W. M., Myeong, J. I., Kim, D. J., Kim, B. H. and Jo, Q. T. (2001). Anaesthetic tolerance of juvenile black rockfish *Sebastes schlegeli,* produced for wild stock enhancement. *Ocean and Polar Research, 23,* 285-290.

Soto, C. G. and Burhanuddin, S. (1995). Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*). *Aquaculture, 136 (1 to 2)*, 149-152.

Stearns, S. C. (1992). *The Evolution of Life Histories.* New York: Oxford University Press, 264 pp., ISBN: 978-0198577416.

Stearns, S. C. (1983). The Evolution of Life-History Traits in Mosquitofish Since Their Introduction to Hawaii in 1905: Rates of Evolution, Heritabilities, and Developmental Plasticity. *Integrative and Comparative Biology, 23 (1)*, 65-75.

Stoskopf, M. K. (1993). Anaeasthesia. In L. Brown, *Aquaculture for Veterinarians: Fish Husbandry and Medicine* (pp. 161-168). London: Pergamon Veterinary Handbook Series.

Summerfelt, R. C., & Smith, L. S. (1990). Anaesthesia surgery, and related techniques. In C. B. Schreck, & P. B. Moyle (Eds.), *Methods for Fish Biology* (pp. 213-272). Bethesda: American Fisheries Society.

Sumpter, J. P. (1990). General concepts of seasonal reproduction. In A. D. Munro, A. P. Scott, & T. J. Lam, *Reproductive seasonality in teleosts:environmental influences.* (pp. 13-32). Boca Raton FL.: CRC Press, Inc.

Sumpter, J. P. (1997). The endocrinology of stress. In G. K. Iwama, A. D. Pickering, J. P. Sumpter and C. B. Schrec (Eds.), *Fish stress and Health in aquaculture* (pp. 95-118). Cambridge: Cambridge University Press.

Sundby, S. (1997). Turbulence and ichthyoplankton: influence on vertical distributions and encounter rates*. *Scientia Marina, 61 (Suppl.1)*, 159-176.

Sutharshiny, S. and Sivashanthini, K. (2011). Lipid Reserves of *Scomberoides lysan* (Pisces: Carangidae) from the Sri Lankan Waters. *International Journal of Biological Chemistry, 5 (3)*, 170-183.

Takano, K. (1964). On the egg formation and the follicular changes in *Lebistes reticulatus*. *Bulletin of the Faculty of Fisheries, Hokkaido University, Japan, 15 (3),* 147-155.

Talling, J. F. (1966). The Annual Cycle of Stratification and Phytoplankton Growth in Lake Victoria (East Africa). *International Review of Hydrobiology, 51 (4),* 545-621.

Trewavas, E. (1983). *Tilapiine fishes of the genera Sarotherodon, Oreochromis and Danakilia.* British Museum of Natural History, London. Cornell University Press. New Yolk, USA: 487 p.

Turner, R. M. (2003). Tales From the Tail: What Do We Really Know About Sperm Motility? *Journal of Andrology, 24 (6),* 790-803.

Tyler, C. R. and Sumpter, J. P. (1996). Oocyte growth and development in teleosts. *Reviews in Fish Biology and Fisheries, 6 (3),* 287-318.

UBOS. (2011). *The 2012 Uganda population and housing cencus Bulletin.*

Unsicker, K. (1975). Fine structure of the male genital tract and kidney in the Anura *Xenopus laevis* Daudin, *Rana temporaria* L. and *Bufo bufo* L. under normal and experimental conditions. *Cell and Tissue Research, 158 (2),* 215-240.

Velaso-Santamaria, Y. and Casallas, C. (2007). Methods for determination of plasma cortisol in fish using competitive enzyme-linked immunosorbent assay (ELISA). *Revista MVZ Cordoba, 12 (1),* 869-877.

Wagner, E., Arndt, R. and Hilton, B. (2002). Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide. *Aquaculture, 211 (1 to 4),* 353-366.

Wallace, R. A. and Selman, K. (1981). Cellular and Dynamic Aspects of Oocyte Growth in Teleosts. *American Zoologist, 21 (2),* 325-343.

West, G. (1990). Methods of Assessing Ovarian development in Fishes: a Review. *Australian Journal of Marine and Freshwater Research, 41,* 199-222.

Weyl, O., Kaiser, H. and Hecht, T. (1996). On the efficacy and mode of action of 2-phenoxyethanol as an anaesthetic for goldfish, *Carassius auratus* (L.), at different temperatures and concentrations. *Aquaculture Research, 27 (10),* 757-764.

Witte, F., Goldschmidt, T., Goudswaard, P. C., Ligtvoet, W., van Oijen, M. J. and Wanink, J. H. (1991). Species Extinction and Concomitant Ecological Changes in Lake Victoria. *Netherlands Journal of Zoology, 42 (2 to 3),* 214-232.

Wootton, R. J. (1998). *Ecology of teleost fishes* (2nd Edition ed.). (R. J. Woptton, Ed.) Dordrecht, The Netherlands: Kluwer Academic Publishers, 386 p.

Wootton, R. J. (1984). Introduction: tactics and stratagies in fish reproduction. In G. W. Potts, & R. J. Wootton, *Fish reproduction: stratagies and tactics* (pp. 1-12). London: Academic Press.

Yamamoto, M. (1964). Electron microscopy of fish development: Changes in the ultrstructure of the nucleus and cytoplasm of the oocyte during its development in *Oryzias latipes*. *Journal of the Faculty of Science of Tokyo University., 10,* 335-346.

Yamanaka, H., Sogabe, A., Handoh, I. C. and Kawabata, Z. (2011). The Effectiveness of Clove Oil as an Anaesthetic on Adult Common Carp, *Cyprinus carpio* L. *Journal of Animal Veterinary Advances, 10 (2),* 210-213.

Yoshizaki, G., Shusa, M., Takeuchi, T. and Patiño, R. (2001). Gonadotropin-dependent oocyte maturational competence requires activation of the protein kinase A pathway and synthesis of RNA and protein in ovarian follicles of Nibe, *Nibea mitsukurii* (Teleostei, Sciaenidae). *Fish Physiology and Biochemistry, 25 (3),* 201-208.

Zahl, I. H., Kiessling, A., Samuelsen, O. B. and Olsen, R. E. (2010). Anesthesia induces stress in Atlantic salmon (*Salmo salar),* Atlantic cod (*Gadus morhua)* and Atlantic halibut (*Hippoglossus hippoglosssus*). *Fish Physiology and Biochemistry, 36 (3),* 719-730.

Zelazowska, M., Kilarski, W., Bilinski, S. M., Podder, D. D. and Kloc, M. (2007). Balbiani cytoplasm in oocytes of a primitive fish, the sturgeon *Acipenser gueldenstaedtii*, and its potential homology to the Balbiani body (mitochondrial cloud) of *Xenopus laevis* oocytes. *Cell and Tissue Research, 329 (1)*, 137-145.