Effect of dietary lipid sources on the reproductive performance of Nile tilapia *Oreochromis niloticus*.

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By

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Declaration

This work has been conducted exclusively by my own research. Work in this thesis has neither been accepted nor is being submitted for any other degree. Work and analysis in this thesis has been conducted independently unless otherwise acknowledged.

Candidate:……..

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Date :……..

Abstract

Traditionally, fish oil (FO) has been used extensively in aquafeeds. The stagnation in global fish oil production coupled with an increased demand for its use in aquaculture feeds, especially salmonid feeds, has greatly inflated fish oil prices. Therefore, in order to sustain the rapid growth of the tilapia industry, the dependence on these commodities in feeds should be reduced through use of cheaper and more sustainable sources of dietary lipids, such as palm oil. This study therefore investigated several, previously poorly understood, effects of palm oil on reproductive performance of the commercial tilapia species, *Oreochromis niloticus*; which currently ranks as second most popular species in world aquaculture.

In the present study broodstock were fed on experimental diets at full and half ration regimes throughout their entire life cycle from exogenous feeding. Studies were conducted in standardised and controlled hatchery conditions, thereby reducing the potential influence of environmental variations. First feeding *O. niloticus* fry were fed on four diets, cod liver oil (D 1), palm oil (D 2), mixed palm and cod liver oil (D 3) (9:1 ration) and a commercial trout diet as control (D 4) (Skretting, U.K.) on a reducing ration based on fish size. The present study investigated the effect of dietary lipid sources on (1) growth performance, (2) biochemical composition of eggs (total lipid and fatty acid composition), (3) morphological parameters of eggs (total and relative fecundity, egg size, egg weight and EW:BW), (4) larval quality (larval length and weight) and (5) oocyte recruitment and its associated sex steroid hormones. Experimental diets and feeding ration significantly influenced ($p<0.05$) the growth performance over a period of 120 days.

Total lipid and fatty acid composition of eggs originating from broodstock fed on palm oil, mixed palm and cod liver oil (9:1) or a control diet were not significantly different ($P>0.05$) when fed at either full (3% $BWday^{-1}$) or half ration (1.5% $BWday^{-1}$). The present study, however, confirmed that fatty acid composition of fish eggs reflected the fatty acid composition of the diet, although specific fatty acids were selectively utilized or retained in the eggs.

The mean inter-spawning interval (ISI) increased with increasing fish size and averaged 14, 19 and 24 days for fish fed on palm oil, mixed palm and cod liver oil or control diets, respectively. The shortest ISI observed was 7 days for fish fed a palm oil diet.

Total fecundity ranged from 660 - 820 eggs/clutch. Mean total fecundity was 750, 820 and 660 eggs/clutch for fish fed a palm, mixed palm and cod liver oil or a control diet, respectively, but these differences were not significant ($P>0.05$). However, relative fecundity and egg weight to body weight rates as a percentage (EW: BW) were found significantly differ ($p<0.05$) between fish fed the control diet and experimental diets. Mean egg diameter (2.2 mm) was not significantly influenced ($p>0.05$) by experimental diets. The egg volume, egg dry and wet weight, fertilisation and hatching rate were also not significantly different between fish fed the experimental diets.

Oocyte development was classified into distinct stages based upon oocyte size, biochemical properties and structure. The recrudescence to these stages was not significantly influenced by broodstock fed experimental diets either at full or half ration. Steroid hormones and histological analyses provided valuable data concerning the oocyte development and recruitment in this species. Levels of 17ß-oestradiol (E2) and testosterone (T) peaked within 6 days of spawning, suggesting that vitellogenesis began as early as day 2 or 3 post-spawning. By day 6, ovaries were dominated by large late-

vitellogenic/maturing oocytes (stages 6 & 7) occupying about 70% of the ovary. Gonadosomatic index (GSI) reached maximal levels by day 6. It is suggested that pre-vitellogenic oocytes are recruited into vitellogenic growth immediately after spawning and complete vitellogenesis on day 6 post-spawning.

Finally, the present study investigated the effect of food restriction at two rations (full and half) on broodstock reproductive performance. *Oreochromis niloticus* were rationed from first feeding and throughout their life-cycle. The dietary regime, full ration (3%) and half ration (1.5%), influenced fish size but despite this variation no significant differences ($p>0.05$) were detected in total lipid and fatty acid composition in the eggs, total fecundity, egg diameter, total egg volume and larval size. These results suggested that despite large differences in food availability throughout their life cycle, investment in reproduction had remained remarkably consistent. It appeared that during food restriction, *O. niloticus* sacrificed body weight and growth so as to maintain reproductive investment.

In summary, this study provides valuable information using a novel experimental design on the effects of dietary lipid sources on reproductive performance of female *O. niloticus.* Substituting palm oil for fish oil as the dietary lipid source and reducing ration by half (1.5% $BWday^{-1}$) had no significant effect on reproductive performance. Therefore it is suggested that under controlled conditions, lipids of non-marine origin, such as palm oil, can be successfully substituted for broodstock diets. Halving feed requirement should also increase profitability of seed production.

KEYWORDS: Tilapia; *O. niloticus*; palm oil; diet; fecundity; spawning periodicity; oocyte recruitment; reproductive performance.

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Glossaries

Glossary of scientific and common names of fish mentioned in this thesis

Note: some species of fish (especially tilapia) do not have widely recognised common names. These species are indicated here by a dash (-) in place of the common name.

Scientific Name	**Common Name**
Acanthobram terrae-sanctae	-
Acipenser transmontanus	White sturgeon
Anguilla anguila	European eel
Brachydanio rerio	Zebrafish
Carassius auratus	Goldfish
Carassius carassius	Crucian carp
Chelidonichtys kumu	Red gurnard
Chromis dispilus	Damelfish
Cichlasoma citrinellum	Midas cichlid
Cichlasoma nigrofasciatus	Convict cichlid
Clarias gariepinus	Afrcan catfish
Clupea harengus	Atlantic herrng
Clupea harengus pallasi	Pacific herrng
Colisa lalia	Dwarf gourami
Cottomephorus grewingki	Yellowfish Baikal sculpin
Cymatogaster aggregata	Sea perch
Cynoscion nebulosus	Spotted sea trout
Cyprinus carpio	Common carp
Dicentrarchus labrax	Sea bass

Esox lucius	Pike
Etroplus suratensis	Pearlspot
Engraulis japonica	Japanese anchovy
Fundulus confluentus	Marsh killifish
Fundulus heteroclitus	Killifish
Gadus morhua	Cod
Gasterosteus aculeatus	Three-spined stickleback
Gobius jozo	Black goby
Haplochromis 'argens'	-
Heteropneustes fossilis	Indian catfish
Hippoglossus hippoglossus	Atlantic halibut
Lates niloticus	Nile perch
Leuciscus leuciscus	Dace
Lepomis gibbosus	Pumpkinseed sunfish
Leptocottu anntus	Staghom sculpin
Limand limanda	Dab
Lutjanus campechanus	Red snapper
Maccullochella macquariensis	Australian freshwater trout cod
Melanogrammus aeglefinus	Haddock
Merlangius merlangius	Whting
Micropogonias undulatus	Atlantic croaker
Micropterus salmoides floridanus	Largemouth bass
Mugil cephalus	Grey mullet
Mycteroperca olfax	Galapagos bacalao
Oncorhynchus gorbuscha	Pacific pink salmon
Oncorhynchus keta	Chum salmon
Oncorhynchus kisutch	Coho salmon

Oncorhynchus mykiss	Rainbow trout
Oncorhynchus nerka	Kokanee salmon
Oncorhynchus rhodurus	Amago salon
Oncorhynchus tschawytscha	Chinook salmon
Oreochromis aureus	Blue tilapia
Oreochromis esculenta	-
Oreochromis leucosticta	-
Oreochromis macrocephala	-
Oreochromis mossambicus	Mozambique mouthbrooder
Oreochromis niloticus	Nile tilapia
Oreochromis spilurus niger	-
Oryzias latipes	Medaka
Pagrus auratus	Snapper
Pagrus major	Red sea bream
Perea jlavescens	Yellow perch
Psetta maxima	Turbot
Petromyzon marinus	Sea lamprey
Pimeophales promelas	Fathead minnow
Pleuronectes platessa	Plaice
Pseudopleuronectes americanus	Winter flounder
Poecilia monacha	Top minnow
Poecilia reticulata	Guppy
Pterophyllum scalare	Angelfish
Rutilus rutilus	Roach
Salmo salar	Atlantic salmon
Salmo trutta	Brown trout
Salvelinus alpinus	Arctic char

Salvelinus fontinalis	Brook trout
Sardinops melanostictus	-
Sarotherodon galieus	-
Sarotherodon leucostietus	-
Sarotherodon melanotheron	Black-chinned tilapia
Scomber scombrus	Atlantic mackerel
Seriola dumerilii	Mediterranean yellowtail
Solea solea	Dover sole
Sparus auratus	Gilthead sea bream
Symphysodon aequi fasciata axelrodi	-
Thunnus albacares	Yellowfin tuna
Tilapia guineensis	-
Tilapia marocephala	-
Tilapia marie	-
Tilapia rendalli	-
Tilapia tholloni	-
Tilapia zilii	Redbelly tilapia
Trichogoita trichopterus	Blue gouram

Glossary of commonly-used abbreviations and acronyms

Abbreviation	Full Term
E1	Oestrone
E2	17 ß-oestradiol
E3	Oestrol
FSH	Follicle stimulating hormone
GSI	Gonadosomatic index
GTH	Gonadotropin
HSI	Hepatosomatic index
LH	Luteinizing hormone
MIS	Maturation-inducing steroid
ND	Numerical density
POF	Post-ovulatory follicle
SE	Standard error
T	Testosterone
VF	Volume fraction
VTG	Vitellogenin
3β-HSD	3ß-hydroxysteroid dehydrogenase
11-KT	l1-ketotestosterone
17α-OH-P	17 α -hydroxy-progesterone
17α-20ß-P	17 α -20ß-dihydroxy-pregnen-3-one
Approx.	Approximately
ANOVA	Analysis of variance
Cm	Centimetre

^{o}C	Degree centigrade/degree Celsius
DCP	Dicalcium phosphate
DHA	docosahexaenoic acid (22:6n-3)
DPH	days post hatch Dicalcium phosphate
EFA	Essential Fatty Acid(s)
e.g.	"exempli gratia" (Latin) translates to "for example
Etc.	"et cetera" (Latin) translates to "and other things"
et al.	"et alii" (Latin) translates to "and others"
FA	fatty acid(s)
FAO	Food and Agriculture Organization
FO	fish oil
G	gram
GLM	general linear model
GSI	Gonadosomatic Index
HCl	hydrochloric acid
HSI	Hepatosomatic Index
PUFA	Poly Unsaturated Fatty Acid(s)
i.e.	"id est" (Latin) translates to "in other words"
Inc.	Incorporated
IOA	Institute of Aquaculture, University of Stirling
ID	identification
KCl	potassium chloride
$KHCO_3$	potassium hydrogen carbonate
Kg	kilogramme

L / l	litre
M	mole / molar
mg	milligram
$MgCl_2$	Magnesium chloride
min	minute
ml	millilitre
mm	Millimetre
No.	number
ng	nanogram
NRC	nutrition research council
Ltd.	Limited
P	probability
PF	post-fertilisation
PIT	passive integrated transponder
PO	palm oil
ppm	parts per million
PUFA	Poly-Unsaturated Fatty Acid(s)
RPM	revolutions per minute
S	second
SD	standard deviation
SE	standard error
SPSS	Statistical Package for the Social Science
T	T-test value
UK	United Kingdom
URL	uniform resource locator
UV	ultra violet
USA	United States of America

vs.	Versus
µg	Microgram
µl	Microlitre
µm	Micrometre
%	Percent

Chapter 1 - Introduction, literature overview and aims of research

1.1 General introduction

1.1.1 Importance of tilapia in aquaculture

Around one billion people worldwide rely upon fish as their source of animal protein with this number increasing due to population growth, especially in developing countries. Whilst recovery programs for capture fisheries have been implemented for global food security, farm-raised fish must help fill the growing gap between aquatic food supply and demand (FAO, 2002). This objective can be achieved by increasing the area and production efficiency of cultured species. Cultured species usually have a limited range of tolerance for environmental factors; therefore they can be cultured only in special areas within an optimum range of environmental factors for their growth. This is particularly emphasised in the case of mouth-brooding tilapia, which is one of the best candidates for aquaculture for the twenty first century, and is currently the second most farmed species group in terms of quantity after carp (Fitzsimmons, 2006) .

Over recent years, the culture of tilapiine fishes (Family: Cichlidae) have steadily increased, making it one of the most commercially important groups of freshwater species in tropical and subtropical aquaculture. It now represents a major protein source in many developed and developing countries. From the 1950's to mid 1970's tilapia were exported from their native regions in Africa, widening their range to over 100 tropical and subtropical countries including the Americas, the Middle East and Asia (Balarin and Haltton, 1979; Jauncey *et al.,* 1985; Kwon *et al.,* 2000; Lowe-

McConnell, 1982; Lowe-McConnell, 2000; Pullin, 1983; Pullin *et al.,* 1986). In particular, the natural distribution of the Nile tilapia (*Oreochromis niloticus* L. 1758) has spread from a region in the Nile valley to Central and Western Africa (via the Chad and Niger basins) and South to the Ethiopian lakes and Lake Turkana (Bromage and Roberts, 1995).

Tilapia species offer immense potential to aquaculture due to a number of biological characteristics: acceptance of formulated feeds, efficient food conversion ratios (Jauncey, 2000), tolerance of handling (Little, 2000), tolerance of high stocking densities (Popma and Masser, 1999), tolerance of marginal water quality (Fitzsimmons, 2000), year-around spawning (Beardmore *et al.,* 2001), a relatively short reproductive cycle, breeding prolifically under culture conditions, strong resistance to disease and infection (Dempster *et al.,* 1995; Legendre *et al.,* 1995), high growth rate (Cross, 1976; Guerrero, 2004), and a high market demand (Harvey, 2005). These attributes facilitate the husbandry of tilapia and their management under farmed conditions. Collectively these attributes have allowed the tilapia to become one of the most successful cultured species (Cross, 1976; Guerrero, 2004).

1.1.2 Importance of biology of Nile tilapia *O. niloticus* in aquaculture

The Tilapiine tribe of more than 70 different species has been reclassified into three separate genera (Trewavas, 1983): Tilapia (substrate spawners), *Sarotherodon* (paternal or biparental mouthbrooders) and *Oreochromis* (maternal mouthbrooders) mainly based on their reproduction, feeding habits and biogeography (Macintosh and Little, 1995; Rana, 1988; Turner and Robinson, 2000). Throughout this thesis, species of tilapia will be referred to by their scientific names since few have widely recognised common names (see Glossary, page xx). However the common name

'tilapia' and/or 'Nile tilapia' is used for convenience throughout the text to describe *O. niloticus*.

The majority of tilapias featuring significantly in aquaculture belong to the *Oreochromis* genus. Unlike salmonids, Chinese and common carp, seed production of tilapia (*Oreochromis* species) still relies on natural spawning under captive conditions in ponds, tanks or raceway culture systems. These mouth-brooding tilapias have adopted the anti-predator tactic of rearing their eggs and fry in the relative safety of the parental buccal cavity (Rana, 1988). This strategy reduces the total fecundity, which is governed by the size of the buccal cavity, which dictates the amount of eggs that the fish will incubate in its mouth. Unlike many other species of teleost, tilapia exhibit high levels of parental care (Fryer and Iles, 1972; Hulata, 1982; Trewavas, 1983).

In this group, males build and defend territories within a defined spawning area; this area is defined as a "Lek" or "arena" (Fryer and Iles, 1972; Macintosh and Little, 1995; Turner and Robinson, 2000). The "Lek" or arena is built by the fish by pushing sediment to the side using the snout to create a small nest or stirring the area with pectoral fins, cleaning a large area, and then ejecting and removing sediment using the fish's mouth (Turner and Robinson, 2000). Once the lek is established, a female enters and starts nuptial courtship with the male. Courtship between resident males and females in the lek is relatively short, lasting only, a few hours (Rana and Macintosh, 1988), and results in batches of eggs being spawned into shallow nests. Eggs fertilised by the male are picked up by the female and incubated in her mouth. Directly after spawning the female leaves the nest and rears her clutch until the fry are free-swimming (Rana and Macintosh, 1988). This is followed by a period of extended

maternal care in which free-swimming fry may seek shelter in the female's mouth. After this, the female begins a convalescence period, in which she feeds intensively for 2 or 4 weeks (Macintosh and Little, 1995). Most *Oreochromis* species exhibit a significant degree of sexual dimorphism and dichromatism, males are longer than female at the same age and they present bright colours when breeding (Macintosh and Little, 1995; Oliveira and Almada, 1995).

Under natural conditions, tilapia have a tendency for seasonality in their reproductive activity. Under controlled conditions, however, this seasonality is eliminated. Fryer and Iles (1972) indicate that, in general, tilapia under natural conditions mature in 1-3 years, but the age of maturation is strongly influenced by the volume of the water where they live. It was reported that *O. niloticus* matured at 39 cm in a large water body such as Lake Rudolf (currently Lake Turkana); however maturation was reached at 17 cm in Lake Edward.

In the tilapia, the onset of maturation under optimal conditions is reached at an early stage, usually when fish have reached an average weight of 15 to 100g, or within at 2 to 4 months of age (Alvendia-Casavay and Carino, 1988; de Graaft *et al.,* 1999; Macintosh and Little, 1995; Mair and Little, 1991; Mires, 1983). Most tilapias are able to produce a successive series of spawns, producing new generations at intervals of 4 to 6 weeks the sexual maturation is reached and if environmental conditions are favourable (Campos-Mendoza *et al.,* 2003; Jalabert and Zohar, 1982; Macintosh and Little, 1995). Nevertheless, tilapia culture is also restricted by their reproductive strategy; this predominantly includes early maturation, lack of spawning synchrony and low fecundity (Campos-Mendoza *et al.,* 2004; Coward and Bromage, 2000; Coward and Bromage, 1999b; Little *et al.,* 1993; Macintosh and Little, 1995; Mair,

1993). Early or precocious maturation triggers significant unwanted reproduction and leads to overcrowding of the growing tanks (Macintosh and Little, 1995; Mair and Little, 1991).

In order to minimise this problem, fish farmers have adopted mono-sex culture using only male populations. Many investigations have demonstrated that male tilapia grow faster than females (Bhujel, 2000; Guerrero, 2004; Mair *et al.,* 1997; Mair and Little, 1991; Mair *et al.,* 1995).

Sexually mature Cichlidae are generally able to undergo successive reproductive cycles at intervals of 4-6 weeks. Theoretically, this should lead to an almost continuous production of fry assuming that seasonal environmental variations remained minimal (Moreau, 1979). Under intensive farming conditions, however, the asynchronous nature of the reproductive cycles of individual broodstock may lead to spasmodic fry production to the detriment of total farm output (Jalabert and Zohar, 1982). Low fecundity and asynchronous spawning patterns by female broodstock inevitably lead to the need for extensive fish holding facilities to even approach satisfactory output and often requires careful and time consuming management of a very large number of brood fish.

1.2 Tilapia production and culture systems

Tilapias are cultured using various methods ranging from traditional pen, hapa, cage and pond culture to more intensive tank and raceway culture systems. The rapid increase in tilapia production worldwide can be explained by their many physiological attributes that make them highly suitable to aquaculture. Tilapias can adapt to a variety of environmental conditions and, in nature, they have colonised widely

different habitats with a varied range of physical characteristics, in temperature and chemical composition of water (Philipart and Ruwet, 1982). Tilapias are thermophilic fishes (Philipart and Ruwet, 1982) but can tolerate a wide range of environmental water temperatures (eurythermal). Some tilapia species can tolerate environmental water temperatures of over 40^{o}C in some instances (Balarin and Haltton, 1979; Wang and Tsai, 2000), although mortality usually occurs at temperatures above 38^{o}C (Chervinski, 1982).

As a general rule, tilapias do not grow well at temperatures below 16^{o}C, exhibit poor survival below 10^{o}C (Chervinski, 1982) and will not spawn at temperatures below 20-23^{o}C (Behrends *et al.,* 1990). However, some tilapia species (*e.g. Oreochromis aureus*, *Oreochromis mossambicus* and *Tilapai rendalli*) can withstand a short term exposure to cold water to even 8-9^{o}C (Chervinski, 1982). In practical terms, few problems with temperature are encountered by tilapia culturists in tropical environments. However, in subtropical and temperature climates culture necessitates the over-wintering of broodstock and fry and some source of warm water must be provided. Over-wintering of tilapia fry has been successfully achieved using heated facilities (Behrends *et al.,* 1990) or underground warm water (Cruz and Ridha, 1994) and in greenhouses (Jiazhao, 1991).

Although tilapias are freshwater species, some tilapias can also be cultured in brackish and even full-strength seawater. *O. aureus* cultured in seawater exhibited growth rates similar to those obtained in freshwater environments (Chervinski, 1982) while the ability of *Tilapia zillii* to acclimate and grow in seawater is even better (Chervinski, 1982). *Oreochromis niloticus* is the least saline tolerant of the commercial species, but

also been reported to grow well at salinities of 18 to 25 ppt (El-Sayed *et al.,* 2005; Suresh and Lin, 1992).

1.3 Factors influencing tilapia reproduction

Fish reproduction is affected by different external or internal factors: the internal factors are more related to the endocrinology of the fish whilst the external stimuli influence their reproductive physiology and behaviour. These parameters include water temperature, water quality (*e.g.* dissolved oxygen, pH, salinity and hardness) photoperiod, and nutritional status of the fish and, in some cases, the rainy season (Bromage *et al.,* 1992; Bromage *et al.,* 2001; Coward and Bromage, 2000). These factors will be described briefly below, with particular emphasis on tilapia species.

1.3.1 Exogenous (external) factors

1.3.1.1 Temperature

Temperature is recognised to have a notable influence on the overall reproductive cycle in most fish species (Brown *et al.,* 2006; Chervinski, 1982), and may also play an important part in the final stages of maturation of many teleosts (El-Gamal *et al.,* 2005; Huet, 1972; Stacey, 1984). It also influences many fluctuations of the teleost reproductive system, for example the rate of secretion and clearance of the pituitary gonadotropin (GtH) (Yaron *et al.,* 2001).

The tropical origin of tilapia is clearly expressed in their ecological and physiological preferences, especially in terms of temperature preference during the reproductive period (Chervinski, 1982). Reproduction occurs successfully above 22ºC, and this is the reason why the natural distribution of tilapias is restricted to the tropical regions (Chervinski, 1982). In some specific areas in the tropics and subtropical regions *e.g.*

high altitude, low temperature can inhibit tilapia reproduction during certain parts of the year. In these cases, the duration of the reproduction season is conditioned and the breeding season occurs within the hottest months of the year (Philipart and Ruwet, 1982).

In high altitudes (1300-2500 m above sea level), *O. niloticus* shows a different pattern of reproduction. The age at first maturity is higher under these conditions; Hanson *et al.* (1983) demonstrated that in *O. niloticus* first maturity occurs after 6 to 9 months or approximately 231 g in weight. Therefore, it seems that temperature plays a crucial role in the regulation of tilapia reproduction (Little *et al.*, 2000).

Temperatures higher than 20^{o}C are required to stimulate tilapia reproduction, however a range of 20-30^{o}C has been reported for *O. niloticus* and *O. aureus*, and 20-35^{o}C for *O. mossambicus* (Philipart and Ruwet, 1982; Rana, 1988; Rothbard, 1979; Rothbard and Pruginin, 1975). High temperature led to a reduction in spawning activity, *e.g.* in *O. niloticus* a temperature of 33-35^{o}C reduced egg quality and hatching success in a hapa-based culture (Little *et al.*, 2000).

1.3.1.2 Salinity

As a general rule, fecundity in tilapia decreases as salinity increases. During a six month period in sea water, *O. niloticus* failed to spawn or build nests and a sharp reduction in GSI was observed (Balarin and Haltton, 1979; Bhujel, 2000). Most tilapia species are very sensitive to high salinities and reproduction is clearly inhibited by high salinities. Possible reasons for this may be osmotic stress on the eggs (Balarin and Haltton, 1979). On the other hand, Chervinski (1982) reported that several species of tilapia breed successfully at high salinities; (*e.g. Tilapai zillii* and *O. mossambicus*);

both species are known to reproduce at high salinities of 10-26 and 35 parts per thousands (‰), respectively (Philipart and Ruwet, 1982).

Tilapias are also found in estuaries and coastal lagoons along the coast of West and East Africa. Some species are endemic in high salinity lakes such as Lake Natron, and Lake Manyara whereas some tilapia species such as *T. sparrmani, O. acrochir, O. andersonii* and *Tilapia rendalli* are less tolerant to salinity and move to rivers or tributaries of lakes or water bodies in order to avoid osmotic stress when salinity levels increase (Philipart and Ruwet, 1982).

A further report stated that, female tilapia broodstock could be kept in saline water during the time of low seed demand and this suppress spawning activity (El-Sayed, 2006; Suresh and Lin, 1992). An increase spawning activity was observed just after fish were re-stocked in fresh water (Balarin and Haltton, 1979; Bhujel, 2000).

1.3.1.3 Dissolved oxygen

Tilapias can survive at low levels of dissolved oxygen (DO) as they can utilise atmospheric oxygen (Chervinski, 1982; Popma and Lovshin, 1996); however, low DO levels at dawn have been associated with reduced growth (Chervinski, 1982). Some indications have been found that low DO (<0.5 mg L^{-1}), generally occurring at dawn in green water systems, has negative impacts on the quantity and quality (Davies *et al.,* 1994). Low dissolved oxygen causes stress, which brings behavioural (*e.g.* gasping), and morphological changes in the fish (*i.e* marked melanin pigmentation in the skin) and a reduced feed intake. Moreover, it increases disease incidence and impairs reproduction, causing oocyte atresia, spawning inhibition, decreased fecundity and hatchability (Wedemeyer *et al.,* 1990).

1.3.1.4 Photoperiod

Photoperiod is the period of time per day that an organism is exposed to light. Seasonal changes in photoperiod are caused by variation in the angle of radiation striking the earth as it orbits the sun (Frantzen *et al.,* 1997). Seasonal photoperiod changes in the tropics are not pronounced but are more evident at higher latitudes. The photoperiod of captive fish in commercial and experimental facilities is artificially manipulated by controlling the timing intensity. Photoperiod is an important factor controlling the seasonal reproductive cycle of many fish species (Bromage *et al.,* 2001; Little *et al.,* 2000; Wootton, 1982).

Delaying spawning and acceleration of maturation in salmonids and cyprinids by manipulating photoperiod has been well documented (Bromage *et al.,* 2001; Bye, 1984), and has been considered as the most thoroughly investigated environmental factor influencing fish reproduction (Hansen *et al.,* 2001; Yaron *et al.,* 1980). Gonadal development and subsequent breeding in tilapia have been reported to be well correlated with duration of sunlight in natural waters (Guerrero, 2004; Hyder, 1970).

Many investigators have used photoperiod manipulation to control and alter the normal spawning cycle in salmon *e.g.* in rainbow trout (*Oncorhynchus* mykiss), (Bromage *et al.,* 1992; Duston and Bromage, 1986; Duston and Bromage, 1988; Duston and Bromage, 1987; Whitehead *et al.,* 1983), Atlantic cod (Norberg *et al.,* 2004), Atlantic salmon (*Salmo salar*), (Hansen *et al.,* 1992; Hansen *et al.,* 2001; Logan and Johnston, 1992) and *O. niloticus* (Biswas *et al.,* 2005; Campos-Mendoza *et al.,* 2004).

1.3.1.5 Rainfall

In tropical and subtropical regions, in which the change in day-length is not strongly marked, tilapia reproduction seems to be influenced by rainfall; in some cases reproductive activity is reported to occur just after the rainy season (Philipart and Ruwet, 1982). Bhuje (2000) reported that an increased seed output was observed during the rainy season probably due to relatively cool temperatures, increased water levels and dilution of hormones or chemical inhibitors and waste metabolites. Hyder (1970) reported that long periods of heavy rainfall caused a negative effect on spawning activity. However, artificial rain, produced by sprinklers in spawning hapas has been reported to increase spawning activity, especially during dry months. In tilapia, higher frequency of spawning (Hulata, 1982) or spawning intensity (Philipart and Ruwet, 1982) and egg output (Welcomme, 1967) have been reported during the rainy season in the tropics. In nature, tilapiine fish are known to breed throughout the year, with minor peaks of activity occurring in relation to environmental factors such as rainfall patterns (Brummett, 1995). In contrast, captive tilapia may spawn throughout the year as long as environmental conditions remain suitable (Brummett, 1995; Fishelson, 1966; Lowe-McConnell, 1959; Lowe-McConnell, 2000).

1.3.1.6 Water change

Regular changes of water within the breeding systems tend to lead to an improvement in reproductive behaviour in tilapias, possibly due to the flushing of metabolite build-up and a subsequent increase in dissolved oxygen levels (Bhujel, 2000; Billard and Breton, 1978; Mires, 1982; Rothbard and Pruginin, 1975). Bhujel (2000) further reported that higher seed production can be obtained from ponds filled with fresh water compared with those ponds maintained with the same water for a long period of time.

1.3.1.7 Food availability and nutritional status of fish

In tilapia culture, the quantity and quality of food affects both the frequency of spawning and the total seed production per spawn (Behrends and Smitherman, 1983; Hughes and Behrends, 1983; Hulata, 2001; Little *et al.,* 2000; Macintosh and Little, 1995; Rana, 1988). It has also been reported that *O. niloticus* broodstock fed with a high protein level (20-25%) exhibit early spawning as well as an improvement in spawning frequency. It is also known that low food ration has induced early maturation and spawning frequency (De Silva *et al.,* 1991). Brooks (1997) indicated that dietary protein and lipids levels influence oocyte development and egg quality. Gunasekera *et al.* (1996) found that *O. niloticus* fed with a high protein level diet spawned a significantly higher number of eggs than those fish fed a low crude protein level.

Gunasekera *et al.* (1996) reported that no differences were found in the size of the eggs between two different protein levels, but larval quality was significantly higher in females fed on a high protein (35%) than in those fish fed on a low protein (20%) diet. The dietary protein levels also affected inter spawning interval (ISI), (*i.e* time between 2 consecutive spawning in the same fish). Fish fed with higher protein levels (35%) spawned every 16-20 days. Similar findings were reported by Bhujel (2001b); the number of spawnings produced by *O. niloticus* was higher in those fish fed higher protein levels. It has been suggested that in most of the hapa-based hatcheries reproductive activity utilising diets containing 25-30% crude protein levels in broodstock. This level of protein in the diet is nutritionally adequate as well as being cost effective (Bhujel *et al.,* 2001a). Santiago (1985) showed that a high dietary protein level (40%) enhanced reproductive performance (total and relative fecundity) in bighead carp (*Aristichthys nobilis*).

In teleosts, numerous nutritional related factors such as feed ration, nutrient levels and composition have been shown to influence various reproductive parameters such as gonadal development, egg quantity and quality, spawning success, hatchability and larval quality (Izquierdo *et al.,* 2001a; Watanabe and Vassallo-Agius, 2003). Food restriction itself can seriously affect spawning success. A reduction in feeding rate has been reported to cause an inhibition of gonadal maturation in several fish species, including goldfish (*Carassius auratus*) (Sasayam and Takahashi, 1972), European Sea bass (*Dicentrarchus labrax*), (Cerdà *et al.,* 1994b) and male Atlantic salmon (*Salmo salar*) (Berglund *et al.,* 1991). In sea bass, after 6 months of feeding broodstock with a half food ration, reduced plasma estradiol levels were seen, growth rates decreased and spawning time was delayed and eggs as well as newly hatched larvae were smaller than those obtained from fish fed full rations. Food restriction was also found to correlate with a low percentage of egg fertilisation and hatchability (Cerdà *et al.,* 1994a). In the tilapia hybrids (*O. niloticus* × *O. aureus*) fed with four different rations (0.5, 1, 2 and 3% of body weight per day) total fecundity and relative fecundity decreased with decreasing food ration size; the females fed on low ration size (0.5% of body weight per day) spawned earlier than those fish fed with the higher rations (Siddiqui and Al-Harbi, 1999)

Lipids and their fatty acid composition have essential and dynamic roles in the maintenance of optimum growth, feed efficiency, health, kidney and gill function, neural and visual development, reproduction, and flesh quality (market size) of finfish species (Higgs and Dong, 2000). Provision of lipids to oocytes, followed by storage and accumulation in the yolk and subsequent utilisation by developing embryos are essential processes in reproduction and development (Brooks *et al.,* 1997). Studies in the past two decades involving a variety of farmed fish species have identified lipid,

and in particular polyunsaturated fatty acids (PUFA), as key nutrients affecting broodstock reproductive performances (Bell and Sargent, 2003; Watanabe and Vassallo-Agius, 2003). Polyunsaturated fatty acid (PUFA) plays an integral role in regulating levels of eicosanoids, which in turn control selected stages of reproduction such as steroidogenesis and ovulation (Sorbera *et al.,* 2001).

1.3.2 Endogenous factors

The endocrine control of reproduction of both female and male fish works in such a way that the signals from exogenous factors and endogenous physiological cycles impact the neuroendocrine system, which in turn regulates pituitary and gonadal functions (Sumpter *et al.,* 1984). The main endogenous hormones which control reproduction in fish are hypothalamic gonadotropin releasing hormone (GnRH), gonadotropin release inhibitory factor (GnRIF), pituitary gonadotropin (GtH), thyroids, gonadal sex steroids, and prostaglandin. Knowledge of these hormones and their affects on the control of reproduction helps design techniques that could be applied to control the reproduction of a captive broodstock.

1.3.2.1 The brain-pituitary-gonadal axis of teleosts

The brain-pituitary-gonadal (BPG) axis is composed of three physiologically and hierarchically connected structures directly involved in the control of reproduction in vertebrates (Gore, 2002; Weltzien *et al.,* 2004). At all levels of this axis there are a number of key hormones being synthesized and released which act on target tissues within the brain, the liver or the gonad, which has been reviewed in fish by Davies *et al.* (1999) and Weltzien *et al.* (2004). In brief, gonadotropin-releasing hormone (GnRH) is produced by a specific group of GnRH neurons in the preoptic area (POA)

of the brain which affect the pituitary directly, thus stimulating production of two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). As these hormones enter the circulation and reach the steroidogenic cells in the gonads (follicular cells in the oocytes and sertoli cells in the testes) they in turn stimulate gonadal development (growth and final stages of maturation) by promoting the production of sex steroids and growth factors. Both sex steroids and growth factors are crucial for the regulation of reproduction, either directly in the gonad (paracrine, autocrine) or through positive and negative feedback mechanisms on the hypothalamus and pituitary (Gore, 2002; Weltzien *et al.,* 2004; Yamamoto *et al.,* 1998; Yamamoto, 2003) (Figure 1.1).

1.3.2.2 Sex steroids

It is now well accepted that oogenesis, especially vitellogenesis and oocyte maturation in many teleost species, is regulated by ovarian steroids (Fostier *et al.,* 1983; Olivereau and Nagahama, 1983). The gonads are the end point of the reproductive cascade and serve two main functions; germ cell development (spermatogenesis and oogenesis) and steroidogenesis/growth factor production. Spermatogenesis and oogenesis mark the beginning of puberty and subsequent reproductive cycles. Sex steroids, such as 11-KT in males and the estrogen 17β-estradiol (E2), which is regulated by aromatase activity, in females have a crucial role in the stimulatory and regulatory effects of these processes, not only at the gonadal level (paracrine) but also as feedback to the brain and pituitary level where they are known to exert their effects by stimulating or inhibiting gonadotropin release (LH and FSH) (Mateos *et al.,* 2002; Pankhurst, 1998; Pawson and McNeilly, 2005; Weltzien *et al.,* 2004; Young *et al.,* 2005).) Figure 1.1.

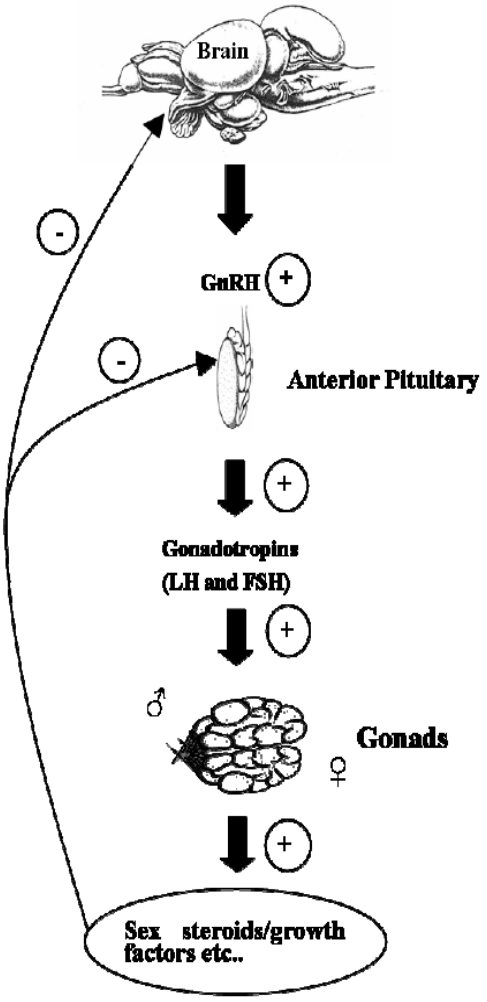


Figure 1.1 Simplified diagram of the BPG axis. The axons of GnRH neurons located in the hypothalamus project to the median eminence where they secrete the decapeptide into the portal vessels of the anterior pituitary. This induces the release of gonadotropins (leuteinizing hormone (LH) and follicle-stimulating hormone (FSH)) into the general circulation, which, in turn, stimulates gonadal functions and the production of sex steroids and inhibiting. Sex steroids and inhibiting exert negative feedback (-) at the level of the pituitary and hypothalamus. Positive (up regulatory) elements are marked by (+). Modified from Weltzien *et al.* (2004) and Cariboni *et al.* (2007).

1.3.2.3 Ovarian recrudescence in teleost fish

In general, gonadal recrudescence is the recruitment of the oocytes after the act of spawning. In teleosts, ovarian development and the ultimate production of mature eggs is a highly complex process, modulated by various environmental and endocrine

pathways such that offspring are produced at times when fry survival is optimal, usually when food availability is highest. In most teleosts ovarian development has been classified as synchronous or asynchronous, and has been classified according to histological appearance of oocytes (Bromage and Cumaranatunga, 1988; Scott, 1987). Fish that are considered synchronous generally have two developing oocyte stages in their ovary at any time; larger secondary oocytes that will be recruited in the coming season, and primary oocytes recruited at later phases (Scott, 1987). Fish, such as salmonids, have two different patterns of spawning, yearly such as in brown trout, (*Salmo, trutta*), (Bagenal, 1969), or once in their life cycle, for example the Pacific pink salmon (*Oncorhynchus gorbuscha*) (Dye *et al.,* 1986). Most fish species reproduce more than once in their life-time and, as such, recrudescence is an important aspect of broodstock management. The effect of temperature on gonadal recrudescence has been investigated in blue tilapia (Terkatin-Shimony *et al.,* 1980) African catfish (*Clarias lazeru*) (Richter *et al.,* 1982) and Indian catfish (*Heteropneustes fossilis*) (Saxena and Sandhu, 1994). The dietary effects on ovarian recrudescence have also been studied for example on two temperate species: winter flounder (*Pleuronectes americanus*) (Burton, 1994), European Sea bass (Cerdà *et al.,* 1994b) and *O. niloticus* (Gunasekera and Lam, 1997).

1.4 Biochemical composition of fish eggs

As discussed earlier, parameters such as fertilisation rate and hatching success are normally used as indicators of egg viability (Bromage, 1988; Kjørsvik *et al.,* 1990; Springate *et al.,* 1984).

Nutritional requirements during embryonic and larval stages are one of the most important challenges in aquaculture (Rainuzzo *et al.,* 1997). Heming and Buddington

(1988) and Lim *et al.* (2005) suggested that the optimal formulations for first-feeding larvae should simulate their yolk composition and reflect the nutrient requirements of broodstock and metabolic capacities of pre-feeding fish, hence determining the egg quality.

Lipids (predominantly triglycerides, neutral lipids, fatty acids), protein (mainly phosphoproteins and lipoproteins), and carbohydrate (glycogen), are the energy reserve that accumulate within oocytes during oogenesis of teleost fish. Carbohydrates are poorly utilised by fish. However, proteins and lipids play a major role in reproduction and are the major components stored in egg yolk. The lipids are also a major source of metabolic energy in gonad formation in female fish and are important materials for the formation of tissue membranes and cells (Falk-Petersen *et al.,* 1986; Sargent, 1995; Tocher and Sargent, 1984b), throughout embryonic development (Bourassa and Terner, 1979; Sargent, 1995; Tocher *et al.,* 1997; Tocher *et al.,* 2002; Tocher and Harvie, 1988).

It has also been reported that lipids are considered to be amongst the most highly essential components of fish eggs as a reserve energy supply (Dabrowski *et al.,* 1987; Evans *et al.,* 1996; Kuznetsov and Khalitov, 1978) Lipids have been shown to increase the viability of rainbow trout larvae (Craik and Harvey, 1984a), wild salmon (Srivastava *et al.,* 1995), Asian sea bass (*Lates calcarifer*) (Nocillado *et al.,* 2000) and Vendace (*Coregonus albula*) (Dabrowski *et al.,* 1987).

Essential fatty acid (EFA) content and profile in fish diet is one of the main nutritional components that affects spawning quality of fish (Fernandez-Palacios *et al.,* 1995). It has been reported that broodstock fed highly unsaturated fatty acid (1.8% n-3 HUFA) in diets, displayed improved fecundity, fertilisation and hatching rate *e.g.* gilthead sea

bream (*Sparus aurata* L.) (Rodriguez *et al.,* 1998) and Atlantic halibut (*Hipoglossus hippoglossus*) (Evans *et al.,* 1996).

1.5 Broodfish, age and size of the tilapia

The size and number of eggs produced by tilapia is affected by female age and size (Alvendia-Casavay and Carino, 1988; Jalabert and Zohar, 1982; Rana, 1988; Wootton, 1979). According to Rana (1988), female size is more important than age in determining egg size. Bigger eggs were positively correlated with larger fry at hatching time and exhibited a greater survival rate. There is a strong correlation between egg size and fry length, suggesting that big eggs will produce larger fry at hatching time. These fry exhibited a high survival rate during starvation compared with fry produced by small eggs (Macintosh and Little, 1995; Rana and Macintosh, 1988).

The wide variations in age at first maturity, fecundity and the frequency of spawning among species and individuals within the species indicate that improvement in broodstock performance can be made by selection (Kirpichnikov, 1981; Mires, 1982; Pante *et al.,* 1990; Pullin, 1982; Pullin *et al.,* 1986). Several spawns may be developed and reabsorbed before the first spawning. The age at which a female first spawns, therefore, might not indicate the time of first maturity (Peters, 1983). Nikolskii (1969) reported that in general, total fecundity (number of eggs per spawning) in teleost fish increases up to a certain age then declines; but relative fecundity (number of eggs per unit weight of female), which is highest at younger ages, declines before absolute fecundity . Moreover, older females spawn less frequently (Nikolskii, 1969) or may stop completely (Lowe-McConnell, 1955; Lowe-McConnell, 2000). In tilapia, females maturing earlier at smaller sizes produce smaller eggs but relatively more

eggs than a larger fish per unit body weight (Hughes and Behrends, 1983; Rana, 1988; Siraj *et al.,* 1983; Smith and Walker, 2004).

In *O. niloticus*, the inter-spawning-interval (ISI) was found to be 15 to 21 days for one and two year old females, respectively. However, three year old broodfish spawned at intervals of between 10 to 20 days. Total fecundity was greater in one year old broodfish, followed by two and three years old fish respectively; egg size was higher in two and three year old fish (Hughes and Behrends, 1983; Rana, 1988; Siraj *et al.,* 1983). A similar pattern was reported by Smith and Walker (2004), who reported that seed production of the hybrid of *O. urolips hornorum* × *O. mossambicus* was significantly higher in one year old than in two year old females. Fecundity is also thought to be strongly influenced by the genetic factors, as well as environmental conditions, especially those affected by nutritional status of fish (Macintosh and Little, 1995; Rana, 1988).

Hatchery operations normally prefer medium size tilapia broodstock (150-200g) and discard females larger than 250g due to husbandry difficulties associated with seed collection. Also, larger broodstock require more space and food, thus affecting the seed production cost. In addition, larger fish are also more susceptible to disease and adverse environmental conditions (Balarin and Haltton, 1979; Bhujel, 2000; Guerrero, 2004; Little *et al.,* 2000; Pullin *et al.,* 1986).

1.6 Egg and larval quality in fishes

Egg quality is presently defined as the potential of an egg to hatch into a viable larva (Brooks *et al.,* 1997; Kjørsvik *et al.,* 1990; Panini *et al.,* 2002). One of the major factors influencing reproduction in tilapia and other species is the quality of the

gametes involved. Factors affecting egg quality are determined by intrinsic properties of the egg itself and by the environmental conditions in which the egg is fertilised and subsequently incubated (Brooks *et al.,* 1997). Some of the factors governing egg quality are known, but most are, unfortunately, unknown. Some of the more well known factors affecting egg and larval quality are diet, photoperiod, physical, genetic and chemical parameters, physical properties of water and husbandry practices (Bromage *et al.,* 1984; Brooks *et al.,* 1997; Brown *et al.,* 2006; Kjørsvik *et al.,* 1990; Nocillado *et al.,* 2000). The nutritional composition and quality of a broodstock diet is an important contributor to egg and larval quality (Bell *et al.,* 1997; Bruce *et al.,* 1999; Cerdà *et al.,* 1994a, 1995).

From the fish farming industries point of view, good quality eggs have been defined as those exhibiting low mortalities during fertilisation, eyeing, hatching and first feeding (Randall and Bromage, 1992). Several factors have been found to influence egg quality, such as spawning season, age, size, and weight of broodfish (Brooks *et al.,* 1997; Craik, 1985; Craik and Harvey, 1984b; Gimenez *et al.,* 2006; Kjørsvik *et al.,* 1990; Lahnsteiner *et al.,* 2009; Lahnsteiner and Patarnello, 2004; Salze *et al.,* 2005). Several authors have reported that availability of food prior to spawning events also has effect on fecundity, egg size, hatchability, the chemical composition of eggs and fry viability, *e.g.* (Boggio *et al.,* 1985; Bromage *et al.,* 1992; Kjørsvik *et al.,* 1990; Springate and Bromage, 1985). Poor egg quality is likely to influence larval survival especially during the transition from endogenous to exogenous feeding (Rainuzzo *et al.,* 1997). Therefore, variable egg quality has been regarded as one of the most important limiting factors for successful mass production of fish.

Bromage (1988), Kjørsvik *et al.* (2003) and Springate *et al.* (1984) suggested that fertilisation rate can be a predictive indictor of egg viability, and batches of eggs with poor fertilisation rates generally affect the later stages of development of the fish species. In some cases there is no correlation between fertilisation rate and good survival and development in later embryonic stages, *e.g.* turbot (*Psetta maxima*) (Fauvel *et al.,* 1992; Kjørsvik *et al.,* 2003).

There is no consistency as to whether egg size is correlated with egg viability. Eggs may be of different size within the species as well as between populations of the same species (Craik and Harvey, 1984b). Such variability could occur due to nutritional conditions of the female during the ovarian process (Bromage *et al.,* 1984; Lancaster and Tyler, 1994). Larger egg size means that larger yolk reserves offer an advantage over smaller eggs and support the larvae until the first feeding, which in turn increases survival in early stages more than smaller eggs do in the wild (Bromage *et al.,* 1992). In hatcheries where the growth condition is optimum, egg size has no influence on the viability of the larvae of rainbow trout as smaller eggs produce viable larvae similar to larger size eggs (Bromage *et al.,* 1992; Springate and Bromage, 1985).

1.7 Seed production of the tilapia

Low egg production per spawning and lack of spawning synchrony amongst tilapia females hinders the management of mass seed production (Jalabert and Zohar, 1982; Little *et al.,* 1993) and this impacts upon the tilapia industry as a whole. As a large number of parental stocks are required in order to meet the demand for seed (Little and Edwards, 1999; Mires, 1982), a hatchery operator has to maximise seed output by exploiting the reproductive potential of his/her broodstock (Macintosh and Little, 1995; Springate *et al.,* 1984). Total seed output from Nile tilapia (*O. niloticus*), a

multiple spawner and maternal mouth-brooder, depends mainly on reproductive life span, fecundity of an individual female and frequency of spawning (Bhujel, 2000; Lowe-McConnell, 1955; Lowe-McConnell, 2000; Nikolskii, 1969). Performance of broodfish in species having parental care may also include ‘‘brooding efficiency’’ (Lowe-McConnell, 1955; Lowe-McConnell, 2000; Welcomme, 1967). Large variations within and between the strains of tilapia have been reported for age at first maturity (Brummett, 1995; Hulata, 1982; Jalabert and Zohar, 1982; Macintosh and Little, 1995), fecundity (Babiker and Ibrahim, 1979; De Silva, 1986; Lowe-McConnell, 1955; Lowe-McConnell, 2000; Pullin *et al.,* 1986) and frequency of spawning (Jalabert and Zohar, 1982; Lowe-McConnell, 2000; Macintosh and Little, 1995; Peters, 1983; Philipart and Ruwet, 1982; Welcomme, 1967). Various factors, namely genetic (Jalabert and Zohar, 1982; Uraiwan, 1988) environmental (Brummett, 1995; Duponchelle *et al.,* 1998; Duponchelle *et al.,* 1999; Duponchelle *et al.,* 2000; Duponchelle and Panfili, 1998; Hulata, 1982) and management techniques (Bhujel, 2000; Little, 1989; Little *et al.,* 1993) affect the performance of Nile tilapia broodfish.

1.7.1 Fish oil in fish feeds

Clearly, aquaculture is heavily dependent on fish meal (FM) and fish oil (FO). The aquaculture sector is at the biggest consumer of fish oil, at about 835,000 tonnes (88.5% total reported fish oil production in 2006) (Tacon and Metian, 2008). Given the fact that supply from wild feed grade fisheries will remain static in the next decade the viability, growth and profitability of aquaculture could be negatively impacted (Pike, 2005; Pike and Barlow, 2003; Sargent and Tacon, 1999; Tacon, 2004; Tidwell and Allan, 2002). Over the past decade, global fish oil production has reached a plateau and is not expected to increase beyond current levels. It is of note that the

prices of FO are projected to rise significantly by 2020 (Delgado *et al.,* 2003). Furthermore, other issues like the contamination of FO with organic pollutants make the use of some FO for aquafeeds problematic. This is highlighted by the increasing international and national demand for safer and higher quality aquatic products (FAO, 2006).

Hence, there is a growing and pressing need for sustainable alternatives to FO and for the reduction of the dependence on FO for fish feeds. In fact, the need for reducing the FO share in aquafeeds has been underlined in numerous reviews, reports and scientific papers and it presents a considerable challenge for the future development of aquaculture (Delgado *et al.,* 2003; FAO, 2006; Naylor *et al.,* 1998; Sargent and Tacon, 1999; Tacon and Metian, 2008; Trushenski *et al.,* 2006). There are numerous lipid sources with a potential use in aquafeeds as substitutes for FO, *e.g.* animal by products, vegetable oils, marine products from lower trophic levels (Moksness *et al.,* 2004; Regost *et al.,* 2003) and transgenic plants (Robert, 2006) and the use of ingredients of plant origin as sustainable alternatives to marine oils in aquafeeds is of great potential. Specifically, plant ingredients have high global availability at competitive prices, as compared to FO, and they have nutritional properties that can largely satisfy the nutritional requirements of the fish (NRC, 1993).

However, their use does present some problems and several challenges have to be met before successful replacement of FO with plant oils is achieved. There are a large number of plant ingredients that have been studied or used as substitutes for FO in aquafeeds. An understanding of the lipid and fatty acid requirements of tilapia is needed before we can embark on a successful program to replace fish oil with vegetable oils in commercial tilapia feeds. Tilapia, like other warm-water fish, are

more inclined to require greater amounts of n-6 fatty acids compared to n-3 fatty acids for maximal growth (NRC, 1993). However, the studies presented in this thesis focused on three dietary lipid sources cod liver oil (CO), palm oil (PO) and mixed palm and cod liver oil (9:1) ratio, on *O. niloticus* reproductive performance with the goal of replacement of fish oil with palm oil. The reason for the chosen 9:1 ratio PO and CO was to reduce the proportion of n-3 to n-6 (M. Bell, personal communication).

1.7.2 Palm oil in Nile tilapia diets

Palm oil (PO) is derived from the oil palm (*Elaeis guineensis* family: Arecaceae). Palm oil is a fruit flesh oil; however, seed oil (palm kernel oil) is also produced (Hertrampf and Piedad-Pascual, 2000). Palm oil has a high availability as its production provides one of the largest vegetable oil (VO) tonnages in the world, along with soybean oil (FAO, 2005; U.S.Department for Agriculture, 2007). Interestingly, it is also predicted to exceed soybean oil production within the next few years to become the most abundant VO in the world (Gunstone, 2001).

Crude palm oil has a very high content of 16:0 and 18:1n-9 (43.5% and 36.6% of total lipid fatty acid composition, respectively) and relatively low levels of 18:2n-6 (9.1%) (Ng *et al.,* 2002; Ng, 2002; NRC, 1993). This fatty acid composition (FA) composition makes PO a good potential candidate to replace FO in diets for *O niloticus* to provide energy for sufficient growth. The use of PO in the diets of tilapia and other species such Atlantic salmon and rainbow trout has been investigated with regards to growth and feed utilisation efficiency, and changes in tissue FA composition and FA metabolism, give promising results (Bell *et al.,* 2002; Caballero *et al.,* 2002; Ng and Chong, 2004; Ng *et al.,* 2003, 2004; Rosenlund *et al.,* 2001; Torstensen *et al.,* 2000).

1.8 General objectives

The overall objective of the present study was to investigate the effects of dietary lipid sources on the reproductive performance of the commercially important Nile tilapia with the goal of substituting FO with sustainable alternatives of plant origin.

Specifically the objectives were to:

- Investigate the effects of dietary lipid sources on egg quality in terms of lipid and fatty acid composition of eggs.
- Elucidate the interactive effects of dietary oil source and ration size on the egg lipids and fatty acid composition over consecutive spawnings.
- Investigate the effect of dietary lipid on spawning periodicity and egg size.
- Elucidate the interactive effects of dietary fat and FA contents and ration size on egg and larval quality in terms of egg and larval size over serial spawnings.
- Investigate the effects of dietary lipid sources on oocyte recrudescence associated with sex steroids.
- Elucidate the interactive effects of dietary oil source and ration size on oocyte recrudescence over consecutive spawnings.

Chapter 2 - General Materials and Methods

2.1 General material and methods

The materials and methods used throughout this study are given in this Chapter, and those specific to particular sections of the thesis are given under the relevant Chapters.

2.1.1 Experimental diets

Diet formulation and preparation, and their chemical analysis were carried out as detailed below:

2.1.2 Diet formulation

Diets were formulated to resemble the nutrient and energy contents of commonly available commercial pelleted diets for tilapias. Prior to diet formulation a number of feed ingredients which have no oil were selected and analysed to establish if nutrient profiles were suitable for possible inclusion. Calculations and estimations were made using an Excel spreadsheet. A number of considerations were taken into account when formulating the experimental diets.

Diets were formulated to ensure that dietary nutrient requirements of tilapia *O. niloticus* for protein, lipid, essential amino acids, fatty acid, vitamins and minerals respectively, were met (National Research Council USA) (NRC, 1993).

The lipid levels of the diet resembled those in commonly available commercially pelleted diets. The fibre levels of diets should be below 8% to avoid problems of palatability and pelleting. The ash levels of diet should be below 10% and their moisture should be suitable (around 12%) for pelletibility.

Three isonitrogenous (40% crude protein) and isoenergetic (20 kJg^{-1}) experimental diets were formulated using the same basal ingredients but varying only the oil source (Table 2.1). Soy protein concentrate (63% protein, GEA Niro, Denmark) was used as a protein source whereas corn starch (Sigma 4126) was used as a source of carbohydrate. Carboxy methyl cellulose (Sigma 5013) and α-cellulose (Sigma 8002) were used as a binder at rates of 3.5% and 2%, respectively. The ingredients of vitamins and minerals were purchased from Sigma and the premixes and also all experimental diets formulated and were made in the nutrition laboratory at the Institute of Aquaculture (IOA), Stirling University. The vitamin premix (Table 2.2) and mineral premix (Table 2.3) were added to the diets at a rate of 2% of total diet for each. Dicalcium phosphate (Sigma 7263) was added to the diet at a rate of 2% of total diet to ensure that there were no deficiencies of calcium and phosphorus at a high inclusion level of soy protein concentrate (vegetable protein source).

DL-methionine (Sigma 9500) was added at a rate of 0.5% to supply adequate amounts of methionine, as this could be a potential limiting amino acid using high levels of soy protein concentrate. Fish hydrolysed protein (CPSD 90, France) was added at a rate of 5% in order to enhance the palatability. Casein was added a rate of 0.5 % to maintain the protein balance. Cod liver oil (pure cod liver oil, Tesco UK), (Palm oil Arous, UK) and the combination of cod liver and palm oil (1:9) ratio were added at a rate of 10% of the total diet as a lipid source (Chou and Shiau, 1996; Stickney and Wurts, 1986). Premixes were made in the nutrition laboratory at the Institute of Aquaculture, Stirling University.

Table 2.1 Composition of experimental diets (g/100g total diet. Composition of the diets (expressed as %).

Ingredients	Diet 1	Diet 2	Diet 3	Source
Soy-protein concentrate	55	55	55	Denmark
Casein	0.5	0.5	0.5	Sigma
Corn starch	17.5	17.5	17.5	Sigma
Cod liver oil	10	---	---	Tesco UK
Palm oil	---	10	---	Arous UK
C& P oil (1:9)	---	---	10	
DCP	2	2	2	Sigma
Fish hydrolysate	5	5	5	DCP 90 France
DL-methionine	0.5	0.5	0.5	Sigma
Vitamin premix	2	2	2	
Minerals premix	2	2	2	
Carboxy methyl cellulose	3.5	3.5	3.5	Sigma
α-cellulose	2	2	2	Sigma
TOTAL	100	100	100	

*C&PO= combination of cod liver and palm oil**DCP= dicalcium phosphate.

Table 2.2 Composition of the vitamin premix used in the experimental diets

Vitamin	Form	mg/kg Premix*	Source
A	Retinol palmitate	1000	Sigma R 3750
D	Cholecalciferol	4	Sigma C9756
E	Tocopherol acetate	7000	Sigma T3376
K	Vitamin K	1500	Sigma V3501
C	Ascorbic acid	37500	Sigma A0278
B_{12}	Cyanocobalamin	1.25	Sigma V2876
Thiamine	Thiamine hydrochloride	4250	Sigma T4625
Riboflavin	Riboflavin	3000	Sigma R4500
Pyridoxine	Pyridoxine hydrochloride	1250	Sigma P9755
Pantothenic acid	Calcium pantothenate	5250	Sigma P9153
Niacin	Niacinamide	12500	Sigma N3376
Biotin	Biotin	90	Sigma B4501
Folic acid	Folic acid	1000	Sigma F7876
Choline	Choline chloride	74050	Sigma C1879
Inositol	myo-Inositol	25000	Sigma I5125
	Ethoxyquin	200	Sigma E8260

Note: the mixture was made up to 1 kg with α-cellulose (as carrier). According to Jauncey and Ross (1982)

Table 2.3 Composition of the minerals used in experimental diets

Minerals	Chemical Formula	g/kg Premix	Source
Calcium orthophosphate	CaHPO4.2H2O	277.78	BDH261172P
Magnesium Sulphate	MgSO4.7H2O	127.5	BDH291172L
Sodium Chloride	NaCl	60	BDH 26281
Potassium Chloride	KCl	50	BDH 101984L
Iron Sulphate	FeSO4.7H2 O	25	BDH 101124L
Zinc Sulphate	ZnSO4. 4H2O	5.5	BDH 102994R
Manganese Sulphate	MnSO4 .4H2O	2.54	BDH 101534M
Copper Sulphate	CuSO4 .5H2O	0.79	BDH 10091
Cobalt Sulphate	CoSO4 .7H2O	0.48	BDH 27801
Calcium Iodate	CaIO3.6H2O	0.295	BDH 27602
Chromic Chloride	CrCl3 .6H2O	0.1275	BDH 27752

Note: Before weighing, all ingredients were ground to a fine powder then weighed onto a balance in a fume cupboard. Ingredients then were mixed and stored in a freezer for a maximum 6 months. According to Jauncey and Ross (1982)

2.1.3 Diet preparation

Three diet treatments were employed in this study (Table 2.1). The ingredients were first mixed for approximately 30 minutes in a Hobart mixter (Belle, Mini 150; England) to ensure that the mixture was well homogenised and then blended by adding 10% oil from cod liver oil (CO), Palm oil (PO) either 1:9 mixture CO and PO together, respectively for a further 15 minutes. Water was added at 20-30% V/W to give a pelletable mixture. The California pellet mill (model CL2, San Francisco, California) was used to pellet the diets. An appropriate die was used to produce pellet of desired sizes of approximately1, 2, and 3 mm.

Table 2.4 The size of pellet fed a different fish size. A water stable-sinking type of pellet was used.

Fish size	Pellet size
First feeding larvae	250-500 μm
Larvae-1g	500-850 μm
1-5g	1 mm
5-35g	2 mm
35g+	3mm

The pellets were then dried by convection at 40ºC overnight in a drying cabinet. The dried pellets then were placed in plastic bags and stored in a freezer at -20ºC until use. The proximate analysis is presented in Table 2.5 and the fatty acid composition of experimental diets is shown in Table 2.6.

2.1.4 Proximate analysis of feed ingredients and experimental diets

Proximate analysis was performed for moisture, protein, lipid ash, crude fibre, nitrogen free extract (carbohydrate) and energy using the following procedures that broadly adhere to AOAC (1995). Samples of feedstuffs were analysed in triplicate. Samples were ground to a powder using a coffee grinder (Moulinex optiblend 2000, France).

2.1.4.1 Procedure for moisture determination

One gram of sample was pre-weighed (W1) on a foil and placed in a drying oven (Gallenkamph) at 110ºC overnight. The sample was removed from the oven, cooled in a dessicator and reweighed (W2). Moisture percentage was calculated according to the formula:

$$\text{Moisture (\%)} = \frac{W1 - W2}{W1} \times 100$$

2.1.4.2 Procedure for crude protein determination

A two hundred mg sample was placed in a Kjeldhal digestion tube. Two Kjeldahl mercury catalyst tablets (Fisher K/0130/80) and 5 ml concentrated sulphuric acid (BDH 45006) were added to the sample tubes. The sample was digested at 400 ºC (Digestion system 40 Tecator 1006 Heating unit, Sweden) for one hour. The samples then were cooled in a fume cupboard for 30 minutes and 20ml of de-ionised water and 5 ml of 1.33M sodium thiosulphate were added to the samples. The sample was then distilled, its ammonia content was liberated and collected in standard boric acid and titrated against 0.2M hydrochloric acid. Both distillation and titration were automated (Kjeldhal Auto 1030 analyser Tecator). A blank tube (free of sample) was prepared and treated in the same manner. A sample of urea (50 mg) was prepared and treated as standard. It was treated prior to any sample in order to check that its nitrogen content is similar to the known value (46.64%) for urea as standard. Protein percentage for a sample was calculated according to the formula:

$$\text{Protein (\%)} = \frac{(\text{sample titre - blank tire}) \times 0.2a \times 14.007b \times 6.25c}{\text{sample weight}} \times 100$$

Where:
(a) Molarity of hydrochloric acid;
(b) Molecular weight of nitrogen;
(c) Nitrogen factor; since protein is assumed to consist of 16% nitrogen.

2.1.4.3 Procedure for lipid extraction using Soxhelt method

One gram of sample was weighed into an extraction thimble and covered with cotton-wool. 40 ml of solvent was added to a pre-weighed cup, which contained five glass balls. Both thimbles and cups were placed in the extraction unit (Soxtec Machine 1043, Tecator Sweden). The sample was subjected to boiling in petroleum ether solvent for 20 minutes and then rinsed for 1-1.5 hours. The solvent was evaporated

from the cup to the condensing column. Cups and thimbles were removed from the extraction unit and the cup was placed in an oven at 110 ºC for one hour. Then the cup was removed from the oven, cooled in a dessicator and weighed. Extracted lipid was expressed as percentage of the original sample and calculated according to the formula:

$$\text{Crude lipid (\%)} = \frac{\text{extracted lipid}}{\text{sample weight}} \times 100$$

2.1.4.4 Procedure for crude fibre determination

For determination of crude fibre, a 0.5-1 gram sample was placed in a pre-weighed (w1) FibreCap capsule. The samples were de-fatted using petroleum ether. De-fatted samples were placed in the extraction vessel (Fiberec system 2022 hot Extractor, Foss Sweden) by adding 350 ml of boiling 1.25% sulphuric acid solution, and sample was digested for 30 minutes and then the process was stopped, the acid was drained out and the sample washed with boiling distilled water. After this, 350 ml of 1.25% sodium hydroxide solution was added, the sample was digested for a further 30 minutes. The alkali was drained out and the sample was washed with boiling distilled water. Finally the capsule was removed from the extraction vessel and oven dried at 110 ºC overnight. The sample was then , cooled and weighed (W3). The capsule was then placed in a pre-dried and pre-weighed (W4) ashing crucible and ashed at 550 ºC in a Muffle Furnace (Gallenkamp Muffle Furnace) for 4 hours, cooled in a desiccator and reweighed (W5). Extracted fibre was expressed as a percentage of the original un-defatted sample and calculated according to the formula:

$$\text{Crude fibre (\%)} = \frac{\text{W3 - (W1} \times \text{C) - (W5 - W4 - D)/}}{\text{W2}} \times 100$$

Where:

W1 is Initial capsule weight (mg)
W2 is sample weight (mg)
W3 is Capsule weight + residue weight (mg)
W4 is Empty ashing crucible weight
W5 is Total ash (mg) + Crucible
C is Blank correction for capsule solubility
D is Capsule ash (mg)

2.1.4.5 Ash

Ash content was determined from the contents of inorganic matter by incineration of the sample at 600ºC. The remaining inorganic materials, which do not burn off, are reduced to their most stable form, oxides or sulphate and are considered as ash.

2.1.4.6 Procedure for ash determination

A one gram sample was weighed into a pre-weighed porcelain crucible and incinerated (Gallenkamp Muffle Furnace) overnight at 600ºC. The crucible was removed from the Muffle Furnace, cooled in a desiccator and weighed. The incinerated sample was expressed as a percentage of the initial (before lipid extracted) sample and calculated using the following formula:

$$\text{Ash (\%)} = \frac{\text{Ash weight (g)}}{\text{Sample weight (g)}} \times 100$$

2.1.4.7 Nitrogen free extract (NFE)

Nitrogen free extract (carbohydrate) was calculated by subtracting the total percent of moisture, crude protein, crude lipid, ash, and crude fibre from 100(%), using the following formula:

NFE (%) = 100- (moisture + Protein +lipid+ ash +fibre)

2.1.4.8 Gross energy

The gross energy was determined directly by bomb calorimetry using an Automatic Adiabatic Bomb calorimeter (Gallenkamp co. Ltd, England). The method is based on the fact that upon combustion in a bomb chamber, the sample is burned and the resulting heat is measured by the increase in the temperature of the water surrounding the bomb.

2.1.4.9 Procedure for gross energy determination

One gram of sample was pelleted firmly using a briquette press and reweighed in a metal crucible. The sample was connected to the firing wire, which was fitted between the electrodes, by cotton thread. The electron assembly was placed into the bomb and then the bomb was tightened. The circuit was rested and the bomb was filled with oxygen to a pressure of 30 bar. The calorimeter vessel was filled with water (total weight 3 Kg) at 21-23ºC and the prepared bomb was placed inside the calorimeter vessel, then the calorimeter vessel was placed into the water jacket. The machine was switched on and kept for 2-3 minutes for the temperature to stabilise. Prior to firing, the initial temperature of the water was checked and recorded and after firing the final temperature was recorded. Finally, the energy content was calculated according to the formula:

$$\text{Gross energy (KJ/g)} = \frac{[(\text{final temp.} - \text{initial temp.}) \times 10.82] - 0.0896}{\text{smaple wieght (g)}}$$ [1]

[1] This is the combined energy value of the Nickel wire and the cotton.

Table 2.5 Proximate composition % of diets

Parameters	Diet 1	Diet 2	Diet 3	Diet 4
Moisture	15.1	14.3	14.2	8.0
Crude protein	40.5	41.0	40.8	40.6
Crude lipid	10.0	9.8	9.7	7.02
Carbohydrate	24.1	22.2	23.1	24.3
Ash	5.3	5.3	5.1	10
Crude fibre	7.7	7.3	7.3	7.8
Grass energy (KJg-1)	20.4	20.4	20.3	23.9

Note: values of the proximate composition and value of energy of the diets represent the mean of triplicate of each diet .

2.1.4.10 Fatty acid composition of diets

Fatty acid composition of the experimental and control diets were analysed to compare the fatty acid profile of diets and the FA composition of eggs originating from broodstock fed the experimental diets. The fatty acid composition of three experimental diets and a commercial diet as control is shown in Table 2.6. The experimental diets differed considerably in their fatty acid composition. Overall, fatty acid compositions of diet 1 containing (cod liver oil), diet 2 (containing palm oil), diet 3 (containing P&C oil 9:1 ratio) and diet 4 control, which were contained by monoenoic fatty acids (39-43.9%). The polyunsaturated fatty acids, particularly *n*-9, DHA, ARA and EPA series dominated in the control diet; whilst the total saturated fatty acids were the major fatty acid class (41.2 and 45.5%) in diets 2 and 3, respectively. The diets containing palm oil (diets 2 and 3) had significantly more palmitic acid (16:0) 35.6 and 38.1% when compared with diets 1 and 4 which contained 16.8 and 14%. ARA was not detected in the palm oil and the mixed P&C oil whereas in diet 1 and 4 it was 0.5 and 0.4% respectively. EPA was significantly lower in these diets, resulting in ARA: EPA ratios close to zero for diet 2 and 3 and 0.1 for diet 1 and 4. The diet 1 and 4 were characterised by very large amounts of DHA (9.9 and 6.3% in diet 1 and 4, respectively) compared with diet 2 and 3 in which

these were not detected in diet 2 and was 0.9% in diet 3. The total polyunsaturated fatty acid dominated in diet 1and 4 (34.8 and 35.0% for diet 1 and 4, respectively) whilst diet 2 and 3 had significantly lower amounts (14.8 and 13.5%, respectively).

Table 2.6: Fatty acid composition of the experimental diets, values are as percentage of total lipid and represent the mean of triplicates

Fatty acid	D1 (CO)	D2 (PO)	D3 C&PO)	D4 Control
14:0	5.1	1.2	1.5	4.6
15:0	0.5	0.1	0.2	0.3
16:0	16.8	35.6	38.1	14.0
18:0	2.9	4.0	5.2	1.9
20:0	0.2	0.3	0.4	0.2
22:0	0.1	nd	nd	0.1
Total saturated	25.6	41.2	45.5	21.1
16:1n-9	7.7	0.4	0.7	0.1
16:1n-7	0.3	0.2	0.9	5.0
18:1n-9	17.1	43.0	36.9	12.7
18:1n-7	3.7	nd	1.2	2.3
20:1n-9	10.3	0.4	1.2	10.6
20:1n-7	0.4	nd	0.1	0.5
22:1n-11	0.1	nd	nd	11.7
24:1n-9	nd	nd	0.0	0.8
Total MUFA	39.6	44.0	41.0	43.9
18:2n-6	5.3	13.7	10.6	16.8
18:3n-6	0.1	nd	nd	0.1
20:2n-6	0.2	nd	nd	0.2
20:3n-6	0.2	nd	nd	0.0
20:4n-6 (ARA)	0.5	nd	nd	0.4
22:4n-6	0.3	nd	nd	0.0
22:5n-6	0.2	nd	nd	0.1
Total n-6 PUFA	6.9	13.7	10.6	17.6
18:3n-3	1.5	0.8	0.7	1.8
18:4n-3	2.9	nd	0.2	1.7
20:3n-3	0.1	nd	nd	0.1
20:4n-3	1.0	nd	nd	0.4
20:5n-3 (EPA)	9.2	0.2	0.7	5.6
22:4n-3	nd	nd	nd	0.0
22:5n-3	1.8	nd	0.1	0.6
22:6n-3 (DHA)	9.9	nd	0.9	6.3
Total n-3 PUFA	28	1.1	2.9	17.5
Total PUFA	34.8	14.8	13.5	35.0
ARA/EPA	0.1	nd	0.0	0.1
DHA/EPA	1.1	nd	1.3	1.1
n-3/n-6	3.8	0.1	0.2	0.9

nd= not detected. D1 containing cod liver oil, D2 palm oil, D 3 palm and cod liver oil 9:1 ration and D4 a commercial diet as control

2.2 Experimental fish

Mixed-sex *Oreochromis niloticus* known as the "Red-Stirling strain" (Ranson, personal communication) were used in this research. They were bred and reared in the tropical aquarium and hatchery complex of the Institute where this research was

carried out as described below. They were originally from Lake Manzallah in Egypt and introduced to the University of Stirling in 1979 and underwent natural selection over the years (Majumdar and McAndrew, 1986; McAndrew *et al.,* 1988).

Throughout this experiment eggs were collected by stripping manually from Nile tilapia (*O. niloticus*) broodstock. The eggs were fertilised, the fertilised eggs washed and placed into round-bottomed plastic containers (Rana, 1986) supplied with clean, U.V. sterilised water and left until hatching (approximately 2 days post-fertilisation) and absorption of yolk-sac (a further approximately 6 days). At this stage fry were transferred and stocked at 210 per tank (11/litre) into 25 litre plastic tanks (12×48×32cm) linked to a recirculation system for on-growing, to investigate their reproductive performance when began to broodstocks. The water temperature was maintained at 27±1ºC and all tanks experienced a constant daily photoperiod regime of 12L: 12D. Water quality was monitored in the system by measuring oxygen, pH, nitrate, nitrite and ammonia levels (C-Test kits, New Aquarium Systems U.K.) weekly. System settling tanks and biological filters were cleaned every two weeks concurrent with a 10-20% water change. Fry were fed 5 times to twice a day with their respective diets of appropriate sizes. The diet sizes were varied according to fry size (see Table 2.4).

Table 2.7 Ration allocation during experimental period (120 days)

Age (days)	Ration allocation (% of body weight/day)	
0-20	30	15
20-40	20	10
40-80	10	5
80-100	5	2.5
100-120 and thereafter	3	1.5
120 and thereafter	3	1.5

2.3 Growth performance

Parameters used to evaluate growth performance in this study were weight gain by fish and specific growth rate (SGR). The SGR is the most commonly used indicator of fish growth.

2.3.1 Specific growth rate (SGR):

The SGR is the instantaneous change in weight of fish expressed as the percentage increase in body weight per day over any given time interval.

It is calculated by taking natural logarithms of body weight, and expresses growth as $\%.day^{-1}$ (Ricker, 1979).

$$SGR = \frac{(\ln FBW - \ln IBW)}{D} \times 100$$

where D is the number of days between weighings.

2.3.2 Feed conversion ratio (FCR)

FCR is defined as the amount of dry feed fed per unit live weight gain. It often serves as a measure of efficiency of the diet. The more suitable the diet for growth, the less food is required to produce a unit weight gain, *i.e.* a lower FCR (De Silva and Anderson, 1995). It was calculated as:

$$FCR = \frac{feedfed_{(g)}}{weightgain_{(g)}}$$

2.4 Fish handling and anaesthesia

All procedures requiring fish handling *e.g.* stripping, tagging weighing, blood sampling etc. were performed under anaesthesia to facilitate easy handling and

minimise stress and scale damage. Fish were anaesthetised by immersion in a 10% (w/v) solution of ethyl 4-aminobenzoate (Sigma Chemicals, Ltd U.K.) diluted in ethanol. The working concentration was 1:10000 in fresh water. Anaesthesia was generally induced within 1-2 minutes. After sampling procedures had been undertaken anaesthetised fish were placed into clean, aerated water until recovery. Full recovery usually occurred within 1-2 minutes.

2.5 Fish Husbandry

All animals were held in accordance with, and experiments performed under the licensing of the Home Office UK (Scientific Procedures) Act 1986.

2.6 Fish holding tanks

The female broodstock were maintained in glass tanks, each tank incorporating two, three or four (depending on fish size) vertical dividers constructed from translucent Perspex, thus creating three or four separately partitioned 'holding spaces' within each tank into which female broodstock could be introduced and maintained individually (Coward and Bromage, 1999b). All fish were maintained in gravity-fed recirculation tanks, faecal traps and filtration units appropriate to incorporated filter brushes and bio-rings (Dryden Aquaculture, UK) to avoid particulate filtration and maximize bio-filtration. Water was pumped from the system collector tank to a sand filter tank and then sent to a header tank (227-l capacity) via a water pump (Beresford Pumps, UK) The water temperature was maintained at 27±1 °C (using a 3-kW thermostatically controlled water heater). Water was oxygenated via airstones in the header tank and each aquarium by a low-pressure blower. The water inflow was constant at 252 l h^{-1} $tank^{-1}$. Water quality was monitored weakly, including dissolved

oxygen (O_2) and water temperature. The levels of pH, nitrate, nitrite and ammonia were evaluated with aquarium water quality kits (C-Test kits, New Aquarium Systems, UK). To maintain good water quality, a partial change of water (10% of total volume) was carried out once a week; the system was refilled with fresh, aerated, and preheated water (Figure 2.1).

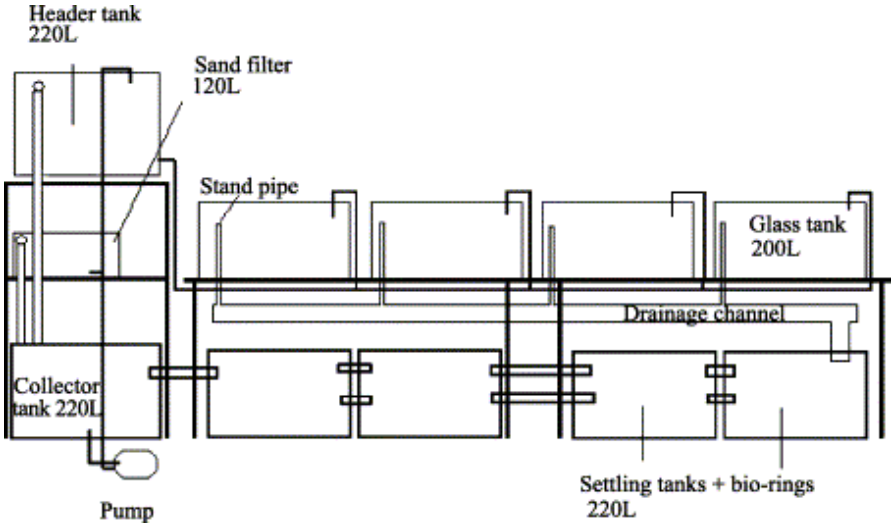


Figure 2.1 Lateral view of the closed recirculating system used to hold experimental fish

2.7 Sampling for oocyte recruitment

The female fish were sacrificed by an overdose of anaesthesia. Sacrificed fish were weighed to the nearest 0.1g (Mettler 400 balance, Fisons Scientific Equipment, U.K.) and then ovaries and livers were dissected from gut. The ovaries and liver were weighed separately to the nearest 0.0001g (Mettler AE 100 balance, Fisons Scientific Equipment U.K.) which was then expressed as a percentage of the whole body wet weight, referred to as gonadosomatic (GSI) and hepatosomatic indices (HSI), respectively, calculated as follows: .

$$\text{(a) GSI (\%)} = \frac{\text{gonad weight}}{\text{body weight}} \times 100$$

$$\text{(b) HSI (\%)} = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

2.8 Tissue fixation

Sample of ovarian tissues were cut to an appropriate size (*i.e.* no greater than 5 mm in thicknesses) to allow adequate fixative penetration. Mature ovarian tissues were fixed in Bouin's fluid for 24 hours as recommended by Drury and Wallington (1980) and immature ovarian tissues were fixed in 10% buffered neutral formalin (BNF). Following fixation in Bouin's fluid, samples were washed thoroughly with several changes of 70% ethanol to remove picric acid and stored in 70% ethanol until required for infiltration. The importance of through washing after fixation in Bouin's fluid cannot be over-emphasised. Residual picric acid can damage tissues resulting in poor quality section. Following fixation, Bouin's and 10% BNF fixed samples were rinsed and stored in 70% ethanol and water, respectively, prior to required for either paraffin wax or resin embedding. The Bouin's fluid and 10% BNF were prepared as detailed in (**Appendix 2**, section 2.1 and 2.2).

2.8.1 Paraffin wax embedding

Methods for paraffin wax embedding and staining were carried out according to the routine procedure used at the Histopathological Laboratory, Institute of Aquaculture, University of Stirling. These methods were modified from Carlton's Histological Technique (Drury and Wallington, 1980).

The rinsed samples were dehydrated in a series of alcohols, finishing with 100% alcohol and cleared in chloroform in an automated tissue processor (Histokinette

2000, Shandon Scientific Limited, England) at the appropriate time interval. The paraffin wax impregnated samples were then replaced into a paraffin bath (Histoembedder R134a, Leica Instrument GmbH, Germany) then blocked with wax in tissue moulds and solidified on a cold plate. The blocks were then trimmed-in using a rotary microtome (Biocut 2035, Leica Instrument GmbH Germany) to remove the excess wax. The trimmed blocks were then placed in:

(a) Water bath (Raymond A Lamb, England) for 30min at 45^{o}C. The blocks were then removed and cooled on a cold plate for a few minutes and then sectioned with a rotary microtome.

(b) Rapid decalcifier solution RDC (TAAB Chemical, UK) for 1-2 hours to decalcify the hard tissue (*e.g.* whole body of fish in this study), depending upon the tissue hardness. The blocks were removed from RDC solution and rinsed in tap water and cooled on a cold plate (RA lamb, England), and sectioned with a rotary microtome set at 5µm.

The sections were mounted on glass slides to prevent the loss of sections during staining; the slides were then dried at least one hour in an oven (Windsor Incubator, Sand Rest, England) at 60^{o}C before staining.

2.9 Sectioning of prepared sample blocks

Blocks were sectioned at a thickness of 5µm with a carbon diamond knife on a retracting microtome (Reichert-Jung 2050, Cambridge Institute Gmbh, Germany). After cutting, sections were floated in a water bath (Raymond a. Lamb, U.K.) at 35^{o}C, transferred to microscope slides and allowed to dry on a hotplate (Raymond A. Lamb, U.K.) set at 40^{o}C for 1-2 hours. Sample details were recorded onto appropriate slides

with a diamond-tipped pen and slides dried overnight in a 60^{o}C oven (Windsor Incubator, U.K.) prior to staining.

2.10 Staining procedures for histological slides

The present study routinely utilised haematoxylin and eosin staining and also, to a lesser extent, a polychrome method.

2.10.1 Haematoxylin and Eosin stain

The haematoxylin procedure is one of the most widely used stains in histological analysis, highlighting most cellular structures and is commonly used as a nuclear stain preceding the staining of cytoplasm and connective tissue by eosin (see Drury and Wallington, 1980).

Where general visualisation of internal ovarian structure was required, batches of 25 slides at a time were placed into a slide rack and stained in accordance with a slightly modified version of haematoxylin and eosin staining schedule used routinely in the histopathology laboratory at the Institute of Aquaculture, University of Stirling as detailed in Appendix Table 3.1.

2.10.2 Polychrome Staining

The polychrome staining technique was utilised in histological studies of fish ovaries and has been used successfully to visualise stages of oocyte development in the ovary of Atlantic mackerel (*Scomber scombrus* L.), yellow fin tuna, (*Thunnus albacares*) or Galapagos bacalo (*Mycteroperca jenyus*) (Coello, 1989), Nile tilapia *O. niloticus* (L) (Srisakultiew, 1993) and also in *Tilapia zillii* (Coward and Bromage, 1998).

Staining of ovarian tissues by this technique not only enables the versatility of discrimination of gross yolk composition from a single histological section but also all other main ovarian components such as atretic oocytes and postovulatory follicles and particularly oocytes undergoing very early vitellogenesis (Coello, 1989). The staining procedure and buffer preparation used in the present study (Appendix Table 3.2) was initially utilised in an earlier study of tilapiine ovaries by Coward and Bromage (1998) and Srisakultiew, (1993), originally modified from that given by Coello (1989). Slides were transferred from phosphate buffer, rinsed with tap water and carefully wiped clean with tissue paper and coverslips mounted with Pertex. Slides were allowed to dry at room temperature overnight before examination.

2.11 Fish Identification

In order to distinguish individual fish or group (section 3.2.3) within a population, individual fish were tagged with an inter-muscular passive integrated transponder (PIT) tag (AVID Tags, Labtrac Ltd., U.K.). Tags were implanted through a small incision made into either the dorsal muscular or the peritoneal cavity (dependent upon fish size). Incision sites were sterilised prior to implantation with 70% ethanol. A tag reader was then used to scan the tag to ensure it was functioning.

2.12 Determination of total and relative fecundity

2.12.1 Total fecundity

The eggs were collected from the Nile tilapia (*O. niloticus*) by stripping under anaesthesia. The fertilised eggs in a 100 mm petri-dish were scanned using a scanner and scanned picture of eggs was counted utilising a micro program of image analysis software program MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001). According to

Rana (1988) total fecundity was measured as the number of eggs obtained from a female. Further details will be discussed in the respective chapters.

2.12.2 Relative fecundity

Relative fecundity (expressed as a number of eggs per gram of female weight) was calculated using the following equation:

$$RF = \frac{TF}{W}$$

Where: RF: Relative fecundity (no. of eggs/BW)
TF: Total fecundity (number of eggs in a freshly spawned clutch)
W: Female body weight (g)

2.13 Determination of mean egg size, diameter and volume of egg measurement procedure

The eggs of *O. niloticus* have an ovoid shape, therefore, in order to evaluate egg size and diameter of the eggs, two parameters (egg long and short axes) were measured. Egg long axis refers to the length of the longest axis and short axis refers to the width, perpendicular to the longest axis of the egg. The diameters of the eggs were easily calculated by this method.

The maximum length of both long and short axes in a sub-sample of 50 randomly chosen eggs from each batch were measured to the nearest 0.001mm under a dissection microscope (Olympus Optical Ltd., U.K.) connected to a video camera with specific calibrations. The mean egg diameter (D) and mean egg volume was measured by image pro software (Macromedia V. 4) and calculated as follows:

$$D = \frac{(L + S)}{2}$$

Where: D=egg diameter (mm)

L= mean lengths of egg long axis (mm)

S= mean lengths of short axis (mm)

$$V = \frac{\pi \times L \times H^2}{6}$$

Where: V= egg volume (mm^3)

L= long axis (mm)

H= short axis (mm)

π =3.14

2.13.1 Determination of total egg volume

Total egg volume (mm^3) was calculated according to the following equation:

TEV = TF× MEV

Where: TEV = Total egg volume (mm^3)

TF = Total fecundity

MEV = Mean egg volume (mm^3)

2.14 Determination of mean individual egg dry weight

A sub-sample of 50 eggs was randomly removed from the spawned egg mass and dried on a pre-weighed aluminium foil weighing boat (weighed to the nearest 0.0001g on a Mettler AEl00 balance (Fisons Scientific Equipment, U.K.)) in a 70°C drying oven. Foil boats were removed 48-60 hours later and placed inside a dessicator containing silca gel (B.D.H./Merck Ltd., V.K.) to cool to room temperature for at least one hour prior to reweighing. Boats and eggs were reweighed to constant weight after similar periods of drying.

2.15 Determination of egg weight as percentage of body weight (EW: BW)

Egg weight as percentage of body weight (EW: BW) was calculated according to Coward and Bromage (1999b) using the equation given below:

EW: BW = (EDW × TF/W) × 100

where:

EDW = egg dry weight (mg)

TF = total fecundity

W = fish body weight (g)

2.16 Estimation of spawning periodicity

Spawning periodicity was estimated using the parameter Inter-Spawning-Interval (ISI) which is the time between two consecutive spawnings in a female.

2.17 Blood sampling

2.17.1 Procedure for collecting plasma

Blood samples were taken from the caudal dorsal aorta with ammonium heparin-coated syringes connected to 23G sterile hypodermic needles and sterilised 1ml syringes (Terumo Eurupe N.V., Leuven, Belgium). Approximately 200-300µl (depending on the fish size) blood was removed from fish. Immediately after sampling, the needle was removed from the syringe and the blood sample was transferred into a clean Eppendorf micro-centrifuge tube (Saratedt Ltd. Leicester, UK) and placed on ice until sampling was completed. Samples were then centrifuged at 13000×g for 5-10 minutes at 4^{o}C in a biofuge (Heraeus Sepatch, Germany). The plasma samples (supernatant) were removed with pipettes and transferred into new clean Eppendorf tubes and stored at –70^{o}C until required for further analysis.

2.18 Extraction of total lipid

Approximately 100 mg of eggs were weighed to the nearest 0.1 mg and used for total lipid extraction. Total lipid extraction was performed using the Folch-Lee extraction method (Folch *et al.,* 1957). The eggs were placed directly in a barrel homogeniser tube containing 16ml chloroform: methanol (C: M) (2:1) and butylated hydroxytoluene (0.01% w/w, BHT) and then homogenised using an Ultraturax^{TM} homogeniser. The homogenate was transferred to stoppered boiling tubes together with 10ml additional chloroform: methanol (C: M) (2:1 v/v). After homogenisation of each sample, the Ultraturax^{TM} probe was rinsed with C: M (2:1, v/v) in order to avoid lipid contamination between the different samples. The stoppered tubes were allowed to stand on ice for approximately 60 minutes to ensure that the lipid was extracted comprehensively. After that 5ml of 88% (w/v) KCl (0.25 volume of KCl) was added to the homogenised egg sample, mixed vigorously on a vortex mixer and left to stand on ice. These organic solvent and aqueous solutions (C: M: aqueous 8:4:3 v/v/v) separated to give a lower chloroform-rich phase containing the lipids and an upper aqueous methanol-phase containing water soluble components. The organic layer that contains the purified lipid was separated from the aqueous layer that contains the non-lipid fraction by centrifugation (Jouan C 412 bench centrifuge) of the tubes for 2-3 minutes at 400×g. The upper aqueous layer was discarded carefully by aspiration, and the bottom layer was transferred to pre-weighed tubes through filter paper (Whatman No.1). The homogenised tubes and the filter papers were rinsed with a small amount of C: M (2:1) in order to minimise losses of the total lipid through the process.

Solvent was evaporated under a stream of oxygen-free nitrogen in a fume cupboard to dryness, and the samples were then desiccated in a vacuum overnight. Lipids were quantified gravimetrically the next morning by reweighing the test tube and the total

lipid content was determined per weight of material extracted. Lastly, the total lipids were redissolved in C: M (2:1 v/v) containing 0.01% butylated hydroxy toluene (BHT 0.01% v/v), a concentration of 10mg/ml, and transferred into a labelled 2ml glass vial using a Pasteur pipette; the vial was flushed with oxygen-free nitrogen and stored in a freezer at -20°C for further analysis.

2.19 Preparation and purification of fatty acid methyl ester

Fatty acid methyl ester (FAME) was prepared from total lipid by acid-catalysed transformation, as described by Christie (2003) and FAME extracted and purified as described by Tocher and Harvie (1988).

Specifically, total lipid (normally 1mg, but up to 50mg) was added to a small test tube along with heptadecanoic acid (17:0) standard (prepared by 1mg/ml in C:M, 2:1, v/v) at 10% of total lipid mass. The solution was mixed vigorously and evaporated under oxygen-free nitrogen in order to remove any organic solvent. After that, 2 ml of methanol-sulphuric acid methylating reagent (1% sulphuric acid in methanol) and 1ml of toluene was added to the tube to assist the dissolving of neutral lipids. The stoppered tubes were shaken vigorously to mix the solvents, flushed with nitrogen and incubated overnight (16hr) at 50°C in a hot-block (Dri-block heating mantel, Techne).

The tubes were then removed from the hot-block, allowed to cool at room temperature, following which 2ml of 2% (w/v) $KOHCO_3$, 1ml of isohexane: diethyl ether (1:1 v/v) containing 0.01% (w/v) butylated hydroxyl toluene (BHT) and 4ml of isohexane: diethyl ether (1:1 v/v) were added. The tubes were shaken and vortex-mixed then centrifuged at 350-400×g for 2 minutes. The upper organic layer was transferred to another clean test tube, while a further 5ml of isohexane: diethyl ether

(1:1v/v) was added to the organic tube, which was again shaken, vortex-mixed and centrifuged as before. The upper layer was added to the other tubes as before and the combined solvent was evaporated under oxygen-free nitrogen and the dry residual in each tube comprised the crude fatty acid methyl ester extracted from the total lipid. The FAME was re-dissolved in 100µl of isohexane.

Methyl esters were purified by TLC glass plates (20×20 cm), pre-coated with silica gel G (Merck silica gel 60). The methyl esters of the sample were loaded on a 2 cm origin marked with a pencil at 1.8 cm above the bottom edge of the plate. The 20×20 cm plate was marked with 4 origins, separated from each other by 1.5 cm with a 2 cm margin from the side edge of the plate. Methyl esters of four different samples were loaded on each TLC plate by means of a Hamilton syringe. The plate was then run in 100ml of isohexane: diethyl ether: glacial acetic acid (90:10:1 v/v) to 1 cm from the top edge of the plate. The plate was then removed from the glass tank and the solvent was allowed to evaporate in the fume cupboard. Fatty acid methyl esters were visualised by spraying the edge of the plate with 1% iodine in $CHCL_3$ (w/v). Spraying was done only on the two origins located at each side of the plate by masking most of the origin off with a blank glass plate, so that only the very edge of the origin was exposed for spraying. That section was then sprayed lightly with the iodine solution. Thus, the FAME band was clearly visible and marked with a pencil. The FAME chromatography is a double band, with saturated and monounsaturated fatty acids forming the upper band and PUFA the lower band. FAME bands were all marked accordingly, and then were scraped from the TLC plate using a razor blade. They were scrapped into test tubes and FAME eluted with 5ml of isohexane: diethyl ether (1:1v/v) plus 1ml of isohexane: diethyl ether (1:1v/v+BHT) , the tubes were then centrifuged and the solvent was transferred carefully, without touching the lower

silica layer, through a pre-washed filter paper (Whatman No.1) into a clean tube. The solvent was evaporated off under oxygen-free nitrogen and the purified FAMEs were transferred to small vials in 1ml isohexane (+BHT). The labelled vials were flushed with oxygen-free nitrogen and stored at -20°C in a freezer for further analysis.

2.20 Separation and identification of fatty acid methyl esters

Fatty acid methyl esters were separated and quantified by gas-liquid chromatography (GLC) using a Carlo Erba Vega 8160 (Milan, Italy) which is equipped with on-column injection (cold) and using a 30m×0.32mm i.d., 0.25 µl film thickness, fused silica capillary column (CP wax 52 CB, Chrompak Ltd, London, UK). Hydrogen was used as a carrier gas at a flow rate of 2.0ml/min (constant flow mode). The oven temperature was programmed to rise from 50°C to 150°C at 4°C/min and from 150°C to 225°C at a rate of 2°C/min and the final temperature of 225°C was maintained for 5 minutes. The samples were loaded by injecting 1 µl of 1 mg FAME/ml isohexane with a syringe through a silicone rubber septum directly into the packing material of the column ("on-column injection"), so that the flow of gas was not interrupted. Individual methyl esters were identified by comparison to known standards (Marine oil) and by reference to published data (Ackman, 1990). Peak areas of fatty acids were quantified with reference to the peak area of 17:0 internal standards (heptadecanoic acid, Sigma Chemical, St, Louis, USA). Peak data was computed automatically by using Chromcard for Windows (version 1.19) computer package (Thermoqust Italia S.Q.A., Milan, Italy).

2.21 Analysis of ovarian morphology and histology

Selected histological sections were photographed primarily using a Zeiss AxioCam MRc digital camera interfacing with an Olympus BH2 compound microscope using a ×0.75 projection lens at ×1.0 magnification and the software program MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001). Microphotographs of gross ovarian morphology were taken with a purpose written macro program within the KS300 (ver. 3.0) (Carl Zeiss Vision GmbH, 1997) image analysis program. Images were calibrated using a stage graticucle 1mm x 1mm (L4334, Agar Scientific,U. K.).

2.22 Determination of total plasma calcium

Calcium analysis was undertaken to measure both total unbound and bound plasma calcium to equate normal basal levels and that involved in vitellogenesis. Plasma calcium levels were determined using an atomic absorption spectrophotometer (Model 2280 AAS, Perkin Elmer, Norwalk, USA). The following protocol was used at all times and carried out as follows;

2.22.1 Assay Buffer

A 1% nitric acid stock solution was prepared by adding 150ml of 69% nitric acid (Analar grade, BDH Chemicals Ltd., Poole, England) to 15 litres of distilled water. This was then stirred by magnetic flea for 15 minutes to ensure thorough mixing. 5 litres of the stock solution was then decanted into a sterile plastic barrel. To this, 50g of lanthium chloride (Sigma Chemical Company Ltd., Dorset, UK) was added to produce a working solution. Lanthium chloride prevents the interference from non-calcium ions during aspiration in the spectrophotometer. The resulting solution was stored at 4°C until required.

2.22.2 Calcium Standards

Using the assay buffer, standards were prepared from a stock standard of calcium chloride (BDH Chemicals Ltd., Poole, England). Fresh standards were prepared for each assay. Two standards were prepared as follows;

(a) 2mg/l standard

200ml of working assay buffer solution was added to a volumetric flask. 400µl was removed using a pipette and replaced with 400µl of calcium standard. The flask was capped and then thoroughly mixed before transferring the solution to a sterile plastic bottle.

(b) 4mg/l standard

200 ml of working assay buffer solution was added to a volumetric flask. 800µl was removed using a pipette and replaced with 800µl of calcium standard. The flask was capped and then thoroughly mixed before transferring the solution to a sterile plastic bottle.

2.22.3 Sample Dilutions

Sample dilutions were carried out depending on the amount of calcium expected in the sample with respect to the stage of vitellogenesis. Samples from fish with low expected calcium levels were diluted to 1:150 in working solution. Plasma samples from later stages of vitellogenesis were diluted to 1:250 in working solution. All dilutions were carried out in polystyrene tubes (LP4 tubes, Thermo Life Sciences; Hants, U.K.).

Plasma calcium levels recorded from the atomic absorption spectrophotometer were multiplied by the dilution used (1:150) and divided by 10 to obtain the total plasma calcium concentration (expressed as mg %). The detail of method using atomic absorption is shown in **Appendix 5**.

2.23 Testosterone and Oestradiol Radioimmunoassay

Plasma samples were analysed for testosterone and oestradiol using a protocol adapted from Duston and Bromage (1987) as follows:

Assay buffer

Fresh buffer was made the day before each assay and stored overnight at 4 º C prior to use. The following chemicals were dissolved at 50 º C for 30 minutes in 250 ml of nanopure water:

4.44 g disodium hydrogen phosphate
2.91 g sodium dihydrogen phosphate
2.25 g sodium chloride
0.25 g gelatine

Radio labels

A primary stock of tritiated [1, 2, 6, 7-^{3}H] testosterone with a specific activity of 70-105 Ci.mmol^{-1} and [2, 4, 7-3H] oestradiol with a specific activity of 85-110 Ci.mmol^{-1} were supplied in 250 µCi quantities (Amersham International Ltd. UK). This primary stock was used to create an intermediate stock by diluting 20 µl in 2 ml absolute ethanol. This was stored at -20 º C in 20 ml high performance glass vials. A fresh working stock solution was then made for each assay from this intermediate stock. The intermediate stcok was diluted in the assay buffer to give an approximate activity of 20,000 dpm/100 µl (50 µl intermediate stock in 10 ml of assay buffer).

Standard (for standard curve)

Stock standard solution was produced by 2 serial dilutions

1- Oneμg/ml dry testosterone in 10ml absolute ethanol (stored at -20 ºC)

2- Dilute 100μl stock (100ng/ml) in 0.9ml absolute ethanol (to make 10 ng/ml)

Antibody

Freeze dried radio rabbit anti-testosterone or anti oestradiol antiserum was reconstituted in 1 ml of assay, buffer mixed, and 100 μl aliquots were transferred into stoppered 3ml polystyrene tubes (Lp3: Luckhames Ltd., UK) and stored at -20 ºC until used. The working solution was prepared by reconstituting 100μl aliquot in 9.9 ml of fresh assay buffer (for 96 tubes = standard curve plus 37 samples in duplicate, or 48 samples).

Standard

A stock solution of 100 $ng.ml^{-1}$ of testosterone or oestradiol was prepared by dissolving 1 mg of testosterone / oestradiol in 10 ml of absolute ethanol. This intermediate solution was stored in high performance glass vials at -20 ºC until required. A working solution of 10 $ng.ml^{-1}$ was prepared by diluting 100 μl of intermediate stock in 0.9 ml of absolute ethanol. A standard curve was prepared from the working standard solution with a range standard from 1000 pg per tube to 1.95 pg per tube. The 1000 pg per tube standard was prepared from 100 μl of working solution with all subsequent standards being prepared from a series of 1:1 serial dilutions (Figure 2.2).

Steroid extraction

Prior to assaying for steroid, the testosterone or oestradiol in plasma was extracted by suspending samples in organic phase solvent (ethyl acetate) as follows:

1. Add 50 μl of plasma sample to each sample tube
2. Add 1ml ethyl acetate to each tube and stopper

3. Spin tubes on a rotary mixer for 1 hour
4. Centrifuge tubes at 430 ×g for 10 minutes at 4ºC
5. Remove and store supernatant (containing organic phase) in clean tubes at 4 ºC until to be used for assay.

Standard Curve

Tube No.	Standard (µl)	+ ethanol (µl)	Standard amount
1&2	100	none	1000
3&4	100	100	500
5&6	100 of 3&4	100	250
7&8	100 of 5&6	100	125
9&10	100 of 7&8	100	62.5
11&12	100 of 9&10	100	31.3
13&14	100 of 11&12	100	15.6
15&16	100 of 13&14	100	7.8
17&18	100 of 15&16	100	3.9
19&20	100of 17&18	100	1.95 (remove 100µl from each)
21&22	None	100	0
23&24	None	100	NSB

Assay protocol

All standards and samples were analysed in duplicate according to the following procedure:

1. Add 50µl sample extract to each 3 ml sample tube

Prepare standard curve as listed above creating a range of standards from 0-10000 pg/100 µl in 3 ml polypropylene tubes. Include two tubes containing only 100 µl absolute ethanol which are used to calculate non-specific binding (NSB's).

2. Dry down all tubes in a vacuum oven at less than 35 ºC (approximately 45 minutes), then cool to 4ºC
3. Add 100µl antibody solution to all tubes except NSB's (tubes 23&24 in standard curve, to these add 100 µl of buffer).
4. Add 100µl of ^{3}H-testosterone or ^{3}H-oestradiol, to all tubes, vortex and incubate at 4ºC for 18hrs.
5. Dissolve 0.48g dextran-coated charcoal in 100ml buffer and stir on ice for 30 minutes, add 500µl of the dextran/charcoal suspension to each tube, vortex and incubate at 4ºC for 15 minutes.
6. Centrifuge the tubes at 770×g 15 minutes at 4ºC.
7. Transfer 400 µl supernatant to 6 ml polyethylene scintillation vials and add 4 ml of scintillation fluid.
8. Place 4 ml of scintillation fluid into three extra vials. Into two of these place 100 µl of tritiated testosterone/ oestradiol to calculated total radioactivity. The final vial is used to calculate background radioactivity.
9. Vortex all vials and count the radioactivity for 10 minutes in a scintillation counter.
10. Place in counter first blank the 2 totals then standard curve

Step 1.) $\left[\left(\frac{\text{pg per tube}}{400}\right)\times 700\right] = \text{pg of testosterone per 100 } \mu\text{l of extract } \left(\text{Equation 1}\right)$

Step 2.) $\left[\left(\frac{\text{Equation 1}}{100}\right)\times 1100\right]\times 10 = \text{pg .ml}^{-1} \text{ of testosterone or oestradiol in plasma}$

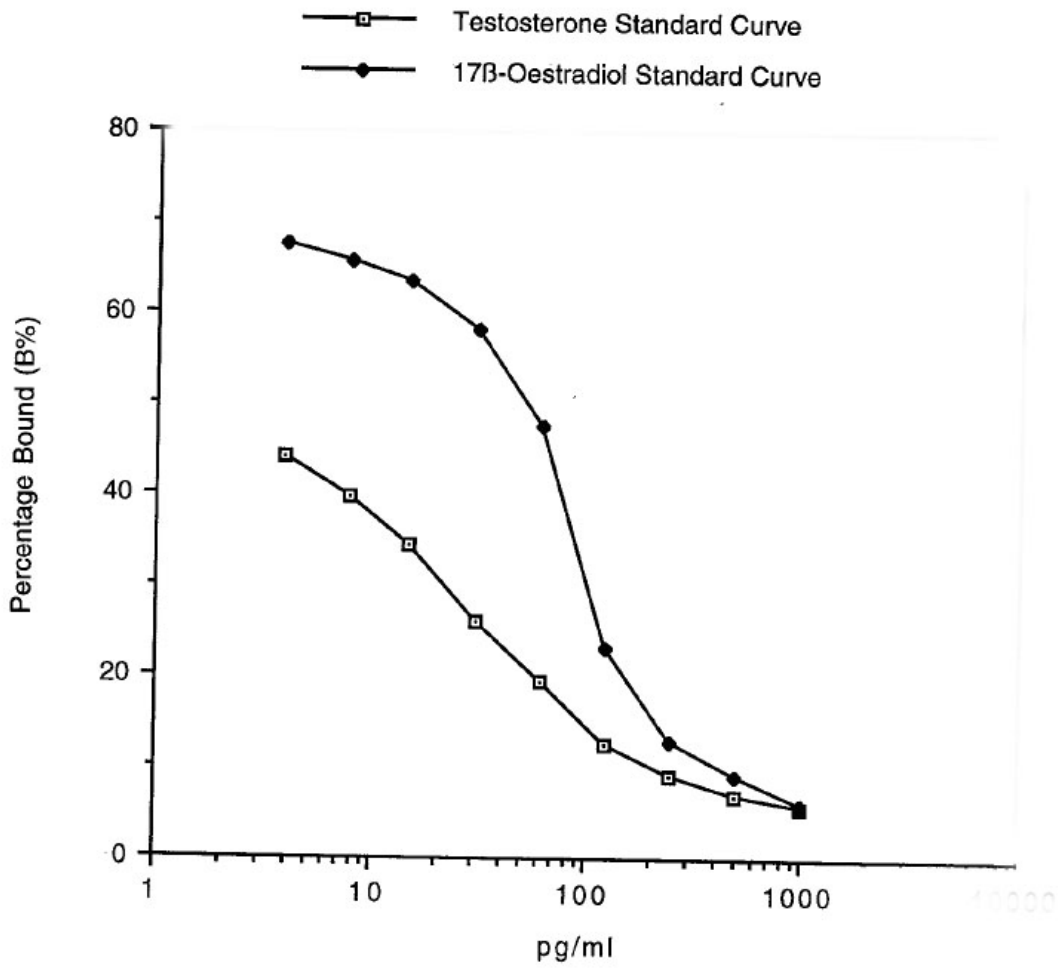


Figure 2.2 Examples of the standard curves obtained for testosterone and 17ßoestradiol using the radioimmunoassay method developed by Duston and Bromage (1987). Concentration of the specified hormone is obtained by intersection of the appropriate standard curve at the point corresponding to the percentage of the radiolabel bound to antibody (% bound) in each sample. Note that each point represents the mean of duplicate standard preparations.

Quality control and validation

The sensitivity of the assay, *i.e.* the minimum amount of testosterone statistically distinguishable from zero, was (1.9 pg.tube^{-1}). Quality controls (QCs) with a testosterone content of approximately (100 pg.tube^{-1}) were used to check the reproducibility of measurements between assays. The inter-assay and intra-assay coefficient of variation were 8.2% and 14.8%, respectively.

2.24 Larvae weight and length measurement

The larvae from each spawning were weighed to the nearest 0.1mg individually after wiping the excess water from each larva using tissue paper with the aid of a small brush. The larval total and standard length were measured on images grabbed using a JVC KY-F30B 3CCD video camera mounted on an Olympus SZ40 dissecting microscope fitted with an extension tube, a ×1.0 additional lens fitted to the objective housing a ×2.5 lens projection lens and examined using ×0.67 magnification and using a purpose written macro program "Tilapia Fish" within the KS300 (ver. 3.0) (Carl Zeiss Vision GmbH, 1997) software. Prior to each set of measurements, the microscope was calibrated using a standard measuring device within the calibration sub-routine of the "Tilapia Fish" macro program.

2.25 Statistical analysis

2.25.1 Estimation of the population mean

The arithmetic mean (X) was used to provide an estimation of the population mean (μ). In all cases the mean was used along with the standard deviation of the mean (SD) to give a representation of the sample distribution. All basic statistics were calculated with the aid of Microsoft Excel 2003. All the data were presented as means ± SD (n=3). The statistical analyses of experimental data were performed using SPSS 15 (SPSS 15 2006) and Minitab (V.15). The graphs were created using Sigma plot 10 (Sigma plot software).

2.25.2 Parametric testing

Parametric tests were performed based on the assumptions that the observations were made at random and the test variances independent. Furthermore, the sample variances must be homogenous and the data normally distributed. Where data failed to meet these requirements, non-parametric tests were employed (section 2.25.5).

2.25.3 Normality testing and Homogeneity of variance

A PP-plot test was used to determine the normality of a distribution. This test compares the cumulative distribution of the data with that of an ideal Gaussian distribution, basing its P value on the largest discrepancy (see **Appendix 6**). Homogeneity of variance was tested using the F-test for comparison of two samples and Bartlet's test for three or more samples. If the calculated F-test value was less than the tabulated value at P=0.05, then the variance was treated as homogenous and if greater as heterogeneous (Zar, 1999).

Where data was examined by a general linear model (section 2.25.5.), and n was typically large, normality and homogeneity of variance were assessed through examination of the residual plots.

2.25.4 Multiple comparisons

Data involving three or more samples, and which met the assumptions set out for parametric testing, was analysed using a three-way analysis of variance (ANOVA) using SPSS or Minitab statistical pakage. In addition, the General Linear Model (GLM) feature was applied to analyse appropriate data sets. The GLM incorporates a number of different statistical models accounting for numerous factor levels including

replication and repeated sampling measures. Where data differed significantly ($P<0.05$), Tukey's multiple comparison post-hoc test were applied.

2.25.5 Nonparametric testing

Data failing to meet the assumptions for parametric tests were analysed using non-parametric statistical methods. A Kruskal-Wallis test was performed using the Instat statistical package (Instat version 3.0.; GraphPad Software Inc., California, USA). Means bearing significant differences ($P<0.05$) were further test using Dunn's multiple comparison post-hoc test.

Chapter 3 - Spawning periodicity, fecundity, egg size and larval quality in *O. niloticus* laboratory stock held under different feeding regimes.

3.1 Introduction

In many cultured fish species, particularly in those new for aquaculture, unpredictable and variable reproductive performance is an important limiting factor for the successful mass production of seed. An improvement in broodstock nutrition and feeding has been shown to greatly improve not only egg and sperm quality but also seed production. Gonadal development and fecundity are affected by certain essential dietary nutrients, especially in continuous spawners with short vitellogenic periods (Izquierdo *et al.,* 2001a). Thus, during the last two decades, more attention has been paid to the level of different nutrients in broodstock diets. However, studies on broodstock nutrition are limited and relatively expensive to conduct.

Few studies have investigated reproductive performance under varying environmental conditions. Cichlidae have been shown to produce many more broods per year in aquaria or culture ponds than under natural conditions in lakes or rivers. The reasons for this are not clear and may vary from species to species (Lowe-McConnell, 1955; Lowe-McConnell, 1982; Lowe-McConnell, 2000). The aim of the present study was to rear the tilapia *O. niloticus* from larvae to broodstock held under different feeding regimes using experimental diets. Thereafter egg and larval quality were investigated from broodstock obtaining in control conditions.

Sexually mature cichlids are generally able to undergo successive reproductive cycles at intervals of approximately 4-6 weeks. Assuming that environmental conditions

remained reasonably constant this would, theoretically, lead to continuous fry production. However, the reproductive cycles of individual tilapia broodstock under intensive farming conditions tend to be asynchronous and lead, inevitably, to episodic fry production (Campos-Mendoza *et al.,* 2004; Coward and Bromage, 2000; Jalabert and Zohar, 1982). Evolution of parental care in tilapia has led to an increase in egg size and corresponding reduction in the number of eggs per clutch (Noakes and Balon, 1982; Rana, 1988). The low number of eggs per spawn, in combination with the asynchronous nature of spawning behaviour in tilapias under hatchery conditions would therefore necessitate the use of extensive fish-holding facilities and time-consuming management of extremely large numbers of fish to maintain a continual supply of sufficient high-quality fry. To minimize the number of broodstock needed to attain target production, factors such as broodstock size have previously been shown to influence reproductive capability (Coward and Bromage, 1999a; Wootton, 1979). Tilapias are multiple-spawners; their fecundity represents only a fraction of their reproductive potential (Coward and Bromage, 1999a; Rana, 1988; Wootton, 1979). Therefore spawning periodicity also needs to be addressed. These requirements may be reduced, in part, by selecting and utilising broodstock exhibiting optimal reproductive traits such as egg size, fecundity, clutch weight and egg to body weight ratio (Coward and Bromage, 2000; Dadzie, 1970; Fishelson, 1966; Little *et al.,* 1993; Rana, 1988; Siraj *et al.,* 1983). Reproductive capacity of broodstock, however, not only concerns fecundity and egg size but also nutritional status, can affect spawning performance and larval quality (Coward and Bromage, 2000; Townshend and Wootton, 1984; Watanabe *et al.,* 1984; Wootton, 1979). Since tilapias are multiple spawners, their fecundity represents only a fraction of their reproductive potential (Rana, 1985).

It is well known that parental care can influence spawning frequency as longer inter-spawning- intervals (ISI) occurr in tilapia with an increase in the levels of parental care (Campos-Mendoza *et al.,* 2004; Rana, 1988; Smith and Haley, 1987; Tacon *et al.,* 1996). Spawning frequency may also be influenced by various manipulative techniques. For example, removal of eggs from mouth-brooding female tilapia can effectively reduce spawning frequency (Campos-Mendoza *et al.,* 2004; Dadzie, 1970; Dadzie and Wangila, 1980; Rana, 1985; Siraj *et al.,* 1983). Under ideal conditions females may spawn every 12-16 days when their eggs are removed (Campos-Mendoza *et al.,* 2004; Coward and Bromage, 2000; Dadzie, 1970; Fishelson, 1966; Little *et al.,* 1993; Rana, 1988; Siraj *et al.,* 1983; Tacon *et al.,* 1996).

The effect of food ration on reproduction under laboratory conditions has been extensively studied in many fish species, such as rainbow trout (*Oncorhynchus mykiss*) reviewed by Randall and Bromage (1992), haddock (*Melanogrammus aeglefinus*) (Hislop *et al.,* 1978; Robb, 1982), stickleback (*Gastehaus aculeatus*) (Wootton, 1973), convict cichlid, (*Cichlasoma nigrofasciatum*) (Townshend and Wootton, 1984), the guppy (*Poecilia reticulata*) (Dahlgren, 1980; Hester, 1964) and *Tilapia zillii* (Coward, 1997). However, although it is believed that food restriction generally reduces total fecundity and may delay maturation and affect egg size and/or weight, no clear pattern of effects has emerged (Cerdà *et al.,* 1994a) and studies specifically on *O. niloticus are* limited. Various studies of the roach (*Rutilu Rutilus*) have also indicated that diminished food supplies reduced fecundity (Kuznetsov and Khalitov, 1978; Mackay and Mann, 1969) and delayed maturation (Mackay and Mann, 1969) but did not affect the egg size (Kuznetsov and Khalitov, 1978).

Mass production of tilapia fry remains the most important prerequisite in the intensification of tilapia culture. Broodstock productivity and nutrition remain the most significant constraints to commercial production costs and therefore knowledge of the factors affecting broodstock productivity is of immense importance to the further development of tilapia culture. However, broodstock nutrition is one of the most important factors limiting fish egg and larval quality (Izquierdo *et al.,* 2001a). In particular, lipid and essential fatty acids (EFA) are one of the nutritional factors, which greatly affect egg and larval quality (Bell *et al.,* 1997; Fernandez-Palacios *et al.,* 1995; Furuita *et al.,* 2000; Harel *et al.,* 1994; Izquierdo *et al.,* 2001a; Navas *et al.,* 1997; Watanabe *et al.,* 1984; Watanabe *et al.,* 1985). Marine fish oils are traditionally used as the main dietary lipid source in many commercial fish feeds. Aquafeeds currently use about 70% of the global supply of fish oil and by the year 2010, fish oil use in aquaculture is estimated to reach about 97% of the world supply (Tacon, 2003). As result of rapid aquacultural development, the industry may not be able to rely on the finite stocks of marine pelagic fish for oil supply of aquafeeds. One of the potential replacements for fish oil in aquafeeds is palm oil.

In this respect, palm oil is similar to other vegetable oils that have been reported in numerous scientific papers by its ability to replace a significant part of fish oil in fish diets without negatively affecting fish growth, feed utilization and survival (Al-Owafeir and Belal, 1996; Bell and Sargent, 2003; Kanazawa *et al.,* 1980; Legendre *et al.,* 1995; Ng *et al.,* 2000; Ng *et al.,* 2003; Ng *et al.,* 2004; Ng *et al.,* 2006; Tortensen *et al.,* 2000; Varghese and Oommen, 2000). In addition, to its low cost and high availability, palm oil also has many additional advantages such as high carotenoid content and vitamin E over other vegetable oils when used in aquafeed formulation (Ng *et al.,* 2003; Ng *et al.,* 2004; Wattanapenpaiboon and Wahlqvist, 2003).

The effect of dietary lipid on reproductive performance of marine species has been reported. The elevation of dietary lipid levels from 12% to 18% in broodstock diets for rabbitfish (*Siganus guttatus)* resulted in an increase in fecundity and hatching (Duray *et al.,* 1994), although this effect could also be related to a gradual increase in the dietary essential fatty acid content. Indeed, one of the major nutritional factors that has been found to significantly affect reproductive performance in fish is dietary essential fatty acid content (Watanabe *et al.,* 1984). Spawning performance (*e.g.* fecundity, hatching and larval survival) in gilthead sea bream *Sparus aurata* was found significantly improved with an increase in dietary n-3 PUFA level up to 1.6% (Fernandez-Palacios *et al.,* 1995), and similar results have been reported in other sparids (Tandler *et al.,* 1995; Watanabe *et al.,* 1984).

The effect of dietary lipid source on spawning performance of tilapias has not been sufficiently studied. Only Santiago and Reyes (1993) studied the effects of dietary lipid source on the reproductive performance and tissue lipids of Nile tilapia, reporting that cod liver oil (rich in *n*−3) resulted in poor reproductive performance, while highest fry production was obtained from fish fed a diet supplemented with soybean oil (rich in n-6 fatty acids). Similarly, El-Sayed *et al*. (2005) studied the effect of dietary lipid source on spawning performance at different salinities and found that tilapia need fish oil for better reproduction performance in brackish water while plant oil (soybean oil) is required for freshwater rearing. However, dietary lipid sources have not been examined under one culture system, including serial spawnings and over the entire life cycle. Consequently, the present study investigated the effect of different dietary lipid sources fed at two rations (3 and 1.5 % body weight /day for full and half ration, respectively) on reproductive traits such as spawning periodicity, fecundity, egg size and larval quality over three consecutive spawnings in Nile tilapia,

O. niloticus, which have been reared for their entire life cycle on their respective dietary regime in recirculating tanks at the Institute of Aquaculture (I.O.A.) Stirling University.

3.1.1 Overall aims of this study were to:

- Investigate the effect of different dietary lipid sources on *O. niloticus* egg and larval viability and quality.
- Investigate the influences of feeding ration size on *O. niloticus* egg and larval quality over three consecutive spawning.
- Investigate the effect of dietary lipid source on reproductive performance of *O. niloticus* for three consecutive spawnings with the goal of substituting palm for fish oil as a lipid source.

3.2 Materials and methods

3.2.1 The effect of dietary lipid sources on growth and early survival of laboratory-held *O. niloticus* stocks at two ration levels

This part of the experiment aimed to produce stocks of male and female *O. niloticus* that had been subject to long-term food rationing that could be used as a stock of full and half rationed fish for use in subsequent experiments. Fish were rationed from first feeding (end of yolk sac – E.Y.S.) for several months until they could be used in subsequent physiological experiments. Owing to a lack of tank space, only two ration levels (full and half) could be studied in duplicate for 4 diets.

Newly hatched tilapia *O. niloticus* larvae were obtained from pure-bred stock held at Institute of Aquaculture, University of Stirling, Scotland. The feeding experiments

were conducted using 13 days post-fertilisation larval tilapia at the onset of exogenous feeding. Sixteen groups of 5 fry from each tank were sacrificed and weighed to the nearest 0.00lg. This data was used to calculate the average initial weight of fry per treatment tank. Initial high ration was set at 30% BW/day and initial low ration at 15% BW/day. These ration levels were 10 and 20% lower than those used by Coward (1997) and Macintosh and De Silva (1984) in a similar study of early growth and survival in *T. zillii* and *O. mossambicus* and *O. niloticus* x *O. aureus* hybrids, respectively. Ration levels were altered as necessary over the course of fry development. Alterations to rations were made when a large amount of excess (uneaten) food was observed in treatment tanks. On these occasions, fish in the high-ration tanks were fed to satiation 3 times during a 24 hour period; this providing an indication of the new high ration size to be allocated. Half ration groups were fed 50% of the ration given to high ration groups.

Food rations for each treatment tank were weighed out in advance in 10 day quantities. Rations were initially provided by grinding the experimental pelleted feed to a suitable particle size (initially 250 -850μm). Particle and pellet size were gradually increased as fish grew (see Table 2.4 in Chapter 2). Daily rations were provided to each treatment group 3 times day^{-1}. Faeces and excess food were siphoned from each tank twice daily. Any mortalities were removed as soon as discovered and rations altered accordingly. Every 10 days, 16 groups of at least 5 fry from each treatment group were sacrificed and weighed to the nearest 0.001g. The length was also measured individually utilising image analysis software (MRgrab 10.0.04 Carl zeiss vision GmbH, 2001) (see section 2.12.1) and the daily ration altered. The fish were then fixed in 10% NBF for gonadal development for later investigation which is not included in the thesis. Every 30 days, the true number of fish remaining in each

treatment tank was ascertained by counting manually. This was to avoid relying merely upon the presence of carcasses as an indicator of mortality. It was assumed that a degree of cannibalism could occur and would thus lead to over-estimations of population size in each tank. Once deemed large enough, fry were transferred into second (bigger) recirculating tanks (incorporating 50 x 38 x 41 cm). Rationing was maintained for a total of 18 months whereupon a series of physiological experiments were commenced. Thus growth data are only provided up to day 120 days here, the ration allocations are shown in (Table 2.7 in Chapter 2). Rationing of the 4 treatment groups was continued so as to provide a stock of rationed fish until terminating the spawning performance and oocyte recruitment sections of experiments.

3.2.2 Feeding procedure

Four diets, including the control with two rations (full and half ration) were used (Table 3.1). Diet 1 (D1) containing cod liver oil (CO) fed one ration only, diet 2 (D2) containing palm oil (PO), diet 3 (D3) containing a combination of palm and cod liver oil (PO & CO) (9:1 ratio) and diet 4 (D4) a commercial trout feed (Skretting UK) containing fish meal and fish oil as control.

Table 3.1 Experimental design

Lipid source of diets	Diet type	Protein source	Ration (%BW day$^{-1)}$		No. of replicate	No. of spawning/fish
			Full	half		
Cod liver oil	D1	Soybean Concentrate	3	No data	2	3
Palm oil	D2	Soybean Concentrate	3	1.5	2	3
Mixed PO*&CO** (9:1)	D3	Soybean Concentrate	3	1.5	2	3
Control	D4	Fish meal	3	1.5	2	3

*PO=palm oil and

**CO=cod liver oil

3.2.3 Fish procurement

Fish in this investigation were maintained in 60 litre holding tanks (50 x 38 x 41 cm) in a recirculating system at the tropical aquarium at I.O.A. University of Stirling. Stocks of *O. niloticus* were obtained from two replicates which had previously been fed with 3 experimental diets and a commercial trout diet (Skretting, UK) as control at full and half ration, respectively, for their entire life from onset of feeding until maturation (see section 3.2.2). Female broodfish were then collected randomly from their successive populations and measured (weight and total length) and tagged with Passive Integrated Transponder-PIT tags (Trovan, UK) under anaesthesia by immersion in 1:10,000 ethyl 4-aminobenzoate (Sigma, UK). The females were then placed into glass aquaria (114×45×42 cm) partitioned with Perspex dividers such that each individual had its own holding space. Fish were allowed to recover completely in clean aerated water prior to being placed to their respective glass tank (section **2.6**). Broodfish were fed according to the feeding protocol. Fish were fed three times daily

(900:1300:1700 hours) at a rate of 3 and 1.5% of BWday^{-1} as full and half ration, respectively with the experimental diets and a commercial pelleted trout feed (Skretting, UK) as a control until 3 successful batches of egg and larvae was obtained from each broodstock.

3.2.4 Spawning investigation

Fish were checked at two hourly intervals during the day for the evidence of spawning. In females undergoing ovulation and oviposition, the genital papilla were considerably swollen and extended. Fish also were observed for brooding behaviour which involved an over-extension of the lower jaw and females cleaning behaviour of a discrete area on the horizontal tank base. Once the indications of spawning were observed broodfish were netted and then anaesthetised, thereafter the genital papillae were blot-dried with absorbent tissue paper and the fish held dorsal side uppermost with the left hand holding the fish and the right hand carefully massaging the fish's abdomen in such a manner that eggs were extruded from the ovary and collected into a clean plastic Petri-dish. Care was taken to avoid water contamination which would otherwise initiate water hardening and precocious closure of egg micropyles. Once eggs were obtained, sperm was collected and pooled from 2-3 anaesthetised males belonging to the same trial (diet and ration) by applying the same stripping techniques as detailed above after first emptying the fish's urea bladder. Sperm released from the testes was collected directly from the genital papillae with the aid of disposable glass heamatocrit tubes (Camlab, U.K.). The females were then measured and weighed prior to returning them into the experimental glass tank and recovered in clean, aerated water and all data recorded.

Eggs were fertilised by adding the pooled sperm to the Petri-dish containing eggs. Gametes were carefully but thoroughly mixed with a fine brush and a tiny volume of water. The mixture was then allowed to stand at ambient aquarium temperature for 4-5 minutes. Water was poured over the tip of the eggs with a 2 ml plastic pipette. After a further standing period of 4-5 minutes, water hardening occurred. In order to count the egg numbers, Petri-dishes containing the fertilised eggs were scanned using a scanner and the scanned pictures analysed using image analysis software (Section 2.21) to determine total fecundity according to Rana (1986) definition where total fecundity is the number of eggs in a freshly spawn batches. Fertilised eggs were then placed in round-bottomed plastic containers (Rana, 1986) supplied with clean, U.V. sterilised water and left until hatch. The inter-spawning-interval (ISI) was determined (section **2.16**) and all the data were recorded.

A sub-sample of 50 eggs per spawn was taken, prior to incubation and each egg individually measured to the nearest 0.1mm (see section 2.13) Since tilapia eggs are ellipsoid-shaped, it was important to measure both axes (long and short axes) in order to calculate egg diameter and volume (see section 2.13) according to the method of Coward and Bromage (1999a).

The fertilisation (%) and hatching rate (%) and inter-spawning-interval (ISI time elapsed between one spawn and the next) were also recorded.

After measuring the egg size, they were weighed and subsequently oven dried at 70ºC for 24h. Mean egg dried weight was determined to the nearest 0.1mg. The EW: BW ratio was also determined (Section **2.15**) according to Coward and Bromage (1999a).

3.2.5 Larval quality

Larvae originating from each individual female were sacrificed by overdose of anaesthetic and weighed to the nearest 0.1mg at 10 days post-fertilisation. Their length was also measured to the nearest 0.1mm utilising MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) (see section 2.12.1).

3.2.6 Statistical analysis

Data were tested for normality and logarithmically transformed if necessary and statistical analyses were performed using SPSS for Windows (version 15) and Minitab (version 15) (section 2.25 in Chapter 2)

3.3 Results

3.3.1 Growth performance

Growth data of rationed fish used in each treatment are provided along with the relevant feeding regime. Growth responses of O. *niloticus* fry are presented as initial and final mean weights, weight gain and specific growth rate in Table 3.2. The growth rate of fish fed diet1 (cod liver oil) was significantly ($p<0.05$) lower than others at either full or half ration. Growth responses were significantly affected by dietary lipid source. In general, during a period of 120 day investigation, growth rate increased with increasing the ration size. Diet 4, a commercial trout diet (Skretting, UK), as control had the highest weight gain (19.76 and 16.21 g), diet 2 (19.53 and 13.12 g) followed by diet 3 (18.3 and 12.96 g) and the least (10.61 and 6.99 g) was diet 1 for full and half ration, respectively. However, in the case of specific growth rate the control was significantly higher (2.6 $\%.day^{-1}$) than diet 1 (2.2 $\%.day^{-1}$). Weight gain of fish fed diets 2 and 3 which had palm and mixed palm and cod liver oil as lipid

source was not significantly different from the control at either full or half rations. With respect to SGR, diets 2 and 3 resulted in the same values as the control. This meant that these diets were not significantly different from the control. Food conversion ratio (FCR) followed the same trend as SGR with the exception of diet 1 which was significantly higher at both full and half ration than other diets.

Table 3.2 Growth and feed utilisation of Nile tilapia (*O. niloticus*) fry fed different dietary lipid sources

Parameters	Diet 1		Diet 2		Diet 3		Diet 4	
	F	H	F	H	F	H	F	H
I_W (g)	0.015±0.02	0.015±0.02	0.015±0.02	0.015±0.02	0.015±0.02	0.015±0.02	0.015±0.02	0.015±0.02
F_W (g)	10.6±1.79^{a}	9.0±2.57^{ab}	19.5±1.5^{c}	13.1±1.2^{d}	18.5±1.36^{c}	13.0±1.76^{d}	19.8±1.45^{c}	16.2±1.37^{cd}
W_G (g)	716.2±59.12	539.4±54.67	2072.2±34.67	1294.4±45.65	1942.9±39.65	1356.91±45.17	2169.6±39.18	1532.54±51.23
SGR (%/day)	3.2±0.23^{a}	2.19±0.26^{b}	2.97±0.09^{c}	2.49±0.13^{c}	2.91±0.17^{c}	2.56±0.13^{c}	2.98±0.16^{c}	2.78±0.14^{c}
FCR	3.4±0.25^{a}	3.9±0.33^{ab}	2.31±0.17^{c}	2.21±0.23^{c}	2.41±0.14^{c}	2.3±0.16^{c}	2.2±0.13^{c}	2.2±0.19^{c}
SR (%)	45±5.77^{a}	40±10.12^{a}	70.1±7.63^{b}	71.2±8.11^{b}	69.9±7.67^{b}	69.3±8.26^{b}	73.2±6.56^{b}	71.9±7.45^{b}

F= full ration, H= half ration, I_W=initial weight, F_W=final weight, W_G=weight gain (as total mean weight), SGR=specific growth rate, SR=survival rate and FCR= food conversion ratio. Values are mean ±SD of two replicate, and values within the same row with different letters are significantly different ($p<0.05$)

3.3.2 Egg and larval quality

All parameters of egg quality of brood-fish fed different dietary lipid sources and ration size over three consecutive spawnings were analysed by three-way ANOVA to establish any interaction between diets, rations and spawning numbers. The analysis model and their significance levels are shown in (Table 3.4).

Table 3.4 shows that the majority of egg quality parameters were not significantly different ($p>0.05$) among the diets, rations and spawning numbers, nevertheless, only one way-ANOVA showed the relative fecundity and EW:BW with the control diet was significantly different from the others diets (Table 3.5).

Table 3.3 Total fecundity, egg weight, egg size and volume data obtained from *O. niloticus* fed different dietary lipid sources

Treatments			Parameters									
Diets	Ration	Sp no.*	Fish weight range (g)	Length (cm)	E.D.[1] (mm)	EV[2] (mm^3)	TF[3]	TEV[4] (mm^3)	RF[5]	EDW[6] (mg)	EWW[7] (mg)	EW:BW[8] (%)
2	full ration	1	116-134	18.3-19.1	2.2±0.15	4.7±0.38	784.4±66.92	4304.3±515.0	6.8±0.69	2.4±0.17	5.6±0.33	1.7±0.22
		2			2.0±0.18	3.7±0.31	717.6±91.06	3644.8±1276.1	5.6±0.69	2.7±0.25	5.8±0.47	1.5±0.15
		3			2.0±0.10	4.1±0.46	777.8±102.9	3226.2±570.0	5.9±0.91	2.8±0.25	6.1±0.55	1.6±0.29
	Half ration	1	132-155	18.6-20	2.2±0.06	5.3±0.38	660.1±59.32	3560.7±478.5	5.1±0.39	2.6±0.10	6.2±0.19	1.3±0.11
		2			2.2±0.07	5.6±0.47	776.4±59.01	4279.2±481.3	5.6±0.48	2.6±0.08	6.2±0.19	1.5±0.14
		3			2.1±0.03	4.9±0.20	820.4±81.70	3990.4±460.3	5.3±0.53	2.7±0.07	6.3±0.21	1.4±0.13
3	full ration	1	155.7-183-3	19.5-20.9	2.2±0.04	5.4±0.37	767.6±44.43	4154.9±439.9	5.3±0.53	2.4±0.06	6.3±0.34	1.3±0.13
		2			2.1±0.11	4.7±0.61	737.3±47.88	3501.9±517.9	4.8±0.50	2.6±0.12	6.0±0.26	1.2±0.09
		3			2.3±0.07	5.7±0.47	889.6±129.6	4877.6±527.8	5.3±1.06	2.5±0.11	6.0±0.23	1.3±0.20
	Half ration	1	114-158.5	17.7-20.3	2.2±0.12	5.3±0.80	846.0±308.1	4337.3±1450.9	7.1±1.93	2.6±0.29	6.2±0.90	1.7±0.30
		2			2.4±0.05	6.3±0.37	902.0±194.7	5552.8±1065.0	6.7±1.63	1.9±0.85	6.7±0.86	1.2±0.69
		3			2.2±0.10	5.2±0.59	894.7±101.0	4633.3±505.4	5.6±0.57	2.5±0.25	6.0±0.84	1.4±0.26
4	full ration	1	205.6-269.2	21.7-23.5	2.2±0.04	5.2±0.30	605.2±64.96	3159.1±382.5	3.5±0.81	2.8±0.25	6.7±0.46	1.0±0.28
		2			2.3±0.07	6.0±0.59	649.8±82.66	3835.1±480.2	3.2±0.97	2.6±0.18	6.6±0.46	0.8±0.16
		3			2.4±0.07	6.3±0.60	678.2±117.2	4081.2±447.7	2.9±0.89	2.9±0.18	7.2±0.36	0.8±0.20
	Half ration	1	142-191.2	18.5-20.3	2.2±0.12	5.0±0.87	637.3±101.0	3421.6±1169.3	4.5±0.42	2.6±0.24	6.1±0.59	1.2±0.21
		2			2.2±0.10	5.0±0.73	640.5±77.12	3043.1±184.0	3.5±0.39	2.6±0.37	6.2±0.72	0.9±0.13
		3			2.2±0.06	5.6±0.45	798.0±114.7	4438.5±750.6	4.2±0.50	2.8±0.33	6.6±0.74	1.1±0.08

* = spawning numbers, [1] Egg diameters, [2] Egg volume, [3] Total fecundity, [4] Total egg volume, [5] Relative fecundity, [6] Egg dry weight, [7] Egg wet weight, and [8] Egg weight to body weight ratio

Table 3.4 Effect of 3 levels diet (D2, 3 and 4) two levels of ration (full and half) and 3 levels of spawning numbers (1, 2 and 3) on the egg quality Nile tilapia *O. niloticus*

Source of variation	d.f.	E. D.[1] (mm)	EV[2] (mm^3)	TF[3]	TEV[4] (mm^3)	RF[5]	E. D. W.[6] (mg)	E.W.W.[7] (mg)	EW : BW[8] (%)
Diet	2	0.21	0.72	0.13	0.29	0.001	0.24	0.31	0.001
Ration	1	0.64	0.89	0.55	0.62	0.38	0.50	0.94	0.57
Spawning No	2	0.99	0.95	0.25	0.52	0.32	0.06	0.40	0.17
Diet × Ration	2	0.27	0.56	0.78	0.81	0.15	0.84	0.35	0.26
Diet × Spawning No	4	0.32	0.63	0.78	0.66	0.78	0.16	0.65	1.00
Ration × Spawning No	2	0.39	0.43	1.00	0.78	0.98	0.51	0.21	0.75
Diet × Ration × Spawning No	4	0.50	0.61	0.81	0.42	0.40	0.07	0.93	0.52

In each row the tests of between-subjects effects of egg parameters shows significant diferences ($p<0.05$) among the values according to Tukey's test.

[1] Egg diameters, [2] Egg volume, [3] Total fecundity, [4] Total egg volume, [5] Relative fecundity, [6] Egg dry weight, [7] Egg wet weight, and [8] Egg weight to body weight ratio.

3.3.2.1 Fecundity and egg size

3.3.2.1.1 Fecundity

A total of 125 spawns were recorded over three consecutive spawnings for all diet treatments at full and half ration. In the CO diet (diet 1) only one fish spawned three times. Due to high mortality of fish during rearing time period and poor egg quality, data obtaining from this group was discarded from analyses see (section 3.3.1).

In total, 43 females were used for this experiment and a total of around 94,000 eggs were collected over three consecutive spawnings in all experiments. In general, each fish produced an average of 2,180 eggs over three spawnings and a mean of 727 in an individual spawning. The minimum number of eggs produced was from group fish fed a full ration of diet 2 (PO) with 311 eggs and the maximum number of eggs was produced by the full ration of diet 3 (PO&CO) with 1,750 eggs. The mean total fecundity of fish fed diet 2 averaged 759.0± 46.7 and 751 ± 39.0; fish fed diet 3 averaged 801.0 ± 50 and 881.0 ± 110 and the fish diet 4 averaged 642.4±48.00 and 692.0 ± 56.4 for full and half ration, respectively, over three spawnings. There were no significant interactions ($P>0.05$) on total fecundity between diet ration and spawning numbers by three-way ANOVA (Table 3.4) and also no significant interactions were observed between diet× ration, diet × spawning number and ration × spawning number by two-way ANOVA (Figure 3.1) . There were no significant differences ($P>0.05$) observed on total fecundity when comparing the mean data between the diets performing one- way ANOVA (Table 3.4).

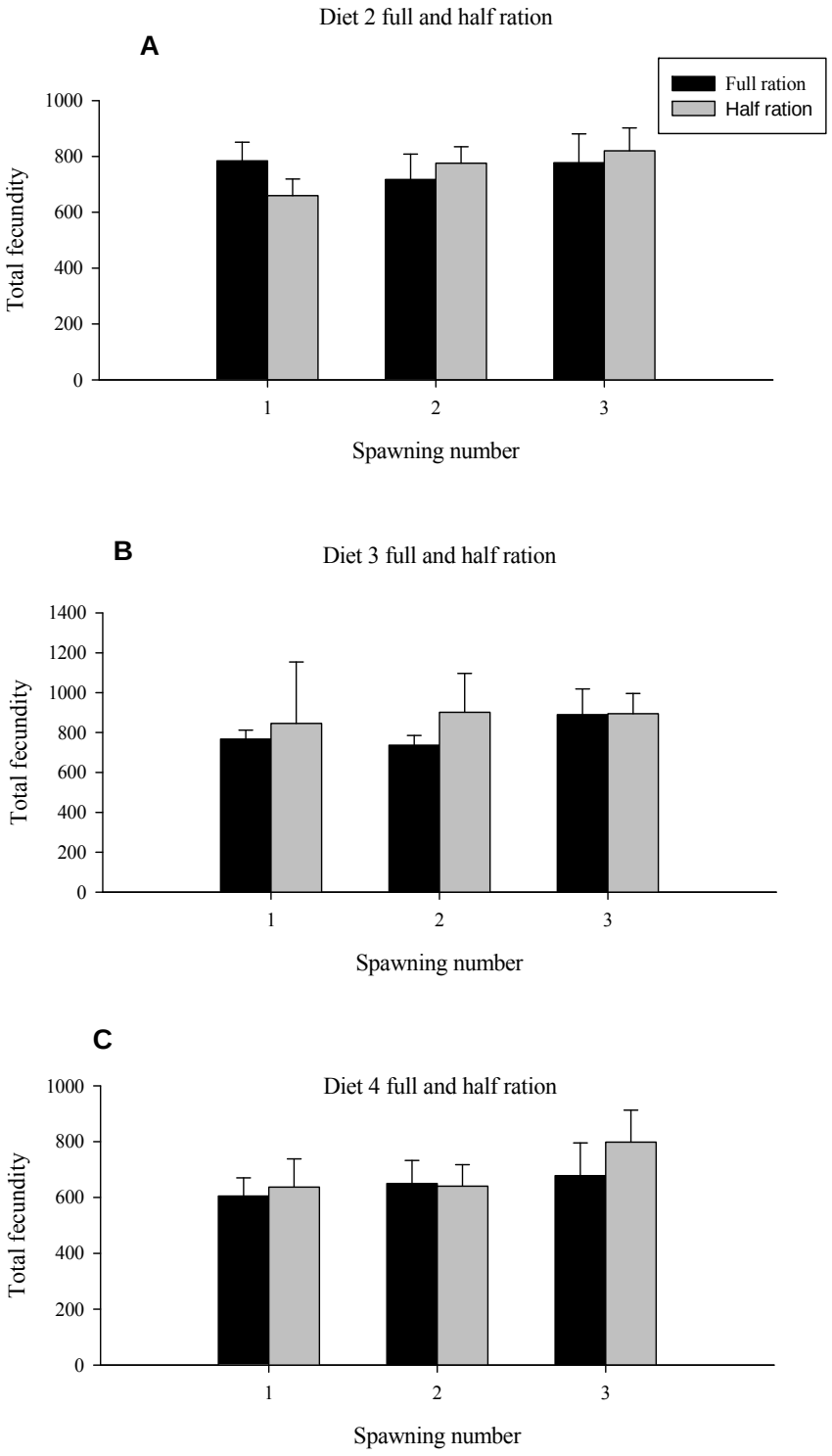


Figure 3.1 Total fecundity of *O. niloticus* fed different diets at full and half ration over three spawnings (data represent as mean ± SEM, n=3). In each graph, the differences between the rations within each spawning are not significant ($p>0.05$). A= diet 2 containing palm oil, B= diet 3 containing palm and cod liver oil and C= diet 4 as a control.

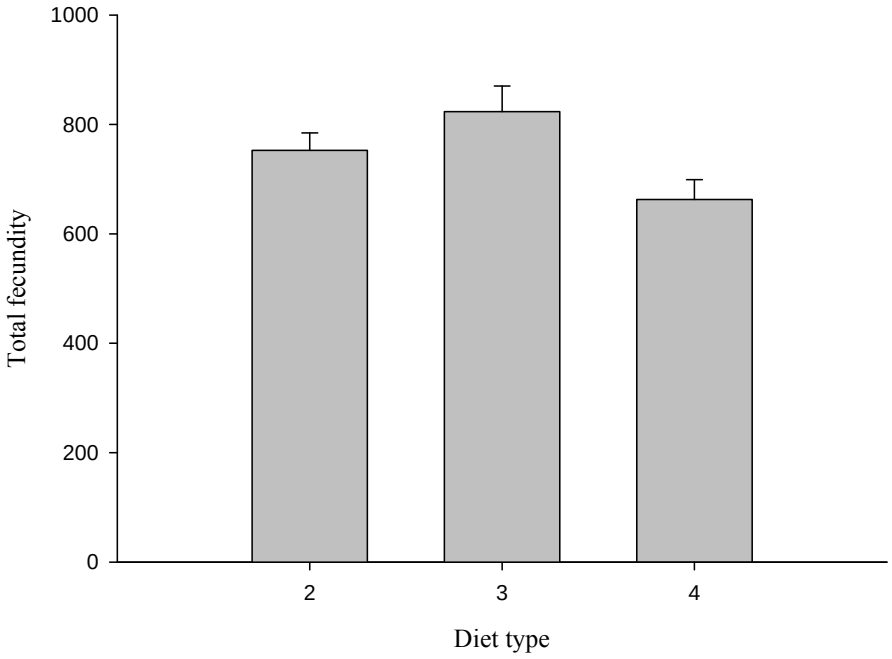


Figure 3.2 Total fecundity of *O. niloticus* fed different lipid source diets (data represents mean ± SEM, n=3). No significant difference (P>0.05) was found between the diet groups by one-way ANOVA.

3.3.2.1.2 Relative fecundity

Relative fecundity ranged from 5.5 ± 0.2, 5.5 ± 0.4 and 3.6± 0.3 number of eggs per body weight (g) for fish fed diets 2, 3 and 4, respectively. However, a significant difference (P<0.05) occurred in relative fecundity for fish fed diet 4 (control) compared to other diets but for fish fed diet 2 and 3 were not significantly different from eachother (P>0.05) (Table 3.5). Similar results were observed when comparing the EW: BW which was 1.4±0.1, 1.3±0.1 and 0.9±0.1 from diets, 2,3 and 4, respectively (Table 3.5).

3.3.2.2 Egg diameter

The mean egg diameter ranged from 2.2-2.4 mm for fish fed diets 2, 3 and 4 at both full and half ration.

Three-way ANOVA showed that diet and ration had no significant effect ($P>0.05$) on egg diameter from fish fed diets 2, 3 and 4 over three spawnings (Figure 3.3). One-way ANOVA also showed that there was no significant difference ($p<0.05$) in egg diameter between broodstock fed diet 2, 3 and 4 (Table 3.5).

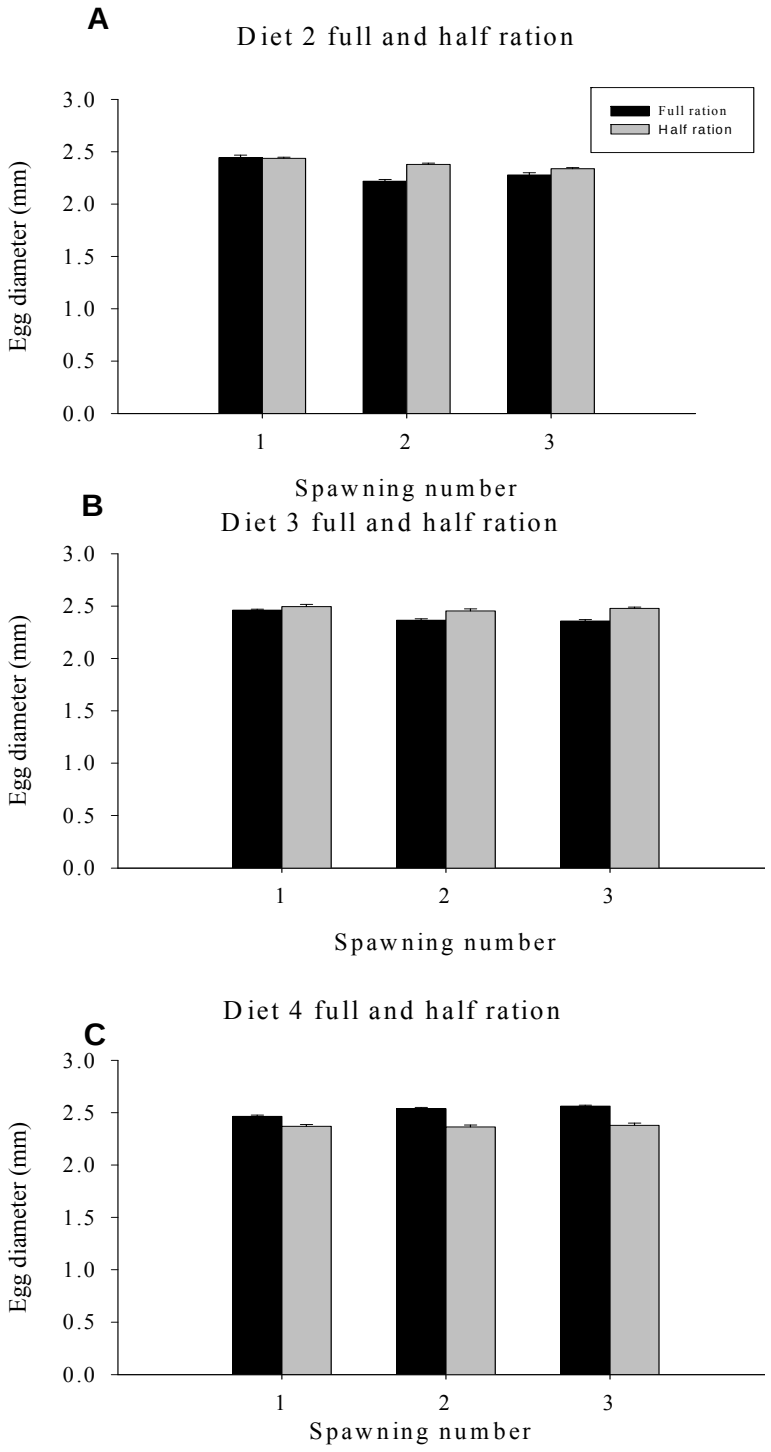


Figure 3.3 Mean egg diameter of *O. niloticus* fed different dietary lipid sources at full and half ration over three spawnings (data presents mean ± SEM, n=3). In each graph, the differences between the rations within each spawning are not significant ($p>0.05$). A= diet 2 containing palm oil, B= diet 3 containing palm and cod liver oil and C=diet 4 as control.

3.3.2.3 Egg volume

Mean egg volume ranged from 6.1 to 7.7 mm^3 and there were no significant interactions ($P>0.05$) with mean egg volume between diets, rations and spawnings from fish fed diet 2, 3 and 4 (Table 3.4) and (Figure 3.4). The egg volume was also not significantly influenced ($P>0.05$) by diets.

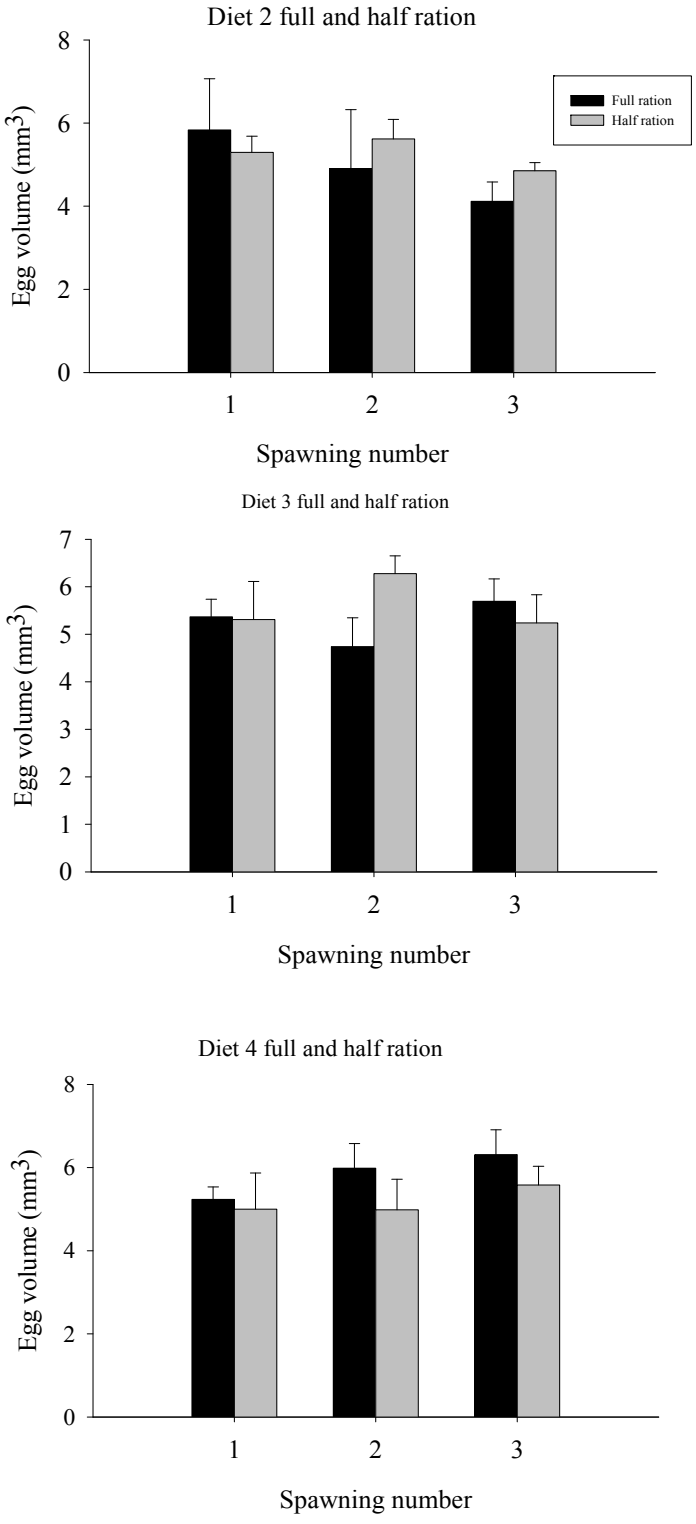


Figure 3.4 Mean egg volume of *O. niloticus* fed different dietary lipid sources at full and half ration over three spawnings (data presents mean ± SEM, n=3). In each graph, the differences between the rations within each spawning are not significant ($p>0.05$). A= diet 2 containing palm oil, B= diet 3 containing palm and cod liver oil and C=diet 4 as control.

3.3.3 Inter spawning intervals (ISI)

The average inter spawning intervals in the present study ranged from 14-24 days. A significant difference ($P<0.05$) was detected when comparing ISI between the diet groups. The longest ISI was found for fish fed diet 4 (control) and the shortest was found for fish fed diet 2 (PO), however, the ISI for fish fed diets 2 and 3 were not significantly different ($P>0.05$) (Figure 3.5).

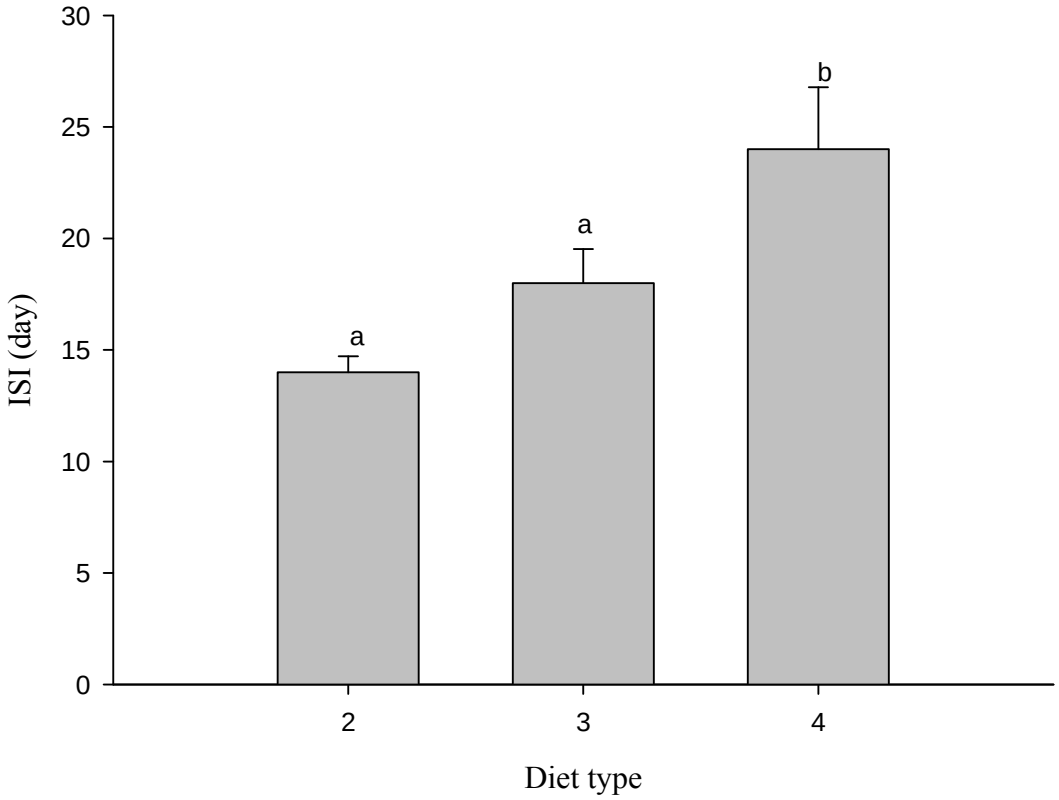


Figure 3.5 Inter-Spawning -Intervals (ISI day^{-1}) in *O. niloticus* broodstock fed different dietary lipid sources. Values are mean ± S.E.M. In each columns means with different superscripts are significantly different ($P<0.05$).

Table 3.5 Spawning performance of *O. niloticus* fed different dietary lipid sources

Parameters	Treatments		
	Palm oil diet (Diet 2)	PO&CO (9:1) diet (Diet 3)	Control (diet 4)
Total fecundity (total egg number/batch)	752.6±32.1[a]	823.3±46.6[a]	662.9±36.1[a]
Relative fecundity (no of eggs/BWg)	5.5±0.2[a]	5.5±0.4[a]	3.6±0.3[b]
Egg diameter (mm)	2.2±0[a]	2.2±0[a]	2.2±0[a]
Egg volume (mm^3)	5.2±0.2[a]	5.4±0.2[a]	5.6±0.2[a]
Total egg volume (mm^3)	3902.7±236.5[a]	4385.7±267.1[a]	3654.6±237.1[a]
Egg dry weight (mg)	2.6±0.1 [a]	2.5±0.1[a]	2.7±0.1[a]
Egg wet weight (mg)	6.1±0.1[a]	6.1±0.2 [a]	6.6±0.2 [a]
EW^1: BW^2 (%)	1.4±0.1[a]	1.3±0.1[a]	0.9±0.1[b]
Fertilisation rate (%)	76.3±1.4 [a]	78.5±1.8 [a]	75.9±2.2 [a]
Hatchability (%)	59.5±1.1 [a]	60.1±1.8 [a]	61.4±1.4 [a]
ISI (day)	14±0.7 [a]	19±1.5 [a]	24±2.7 [b]

In each row, means with different superscripts are significantly different (ANOVA, Tukey's test, $P < 0.05$). Data are means ± SEM of two replicates. 1= egg dry weight, 2= fish body weight

3.3.3.1 Larval quality

Larval batches from each broodfish were recorded individually for three consecutive spawnings for fish fed diets 2, 3 and 4. Mean values of larval length and weight from fish fed different diets rations over three spawnings were analysed using GLM tree-way ANOVA. No significant interactions (P>0.05) were observed between diets rations and spawnings in larval lengths and weights. However, one-way ANOVA showed that mean larval length and weights obtained from fish fed

diet 2 were significantly different (P<0.05) from fish fed diet 3 and 4, while the differnces between fish fed diets 3 and 4 were not significant (Table 3.6).

Table 3.6 Larval performance of Nile tilapia (*O. niloticus*) fed different dietary lipid sources.

Parameters	Treatments		
	Diet 2	Diet 3	Diet 4
Larvae length (mm)	9.3±0.6^{a}	9.6±0.67^{bc}	9.5±0.7^{c}
Larvae weight (mg)	9.8±1.4^{a}	10.2±1.6^{bc}	10.3±1.6^{c}

Values are means ± S.E.M. In each row means with different superscripts are statistically different (ANOVA, Tukey's test, P<0.05).

3.4 Discussion

One of the principal objectives of the present study was to investigate whether fish oil based diets, commonly used by the industry, can be substituted with alternative oil sources. Fish oil is produced from small marine pelagic fish and represents a finite fishery resource (Ng *et al.,* 2003). Due to several factors, such as over-fishing, resulting in dwindling catch and environmental changes which necessitate tight regulations, future demand for wild-caught fish will exceed supply (Sargent *et al.,* 1999). Hence the need to evaluate potential substitutes for fish oil, an important ingredient in the formulation of aquafeeds. Palm oil, currently the second most abundant vegetable oil in the world, presents a viable alternative to fish oil in aquafeeds (Ng *et al.,* 2003, 2004).

Fish meal based diets contain up to 9% fish oil (De Boer and Bickel, 1988). For this study, therefore, to avoid any effect of fish oil in the experimental diets, the protein source of diets was changed to soy protein concentrate containing 65% protein and a trace amount of lipid.

Previous studies revealed that palm oil could be used as dietary lipid source with no negative effect on fish growth (Al-Owafeir and Belal, 1996; Bell *et al.,* 2002; Legendre *et al.,* 1995; Ng *et al.,* 2000,, 2001, 2003, 2004, 2006; Ng and Low, 2005; Tortensen *et al.,* 2000). However, limited information is available on the effect of lipid sources on tilapia reproductive performance. The present study is the first attempt to investigate the effect of dietary lipid source on the reproductive performance of tilapia fed solely their respective experimental diets for their entire life cycle. The results of the present study show that tilapia broodstock can be maintained and spawned successfully on different dietary lipid sources (Palm and

mixed PO & CO). In addition, reducing the ration level of the diet from 3 to 1.5% did not influence spawning or negatively affect spawning performance (Table 3.4). The spawning performance of the Nile tilapia fed the two formulated dietary lipid sources (Palm and mixed PO&CO) was comparable to those fed the control diet (Table 3.5). No significant differences were found in total fecundity, egg wet and dry weights, egg diameter and volume, fertilisation and hatching rate of fish fed diets 2, 3 and 4. Only the relative fecundity in the control diet group (D 4) differed from the other two experimental diet groups; the reason for this was because of the larger size of broodstock. The fish fed diet 1 (cod liver oil) had a high mortality during the on-growing stage and only one fish spawned throughout the experiment and poor egg quality; the growth gain was lower than other diets (section 3.3.1), and this might be due to the high concentration of (n-3) PUFA in cod liver oil. The results of the present study are in agreement with previous studies (Kanazawa *et al.,* 1980; Ng *et al.,* 2001, 2004; Takeuchi *et al.,* 1983) which reported that depressed growth of tilapia with oil having high levels of n-3 PUFA and Santiago and Reyes, (1993); Watanabe, (1982) whom found that fish fed a cod liver oil diet had poor egg quality. In contrast, the reasons for growth gain obsrevd lower could be due to the palatability of the diet which consisted of soy protein source and pure cod liver oil, or the high n-3 PUFA of cod liver oil. However, further investigations are required to support this assumption.

Fertilised eggs of *O. niloticus* usually take about 4 days to hatch at 28°C and the development time to absorb the yolk-sac takes about 6 days (Coward and Bromage, 1999a; Macintosh and Little, 1995). In the present study, eggs from all treatments were kept at 28±1°C and 3-4 days were required for hatching and a further 6 days to absorb the yolk-sac for all diet treatments. That meant for the embryo development

and larva yolk-sac absorption, the experiential diets (PO&CO) were the same as control diet at either full or half ration.

Total fecundity for fish fed the mixed palm and cod liver oil diet was slightly higher than palm oil diet or control (Table 3.5). This could be due to the ratio of n-6 and n-3 (9:1). However, the results indicated that tilapia need small amount of n-3 for growth and enhancement of reproduction performance; a similar result was found by Watanabe (1982) with Nile tilapia fed a basal diet supplemented with soybean oil (high in n-6 fatty acids) displaying a higher fecundity, spawning frequency and fry production but was relatively low in fish fed a 5% cod liver oil supplemented diet (high n-3 fatty acids). Similarly, Kanazawa *et al.* (1980) and El-Sayed and Garling (1988) found that *T. zillii* reared in freshwater required *n*−6 fatty acids for optimum growth. In support, Huang *et al.* (1998) found that the growth rates of hybrid tilapia (*O. niloticus* × *O. aureus*) reared in freshwater and fed soybean oil and fish oil were similar, and were both better than that of fish fed lard or PUFA. Similar results were reported by Chou and Shiau (1999) ; Lu and Takeuchi (2004) ; Santiago and Reyes (1993) ; Stickney and Wurts (1986) and also previous study reported that tilapia, like other warm water fish, are more inclined to require greater amounts of n-6 fatty acids compared to n-3 fatty acids for maximal growth (NRC, 1993).

Larval quality

In general, several authors demonstrated that nutrition, in particular lipid and essential fatty acids, affect larval quality (Bell *et al.,* 1997; Fernandez-Palacios *et al.,* 1995; Furuita *et al.,* 2000; Harel *et al.,* 1994; Izquierdo *et al.,* 2001a; Navas *et al.,* 1997; Watanabe *et al.,* 1984, 1985). The authors also demonstrated that embryo

growth and free-swimming larval weight and length may be affected by their parental diets. The present study showed that the weight and length of larvae originating from broodstock fed experimental diets (PO and mixed P & CO) were similar to those fed control diet, with the exception of the palm oil diet, where larval weight and length were slightly lower than other diet groups. The reason for the lower weight and lengths from fish fed palm oil diet is unknown. However, this significant level could be due to genetic differences within the broodstock or other parameters which would require further investigation.

Inter spawning interval (ISI)

The shortest ISI was observed in the group of fish fed the palm oil diet and the longest in fish fed the control diet. In the present study, there was no significant relationship between egg size and ISI, but it was apparent that larger females had longest ISI and conversely the smaller females the shortest ISI; similar results were found by Campos-Mendoza *et al.* (2004); Siraj *et al.* (1983) and Rana (1986). The reason for the longest ISI in larger females may due to the fact that large fish require more energy for maintenance and growth rather than producing eggs.

Conclusion

In conclusion, the results of this study suggest that, under controlled conditions, lipids of non-marine origin, such as palm oil, fed to broodstock has no detrimental effect on egg and larval viability and performance and that broodstock fed ration of 1.5% BW/day is adequate.

Chapter 4 - Total lipid and fatty acid composition of eggs in serial spawnings of *O. niloticus*

4.1 Introduction

An eggs' potential to develop into viable fry is influenced by physical, genetic and chemical parameters, as well as the initial physiological process occurring within the egg. If one or more of the essential factors is lacking, or is incomplete, embryonic development may fail at some stage. Thus, egg quality should be regarded as determined when the egg has been deposited by a female and the fertilisation process is completed (Kjørsvik *et al.,* 1990).

4.1.1 Vitellogenesis

Females of egg-laying fish enter a phase of oocyte maturation in preparation for ovulation and spawning. Under the control of the hypothalamus and the pituitary gland, the growing follicles surrounding the oocytes synthesise and secrete sex steroid hormones into the blood that affect the oocyte developmental process (Norberg, 1995; Norberg *et al.,* 1991; Patiño *et al.,* 2003; Redding and Patiño, 2006; Riple *et al.,* 1999). One of the primary targets of these steroids, particularly 17β-estradiol (E2), is the liver. This organ, which possesses highly specific binding proteins for E2, in turn responds to such hormonal stimulus by synthesising and exporting vitellogenin via the blood to the gonad (Hu *et al.,* 2009; Hyllner *et al.,* 1994; Lubzens *et al.,* 2009). Vitellogenin, which is a protein chain with a size of 130-200 KDa, made up of various classes of compounds and is selectively accumulated within the developing oocytes (Kishida and Specker, 1993). It also carries copious amounts of lipid material, carbohydrate components, phosphate

groups and mineral salts. Within the oocyte, the vitellogenin is accumulated as egg-specific yolk constituents, such as phosvitin and lipovitellin (Cerdà *et al.,* 1994a, b, 1995). Maternal production of vitellogenin and the deposition of adequate supplies of yolk are essential to subsequent embryonic and larval survival. The bulk of the (n-3) polyunsaturated fatty acids (PUFA) rich of phospholipid in eggs are located in lipovitellin. In trout, for vitellogenesis to proceed normally, a plentiful exogenous source of food is obligatory during the vitellogenic phase (Bromley *et al.,* 2000). Vitellogenesis is identified as the period during oogenesis where essential fatty acids (EFA) are incorporated most effectively into developing oocytes (Navas *et al.,* 1997). In recent years, histochemical, biochemical and immunohistochemical studies have shown that ovarian follicular cells play a significant role in both vitellogenesis and final oocyte maturation in most teleost species (Ravaglia and Maggese, 2003). The terminology and biological functions of the egg envelope vary in different vertebrate groups. In teleosts, the envelope is often referred to as the vitelline envelope, and forms a tough protective coat around egg and embryo. The egg envelope has several roles: attraction of the spermatozoa, prevention of polyspermy and protection of the egg or embryo.

4.1.2 Biochemical composition of tilapia eggs

Identification of a method to reliably discriminate between low- and high-quality egg batches will be vital to reduce hatchery production costs, by ensuring that time and resources are not wasted on egg batches with low survival and/or performance potential. Several morphological (Kjørsvik *et al.,* 1990; Lu and Takeuchi, 2004; Thorsen *et al.,* 2003) and biochemical parameters such as lipids (Bell and Sargent, 2003; Lu and Takeuchi, 2004; Sargent, 1995; Tveiten *et al.,* 2004), amino acids

(Ronnestad and Fyhn, 1993) or vitamins (El-Gamal *et al.,* 2007; Mæland *et al.,* 2003; Ronnestad *et al.,* 1997; Ronnestad *et al.,* 1999) have been considered as indicators of egg quality. Recently, several compounds and enzymes involved in carbohydrate metabolism have been identified as good markers of egg quality in species such as *Sparus aurata* and *Puntazzo puntazzo* (Lahnsteiner and Patarnello, 2004). Lipids and fatty acids have also been considered to compare egg quality between wild and captive broodstocks (Lu and Takeuchi, 2004; Peleteiro *et al.,* 1995; Rodriguez *et al.,* 2004; Salze *et al.,* 2005).

The biochemical composition of a healthy egg should reflect the nutritional requirements of the embryo for growth. Some components such as essential fatty acids and amino acids are known to be "essential" for an organism (*e.g.* the organism is unable to synthesise the nutrient) and these components have to be present in optimum amounts to satisfy biological demands. Biochemical egg quality assessment is therefore a good criterion to determine whether eggs are of good quality prior to the fertilisation process. Thereafter, larval growth and survival depend on the availability of exogenous food in sufficient quantity and of adequate quality after the yolk resorption (Kjørsvik *et al.,* 1990, 2003).

4.1.3 Lipids

Lipids are a large group of heterogeneous compounds, with a common property of insolubility in water, but soluble in organic solvents such as chloroform, hydrocarbons or alcohols (Gurr *et al.,* 2002). Lipids play multiple important roles such as providing efficient energy reserves that can store more energy per unit weight than proteins and carbohydrates (Castell *et al.,* 2004; Hu *et al.,* 2009), participating in physiological processes (*e.g.* vitamins, hormones and pigments),

acting the protein kinase during the cellular signal transfer (Ermakova *et al.,* 1999), being precursors to essential substances as eicosanoids (Castell *et al.,* 2004; Hu *et al.,* 2009).

Animal lipids, including fish lipids, can be divided into two broad classes; neutral and polar lipids. Neutral lipids, that are completely soluble in non-polar solvents, are composed principally of triacylglycerols (triglycerides). Wax esters constitute another class of neutral lipid, consisting of a single molecule of fatty acid esterified to a single molecule of fatty alcohol, which can be present in considerable amounts in body tissues and the egg of some species (Tocher, 2003). Polar lipids are composed principally of phospholipids. The most important simple lipid (*i.e.* a lipid that does not contain fatty acids) in all animals, including fish, is cholesterol. This is the most common form of the tetracyclic hydrocarbon compounds, collectively called sterols. It may exist in an unesterified form as an essential component of cell membranes, or in a neutral lipid storage form esterified to a fatty acid (Tocher, 2003).

Lipids and their fatty acids have a number of biological functions in fish, such as acting as substrates for catabolism, structural components in cell membranes and as precursors for chemical messengers (Tocher, 2003; Tveiten *et al.,* 2004; Tveiten and Scott, 2007). In biomembranes, fatty acids dominate most of the biological functions like fluidity and flexibility, which determines further physiological metabolism such as sperm motility and fertilisation capacity (Hu *et al.,* 2009; Ladha, 1998). Fatty acids and amino acids are the major source of metabolic energy in fish throughout their life cycle, acting also as a major source of metabolic energy for reproductive activity (see Figure 4.1), egg viability and the survival of offspring

(Henderson and Sargent, 1984; Tocher, 2003; Watanabe *et al.,* 1984, 1996, 1999). The fatty acid composition must satisfy embryonic nutritional needs for development and subsequent growth. It is essential that lipid deposited in the egg fulfils the nutritional requirements of larvae during their early stages of development until feeding begins (Craik, 1982, 1985; Craik and Harvey, 1984a, b).

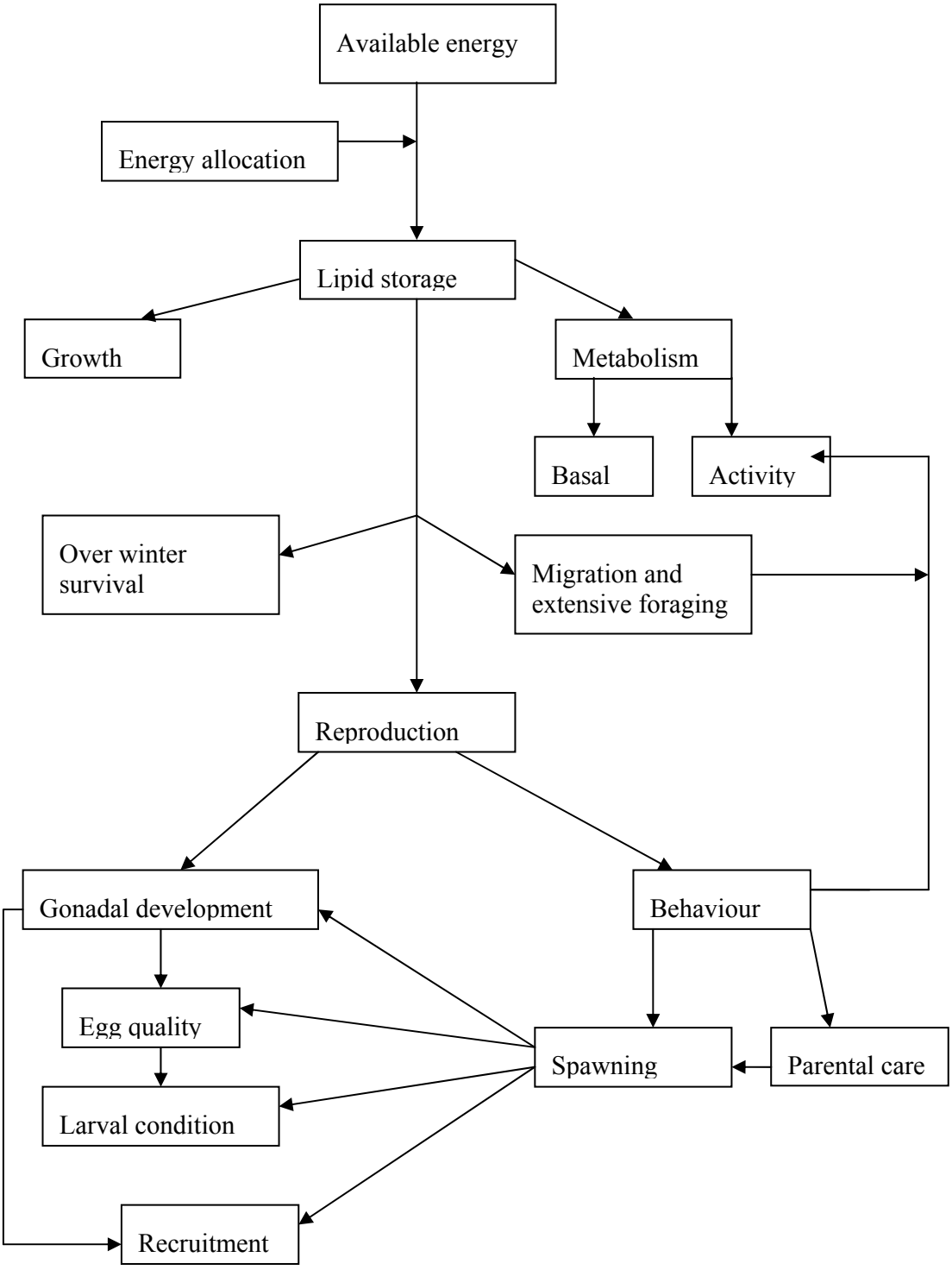


Figure 4.1 General allocation strategy of available energy into the main functional processes of growth, metabolism, lipid storage, and reproduction for fish species, Adams (1998)

4.1.4 Role of lipids in egg development

Lipids and their constituent fatty acids, together with the metabolic derivatives of some of the latter, termed eicosanoids and other associated compounds, play essential and dynamic roles in the maintenance of optimum growth, health (*i.e* immunocompetence and cardiovascular function), kidney and gill function, neural and visual development, reproduction and flesh quality of finfish species (Balfry and Higgs, 2001; Higgs and Dong, 2000).

Lipids are important nutrients for successful embryonic development in fish. Egg lipids play several critical roles in the development of fish embryos. They serve as a major energy store, as precursors for chemical messengers and micronutrients, particularly phospholipids, which enable the growth of the fish embryo (Boulekbache, 1981; Sargent, 1995; Terner, 1979). Teleost oocytes accumulate large amount of lipids in addition to the polar lipids accumulated through vitellogenin. In the eggs, several classes of lipid are represented, where strong variations exist among different fish species (Sargent, 1995).

Lipids are found in two compartments in the eggs. The lipoprotein yolk (LPY) contains mostly phospholipid, along with some neutral lipids and the oil globule (Wiegand, 1996, Wiegand *et al.,* 1999). The oil comprises of neutral lipids, such as triacyglycerol (TAG) and sterol and/or wax esters, (Sargent, 1995; Wiegand, 1996). The main phospholipid in eggs is invariably phosphatidylcholine, and eggs with short and long incubation times have low and high levels of triacylglycerols, respectively (Tocher, 2003; Tocher and Sargent, 1984a).

4.1.5 Fatty acid composition

Fatty acids are chains of carbon molecules with a methyl group and carboxyl group at the two opposite ends, and varying numbers of double and single bonds between the carbon atoms (Figure 4.2). Fatty acids are one of the most important components in the organism. According to the number of double bonds, fatty acids can be divided into subtypes such as saturated, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). The fatty acid composition in many animals has been identified, and has been found to vary dramatically among tissues (Hu *et al.,* 2009). This suggests specific roles for different fatty acids in life systems. There is a dietary requirement for particular polyunsaturated fatty acids (PUFA) in all vertebrate species studied. These are known as essential fatty acids (EFA), and include members of the (n-3) and (n-6) series; (see Figure 4.3 for an explanation of fatty acid nomenclature) in particular, linolenic acid (18:3n-3), linoleic acid (18:2n-6), and their derivates, 22:6n-3 docosahexaenoic acid (DHA) and 20:5n-3 eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (20:4n-6) (Sargent, 1995). The EFA requirements of fish have been reviewed on many occasions (Hu *et al.,* 2009; Izquierdo, 1996; Sargent, 1995; Sargent *et al.,* 1999).

The pathways of DHA, EPA and ARA synthesis in freshwater and seawater fish are shown in Figure 4.3. In freshwater fish such as tilapia, linolenic acid is transformed into EPA and this to DHA. In a similar fashion, linoleic acid is converted into ARA. However, the Δ5-desaturase enzyme is required for the conversion of α-linolenic acid to EPA, and 18:2n-6 to ARA. Thus, in freshwater fish, linoleic are transformed into ARA and linolenic into EPA and DHA (Bruce *et al.,* 1999; Olsen *et al.,* 1990; Sargent, 1995; Takeuchi *et al.,* 1979; Takeuchi *et al.,* 1980; Takeuchi *et al.,* 1983; Teshima *et al.,* 1982). However, in freshwater fish, linoleic and linolenic are

essential fatty acids whereas in marine species, DHA, EPA and ARA are the essential fatty acids (Sargent, 1995) (see Figure 4.3).

EPA or eicosapentaenoic acid

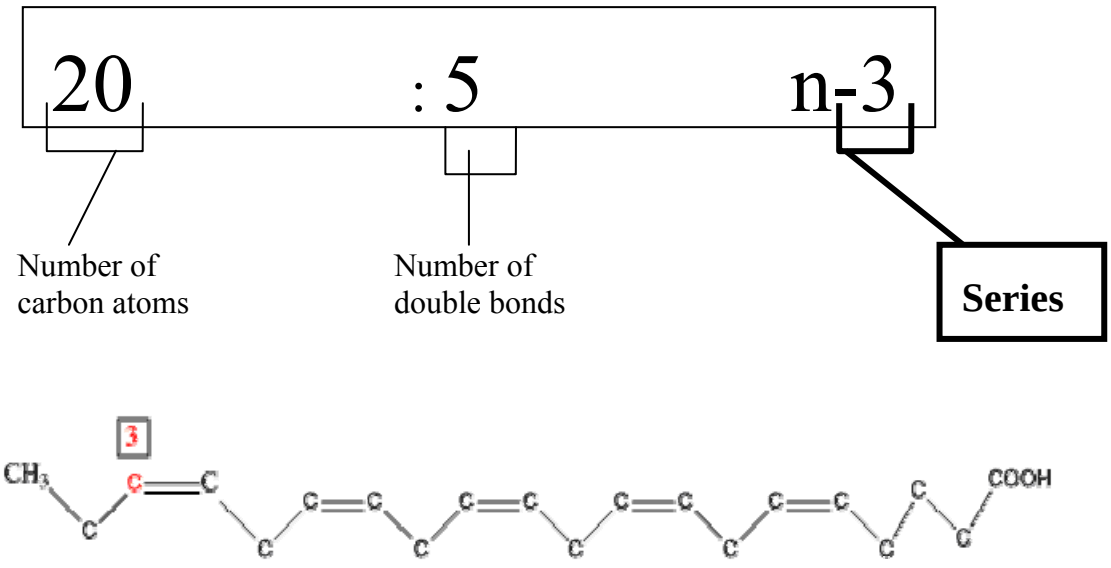
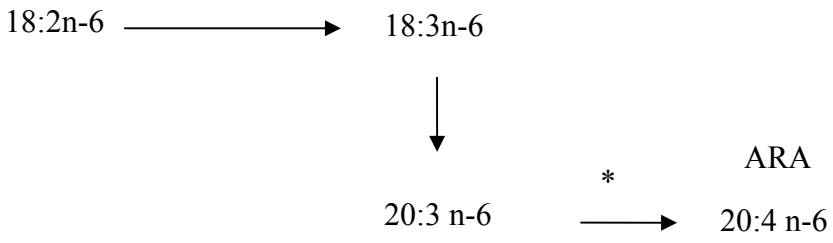


Figure 4.2 Diagrammatic representation of EPA, an essential fatty acid in fish.

Fatty acids are chains of carbon molecules with a methyl group and a carboxyl group at the two ends, and varying numbers of double bonds between carbon molecules (modified from Bruce *et al.* (1999).

Linoleic acid



Linolenic acid

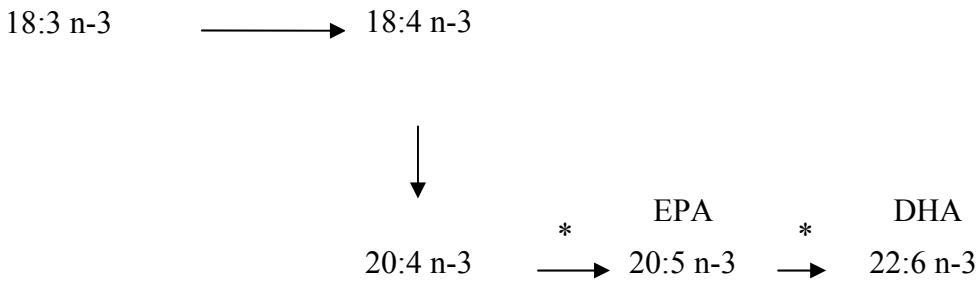


Figure 4.3 Diagrammatic representation of the production pathways for DHA, EPA and ARA. In freshwater fish linolenic acid is, transformed into EPA and this to DHA. In a similar fashion, linoleic acid is transformed into ARA. In marine species, lack of Δ5-desaturatase enzyme required for the conversion of α-linolenic acid to EPA, and 18:2n-6 to ARA, at the steps marked with *. Thus, in freshwater fish linoleic and linolenic are the EFA, whereas in marine species, DHA, EPA and ARA are the EFA (modified from Bruce *et al.* (1999)).

Since fatty acids are provided from food, dietary fatty acids have a great effect on the fatty acid composition of tissues and organs, and thus their normal functioning (Hu *et al.,* 2009). The fatty acid profiles of neutral lipids in eggs tend to vary more than those in fish diets (Almansa *et al.,* 1999; Bell *et al.,* 1997; Wiegand, 1996).

Fatty acids mobilised from the neutral lipid reserves of female broodstock adipose tissue during gonadogenesis are transferred via the serum vitellogenin to the developing eggs in the ovary. Thus, the essential fatty acids vital for early survival and development of newly hatched larvae are determined by the lipids derived directly from the dietary input of broodstock in the period preceding gonadogenesis (Agius *et al.,* 1999; Almansa *et al.,* 1999; Bell *et al.,* 1997; Fernandez-Palacios *et al.,* 1995; Kjørsvik *et al.,* 1990; Mazorra *et al.,* 2003; Mourente *et al.,* 1993; Sargent, 1995; Verakunpiriya *et al.,* 1996). Vitellogenin is synthesised mainly in the liver, under the regulation of 17β oestradiol (E2) but vitellogenin synthesis can also be induced by several other hormones (Babin *et al.,* 2007; Hyllner *et al.,* 1994). The ovary of some spawning fish is very active in terms of vitellogenesis, as shown by the significant levels of E2 in blood plasma (Kjesbu, 1996). Vitellogenesis is identified as the period in the sea bass reproductive cycle during which EFA is incorporated most effectively into the developing oocytes (Bell *et al.,* 1997). PUFA are precursors of physiologically active molecules, such as prostaglandins and other eicosanoids and structural components during organogenesis (Bell *et al.,* 1992; Sargent, 1995).

4.1.6 N-3 PUFA, EPA and DHA

Up to 75% of the EFA mobilised, preferentially saturated and monounsaturated fatty acids, can be catabolised to provide metabolic energy for egg lipoprotein biosynthesis. The remainder, preferentially (n-3) polyunsaturated fatty acids (PUFA) and especially docosahexaenoic acid DHA (22:6n-3), are incorporated into the phospholipid-rich vitellogenin. The (n-3) PUFA are composed principally of DHA and eicosapentaenoic acid EPA (20:5n-3), in a ratio of approximately 2:1

(Sargent, 1995). The fatty acids of both phospholipids and triacylglycerols (TAG), including their (n-3) PUFA, are catabolised to provide metabolic energy for the developing embryos and early larvae, but the chief role of (n-3) PUFA is in the formation of cellular membranes. Because of the unusual richness of DHA in neural cell membranes, this fatty acid appears to have a critical role in the formation of the brain and the eyes, which constitute a large fraction of the embryonic and larval body mass (Tocher and Harvie, 1988). This observation is illustrated by the high levels of phosphoglycerides, rich in DHA, in fish tissue especially neural tissues; approximately 40% of the dry matter of fish brain is lipid, around 10% of which is DHA (Sargent, 1995).

4.1.7 Total n-6 fatty acid

Lipids and their fatty acid composition, particularly the n-6 PUFA, play an important role in reproduction in freshwater fish. Tilapia species are one of the few fish reported as having a strict requirement for only 18:2n-6 (Kanazawa *et al.,* 1980; Lu and Takeuchi, 2004; Takeuchi *et al.,* 1983). Indeed, it has been shown that high levels of dietary 18:3n-3 can actually inhibit growth in blue tilapia *O. aureus* (Kanazawa *et al.,* 1980; Ng *et al.,* 2001; Ng, 2004; Ng *et al.,* 2004; Stickney and Wurts, 1986; Takeuchi *et al.,* 1983). In contrast, hybrid tilapia (female *O. niloticus* crossed with male blue tilapia *O. aureus*) required both 18:2n-6 and some n-3 PUFA for maximal growth (Chou and Shiau, 1999).

Fatty acid inclusion in diets for freshwater fish broodstock has been shown to effect the reproductive performance of females (Gomes *et al.,* 1993; Santiago and Reyes, 1993) and alter the fatty acid composition of eggs (Hardy, 1999), with consequences for egg quality (Czesny *et al.,* 2000; Pickova *et al.,* 1999, 2006).

It was observed that eggs from rainbow trout broodstock fed a corn oil diet containing high levels of 18:2n-6 and low levels of n-3 PUFA, showed no difference in fecundity or subsequent egg viability compared with eggs originating from fish fed a cod liver oil diet (Corraze *et al.,* 1993b). In contrast, in Nile tilapia broodstock fed various dietary oil sources, the best reproductive performance was obtained from fish fed a soybean oil diet, with a high n-6:n-3 PUFA ratio in the eggs, whereas, fish fed cod liver oil had the best weight gain but the poorest reproductive performance and the lowest n-6:n-3 PUFA ratio in the eggs (Santiago and Reyes, 1993).

4.1.8 Overall aims

The overall aims of this study were to:

- Investigate the feasibility of replacing fish oil with palm oil in fish feed.
- Investigate the effect of different sources of dietary lipid on *O. niloticus* egg lipid and fatty acid composition.
- Investigate the influence of feeding ration of the experimental diet on broodstock egg lipid and fatty acid profiles.
- Study the effect of feeding ration on *O. niloticus* egg fatty acid composition over serial spawnings.

4.2 Materials and Methods

4.2.1 Egg sampling

Total lipid content and fatty acid composition of *O. niloticus* eggs was measured from fish fed four experimental diets at two rations: full ration (3 % of body weigh) and half ration (1.5% of body weight) and experimental groups duplicated. In order to investigate the effects of diet on egg quality, egg samples were pooled into eight groups. The protocol used for egg sampling is illustrated in Figure 4.4.

Eggs were stripped manually from females fed different diets and rations over three consecutive spawnings as discussed in section 3.2.4.

Approximately 100 eggs were removed randomly from each batch of eggs; 50 eggs were used for egg size measurement and the balance of eggs was used for lipid analysis and fatty acid composition (see Figure 4.4). The excess water on eggs was removed by placing eggs in 200µm plankton net and dabbing net with eggs on absorbent tissue paper to remove water. The eggs were then placed in plastic tubes and stored at –70°C until further analysis (see Figure 4.4).

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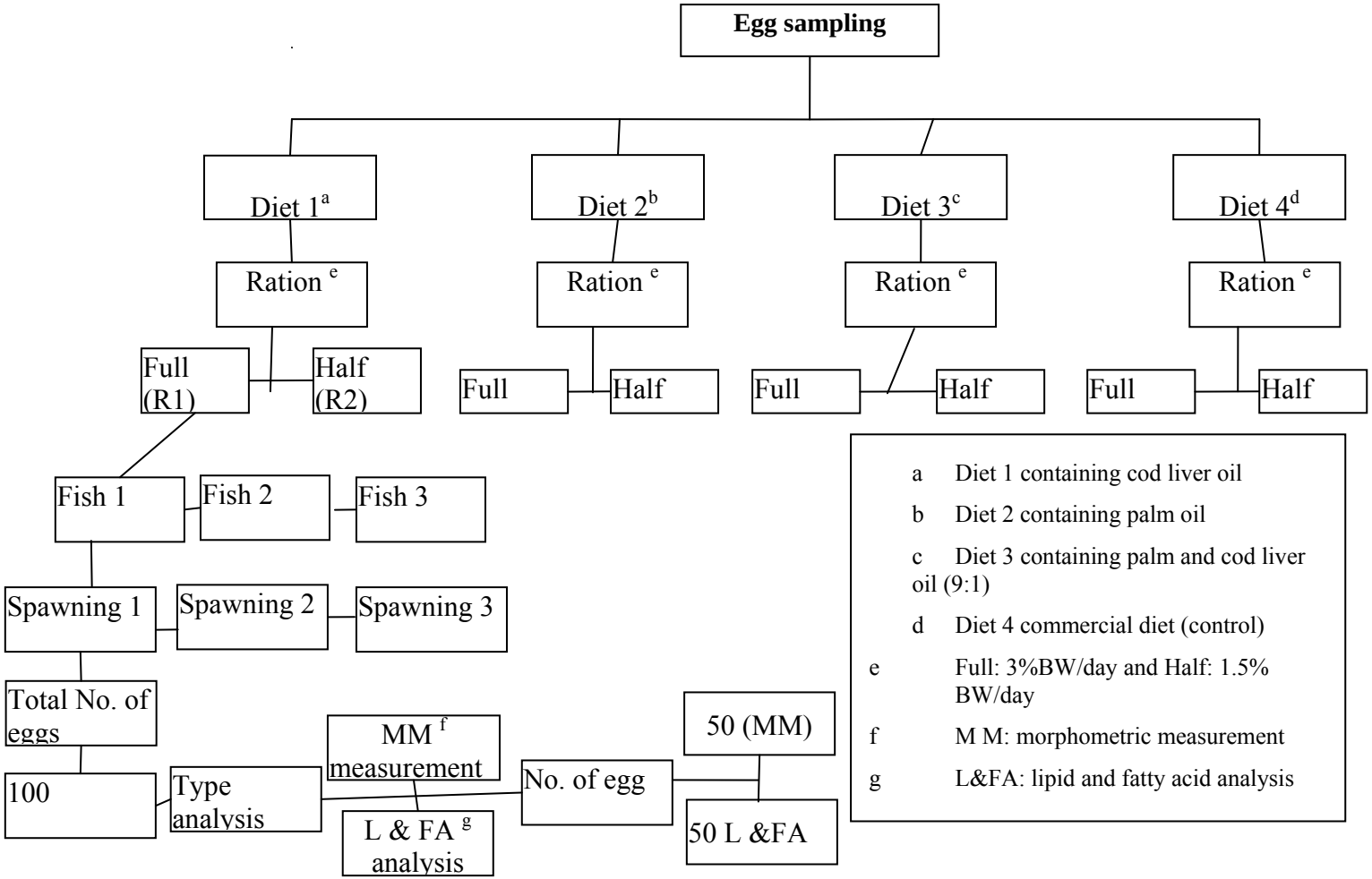


Figure 4.4 Protocol of sampling for egg quality assessment

4.3 Lipid extraction

Total lipid from fish eggs and diets were extracted according to the method of Folch *et al.* (1957). Fish eggs were sampled as detailed in section **2.18**. Approximately 100 eggs were removed randomly from each batch for egg size measurements and lipid analysis. The excess water from eggs were removed using 200µm plankton net as described in section 4.2.1, and eggs placed in Ependorf tubes and stored at –70°C until further analysis.

4.3.1 Fatty acid analysis

Fatty acids were determined by gas chromatography, after preparation of methyl ester derivatives of the fatty acid components. Fatty acids of total lipid samples were converted to methyl ester by acid-catalysed trans-esterification, according to Christie (2003). The preparation of purification of the fatty acid methyl esters (FAME) were performed and described in sections **2.19** and **2.20**.

4.3.2 Statistical analysis

Total lipid and fatty acid compositions of eggs were pooled for statistical analysis using SPSS version 15 for Windows statistical software package (SPSS Inc., Chicago, USA). The data was validated for normality using p-p plots in SPSS (see **Appendix 6**). Data were analysed by three-way ANOVA using the General Linear Model (GLM) and the post-test means comparison was performed by Tukey's test. If three-way ANOVA was significant, the data was then analysed using Minitab version 15 for Windows (Minitab® Statistical Software) to discriminate the significant levels between and within the diets rations and spawnings. The

significant level was set in either SPSS or Minitab at P<0.05. Minitab was used when three-way ANOVA using SPSS showed that significant interaction occurred between eithr diets, ration and spawnings for three-way or diet× ration, diet ×spawning and ration× spawning for two-way ANOVA.

4.4 Results:

4.4.1 Total lipids

In total, 21 female *O. niloticus* fed different diets and rations were monitored over three consecutive spawnings. Total lipid in eggs from broodstock fed different diets and rations are presented in Table 4.1; no significant differences (P> 0.05) were observed between total lipid (TL) of eggs from fish fed on full ration of diet, 1, 2, 3 and 4 for three consecutive spawnings. The mean values of TL in eggs originating from fish fed diets 2, 3 and 4 at half rations were also not significantly different (P>0.05) from each other. Due to insufficient data from fish fed a half ration of diet 1, however, the half ration of this diet is excluded from the anlysis.

Total lipids in eggs from fish fed with full and half ration of the experimental diets over three spawnings were compared. There was no significant difference (P> 0.05) between total lipid of eggs from fish fed diet 2 (PO) at full and half ration over three consecutive spawnings. Moreover, there were no significant differences (P>0.05) in TL of eggs from fish fed diet 3 (PO & CO) and diet 4 (Control) between full and half rations over three spawnings. With the exception of the second spawning in both egg batches from fish fed diets 3 and 4 at half ration which was significantly different (P<0.05) (Figure 4.5).The full comparison details of mean TL between

diets, rations and spawnings; and their significant levels are presented in Appendix Table 4.1.

Table 4.1 Total lipid of *O. niloticus* eggs from fish fed different diets and rations over three consecutive spawnings (data expressed as percentage, means ±SD, n=3)

Diets	Rations	Spawning number		
		1	2	3
1	full	14.2±0.9	13.3±0.3	12.9±0.6
	half	no data		
2	full	12.7±0.7	14.1±1.4	15.1±2.3
	half	15.2±1.4	14.1±2.0	13.4±2.6
3	full	15.9±2.7	14.8±1.5	13.8±1.1
	half	13.1±1.8[a]	11.6±1.4[b]	12.9±2.2[a]
4	full	13.9±1.2	11.9±1.3	14.3±1.4
	half	14 ±1.4[a]	15.5.±0.6[b]	13.3±1.3[a]

Means with different superscript between the row are significantly different ($p<0.05$). Diets 1 containing cod liver oil, 2 palm oil, 3 palm and cod liver oil (9:1) and 4 a control (commercial diet). Full and half ration are 3 and 1.5 5 of BW, respectively.

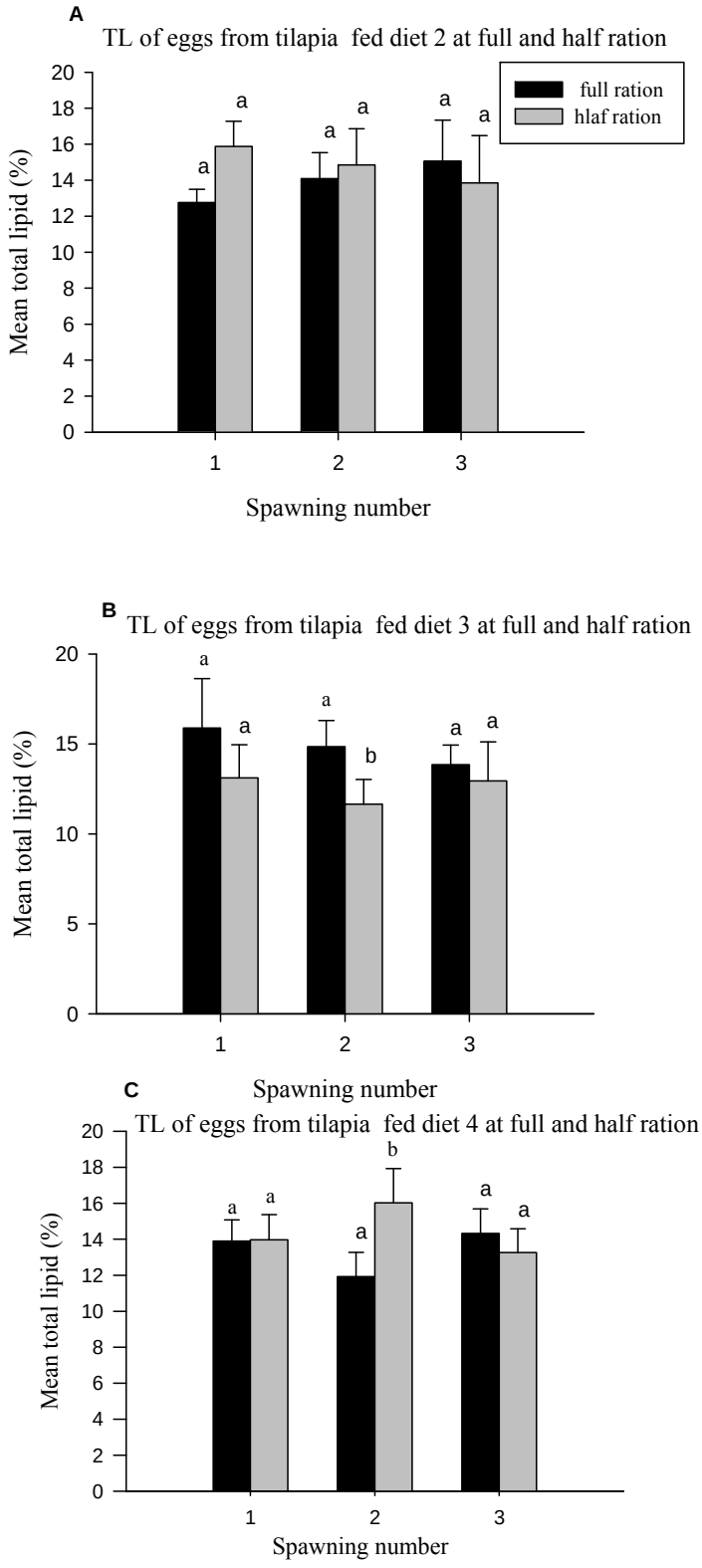


Figure 4.5 Total lipid (TL) of *O. niloticus* eggs originating from broodstock fed different dietary lipid sources at full and half ration over three spawnings (data represented as mean ± SD, n=3). In each graph, bars with different letters are significantly different ($P<0.05$). A= diet 2 containing palm oil, B= diet 3 containing palm and cod liver oil and C= diet 4 as a control.

4.4.2 Fatty acid composition of eggs

A total of 126 samples for FA composition of eggs from three consecutive spawnings was analysed to investigate differences of FAs in eggs from fish fed different diets and rations over three spawnings. The major FAs were analysed by GLM of ANOVA using SPSS and Minitab (see chapter 2 section **2.25**). The data were then compared between full and half rations for three consecutive spawnings.

The fatty acids were measured as a percentage of total lipid. In general, the most abundant fatty acids (as % total lipid area) in tilapia eggs from fish fed the experimental diets were: palmitic acid (16:0), oleic acid (18:1n-9), docosahexaenoic acid (22:6n-3), palmitoleic acid (16:1n-7) and linoleic acid (18:2n-6). Fatty acid composition of eggs derived from fish fed with diet 1 and their significant differences between three spawnings are presented in Table 4.2. Due to insufficient data from fish fed diet 1, this diet group has been discarded for fatty acid analyses with the other groups.

There were no significant differences ($P>0.05$) in SFA, ARA, EPA and DHA between the three spawnings of females fed diet 1 (cod liver oil) at full ration. However, a significant difference ($P<0.05$) occurred in total monounsaturated fatty acids, PUFA, n-3 and n-6, only on the third spawning. The mean value of fatty acids in eggs originating from fish fed diet 1 is shown in Table 4.2.

Table 4.2 Fatty acid composition of eggs from *O. niloticus* broodstock fed diet 1 (cod liver oil) at full ration

Fatty acid	Spawning number		
	1	2	3
14:00	3.3±0.1	2±1.7	4.2±0
15:00	0.4±0.1	0.3±0.1	0.5±0
16:00	21.0±1.1	21.9±3.2	21.6±0.6
18:00	6.5±1.3	7.2±0.9	5.4±0.1
20:00	0.2±0	0.4±0.3	0.2±0
22:00	0.2±0.2	0.4±0.3	0.2±0.2
Total saturated	31.5±2.2	32.1±5.6	32. ±0.6
16:1n-9	1.0±0.5	1.2±0.1	1.7±0
16:1n-7	7.0±1.2	6.6±1.1	8.9±0.7
18:1n-9	20.8±0.6	17.7±2.3	22.8±0.5
18:1n-7	3.9±0.6	3.6±0.4	4.6±0.2
20:1n-9	3.4±0.3	2.6±0.2	3.5±0.1
20:1n-7	0.3±0	0.2±0	0.4±0
22:01	1.0±0.1	0.7±0.2	0.8±0
24:1n-9	0.2±0	0.2±0.1	0.2±0
Total monounsaturated	37.5±2.6[a]	32.5±3.4[a]	42.3±0.3[b]
18:2n-6	6.0±2.2	4.3±0.5	3.7±0.1
18:3n-6	0.6±0	0.4±0	0.7±0
20:2n-6	0.6±0.1	0.4±0	0.6±0
20:3n-6	0.67±0.3	0.5±0	0.7±0.1
20:4n-6 (ARA)	1.7±0.4	1.5±0.1	1.5±0.1
22:4n-6	0.5±0.2	0.3±0	0.3±0
22:5n-6	0.4±0.2	0.3±0	0.3±0
Total n-6 PUFA	10.6±3.3[a]	11.8±6.5[a]	7.6±0.1[b]
18:3n-3	0.6±0.1	0.7±0.5	0.4±0
18:4n-3	0.3±0.1	0.3±0	0.2±0
20:4n-3	0.2±0.1	0.2±0.1	0.2±0
20:5n-3 (EPA)	1.0±0.5	1.0±0.1	0.7±0
22:5n-3	3.1±0.9	3.4±0.4	2.5±0.1
22:6n-3 (DHA)	15.4±1.3	15.8±1.9	14.1±0.7
Total n-3 PUFA	20.7±2.9[a]	21.2±1.8[a]	18.2±0.8[b]
Total PUFA	30.1±1.1[a]	33.1±5[a]	25.7±0.9[b]
AA/EPA	2.1±1.2	1.6±0.1	1.9±0
DHA /EPA	17.1±5.6	16.7±0.5	18.5±0.6
n-3/n-6	2.3±1	2.2±1	2.4±0.1

Value given as means (percentage of total lipid) ± SD., n=3. Means with different superscripts between rows are significantly different ($p<0.05$).

4.4.3 Fatty acid composition of eggs from broodstock fed different rations (full and half) over three consecutive spawnings.

The major fatty acids in eggs from fish fed the 3 diets (diets 2, 3 and 4) were analysed to investigate differences between full and half ration feeding regimes over three consecutive spawnings. Due to insufficient data, diet 1 was excluded from these analyses.

4.4.3.1 Total saturated fatty acids (SFA)

Feeding ration had no significant bearing on mean SFA of eggs from broodstock fed diets 2, 3 and 4 at full and half ration over three spawnings.

4.4.3.2 Monounsaturated fatty acids (MUFA)

Mean MUFA level in eggs originating from tilapia broodstock fed diet 2 was 41.3 and 44.6%, at full and half rations, respectively. Similarly, the mean MUFA for eggs from broodstock fed on diet 3 at full and half ration was 42.4 and 42.6%, respectively, and for eggs from fish fed diet 4 was 36.6 and 39.3%, at full and half rations. The level of MUFA was not significantly different ($P>0.05$) between full and half rations in eggs from fish fed diets 2, 3 and 4 over three spawnings (Figure 4.6).

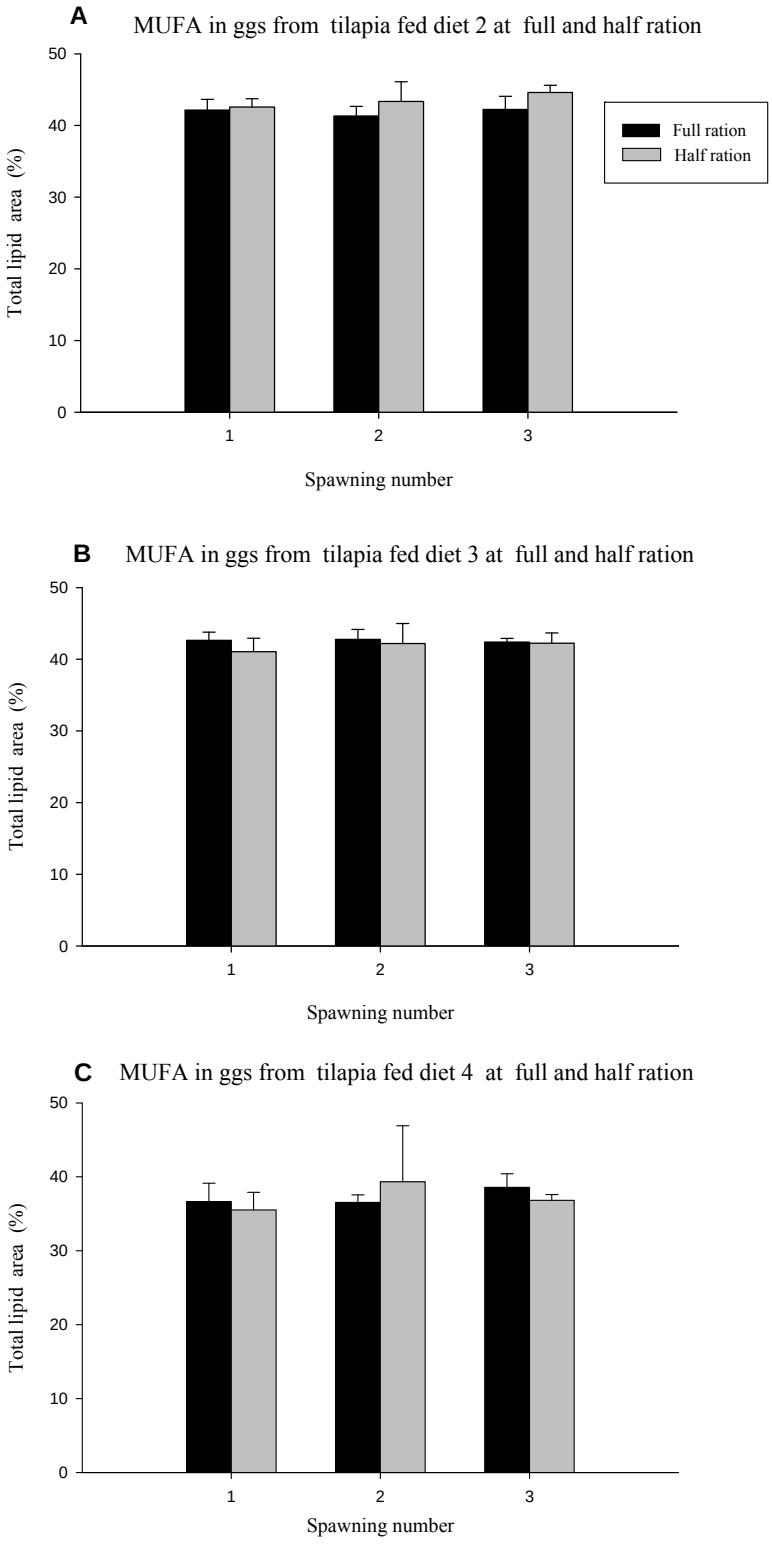


Figure 4.6 Monounsaturated fatty acid (MUFA) in eggs originating from *O. niloticus* fed different dietary lipids at full and half rations over three consecutive spawnings. Values are mean ± SD, n=3. In each graph, the differences between rations but within each spawnings are not significant (P>0.05).

4.4.3.3 Arachidonic acid (ARA)

The FA composition of eggs from tilapia fed with diet 2 at full and half ration is shown in Table 4.3. The ARA level in eggs from fish fed diet 2 ranged between 3.1 - 3.5 %, in eggs from fish fed diet 3 ranged between 1.9 - 3 %, followed by in eggs from fish fed diet 4 ranged between 1.8 - 2.1 % at full and half rations. However, the ARA levels were not significantly different ($p>0.05$) in eggs from fish fed diets 2, 3 and 4 at either full or half ration over three spawnings (Figure 4.7)

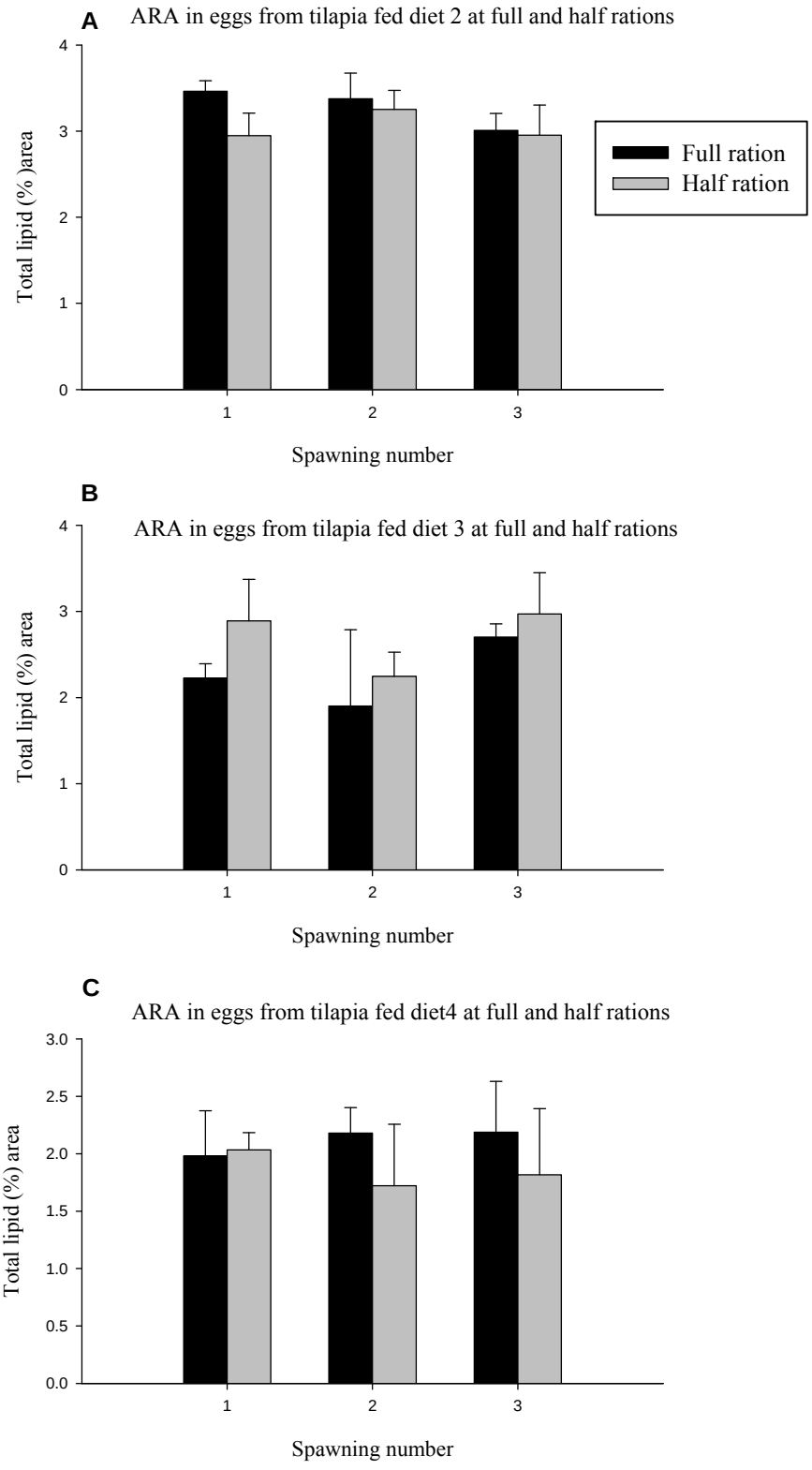


Figure 4.7 Arachidonic acid (ARA) in eggs originating from *O. niloticus* fed different diets at full and half ration. Data presented as %, mean ± SD, n=3. In each graph, the differences between rations but within each spawnings are not significant (P>0.05).

4.4.3.4 Total n-6 fatty acid

The mean total n-6 level of eggs from broodstock fed diet 2 at full ration were 14.6, 14.3 and 13.3% over three sawnins, whilst at half ration the level were 13.19, 14.8 and 13.3% over three spawnings (Table 4.3). The mean values of total n-6 of eggs between the two rations were not significantly different ($p>0.05$) for three consecutive spawnings (Figure 4.8 A).

The mean values of total n-6 of eggs originating from fish fed diet 3 at full ration were 10.6, 11.1and 12.0%over three spawnings, and at half ration were 13, 11.7 and 12.3% (Table 4.4). The inclusion of n-6 FA in eggs from both full and half rations were not significantly different ($p>0.05$) over three spawnings with the exception of the first spawning, in which the effect of full ration was significantly ($p<0.05$) lower than that of half ration fish (Figure 4.8 B).

The total n-6 values of eggs from fish fed diet 4 at full ration were 12.3±0.5, 11.40±0.2 and 11.6±1.2% over here spawnig, at half ration were 12.6±, 10.2±1.4 and 11.3±1.0%. The total n-6 level in eggs decreased in the second spawning and subsequently increased in the third spawning, however, there was no significant difference ($p>0.05$) between full and half ration over the three spawnings (Figure 4.8 C).

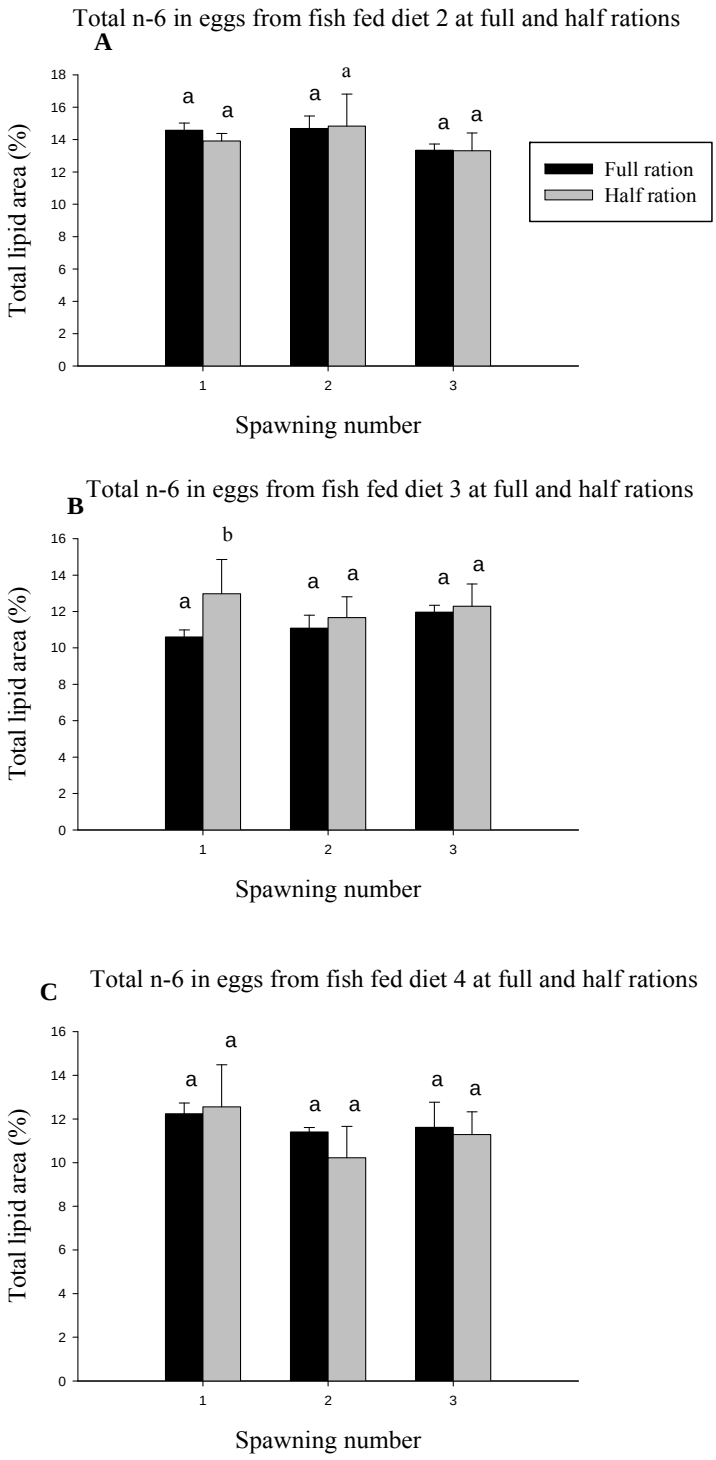


Figure 4.8 Total n-6 fatty acid in eggs originating from *O. niloticus* fed different diets at full and half ration. Data presented as %, mean ± SD, n=3. In each graph, bars with different letters are significantly different ($P<0.05$). A= diet 2 containing palm oil, B= diet 3 containing palm and cod liver oil and C= diet 4 as a control.

4.4.3.5 EPA

The mean values of EPA in eggs from broodfish fed diets 2, 3 and 4 at full and half ration ranged between 0.1-0.19%, over three consecutive spawnings. The mean EPA in eggs from fish fed diets 2, 3 and 4 were not significantly different (P>0.05), when comparing the EPA level between the two rations over three consecutive spawnings (Figure 4.9).

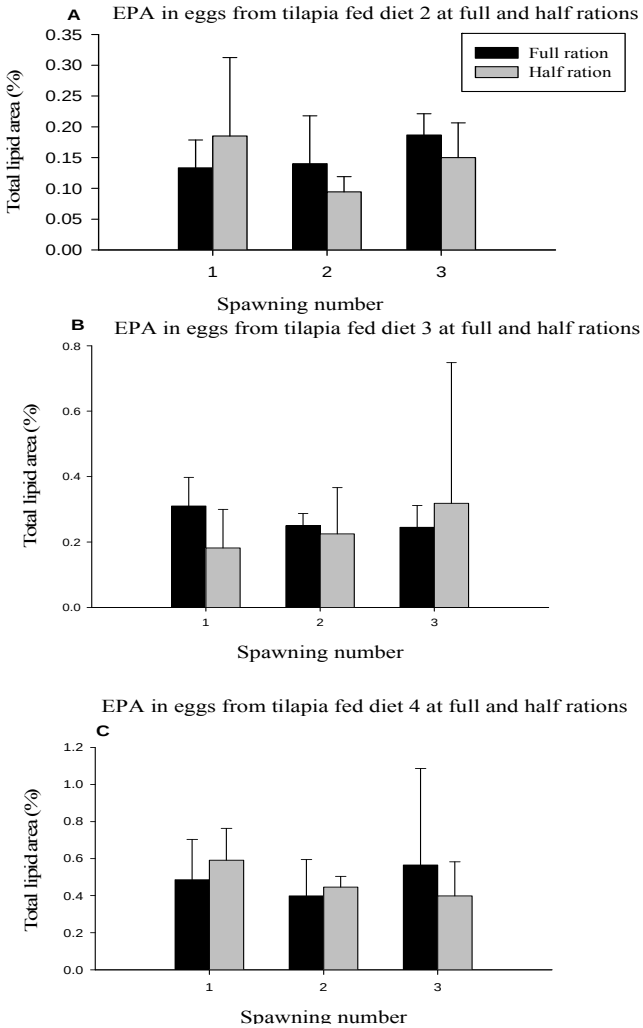


Figure 4.9 EPA Fatty acid in eggs originating from *O. niloticus* fed different diets sources at full and half ration. Data presented as %, mean ± SD, n=3). In each graph, the differences between the rations within each spawning are not significant (P>0.05).

4.4.3.6 DHA

The DHA level in eggs from tilapia fed diet 2 ranged between 5.7-5.8%, whilst in eggs from fish fed diet 3, this level ranged between 8.3-8.7%, for full and half ration over the three spawnings. The mean DHA in eggs from fish fed diet 2 and 3 were not significantly different ($p>0.05$) between full and half ration groups (Figure 4.10 A and B). The, mean DHA in eggs from fish fed diet 4 at full and half ration ranged between 11.5-15.1%, for three consecutive spawnings (Figure 4.10 C). However, the mean DHA in eggs from full ration fish on first spawning was significantly ($P<0.05$) lower than the first spawning of half ration fish, but there were no significant differences ($P>0.05$) observed between second and third spawning at either full or half ration.

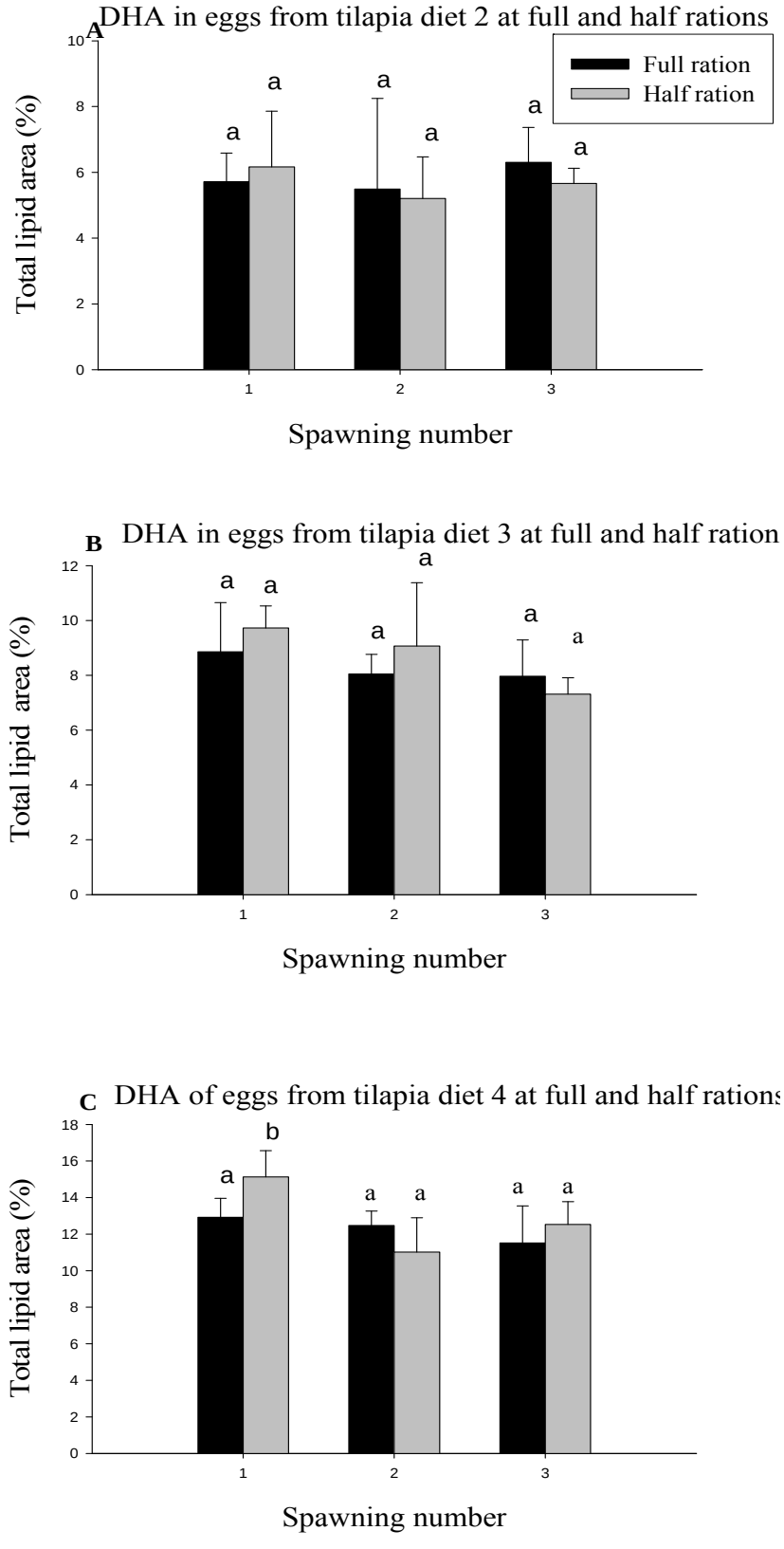


Figure 4.10 DHA fatty acid in eggs originating from *O. niloticus* fed different diets at full and half ration. Data presented as %, mean ± SD, n=3. In each graph, bars with different letters between ration but within spawning are significantly different ($P<0.05$).

4.4.3.7 Total n-3 PUFA

The mean total n-3 PUFA in eggs from fish fed diet 2 at full and half ration ranged between 6.7-7.2-%, and in eggs from fish fed diet 3 at full and half ration ranged between 10-10.3%, over three spawnings, however, there were no significant differences ($P>0.05$) occurred between full and half ration in eggs from fish fed diets 2 and 3 (Figure 4.11 A, B). Nevertheless, the total n-3 PUFA in eggs from fish fed diet 4 at full and half ration ranged between 14.2-19.4%, for the three consecutive spawnings. In terms of total n-3 PUFA of eggs from fish fed diet 4, there were no significant differences ($P> 0.05$) observed between the two rations, with the exception of the first spawning which was significantly higher ($p<0.05$) than in eggs from full ration fish (Figure 4.11 C).

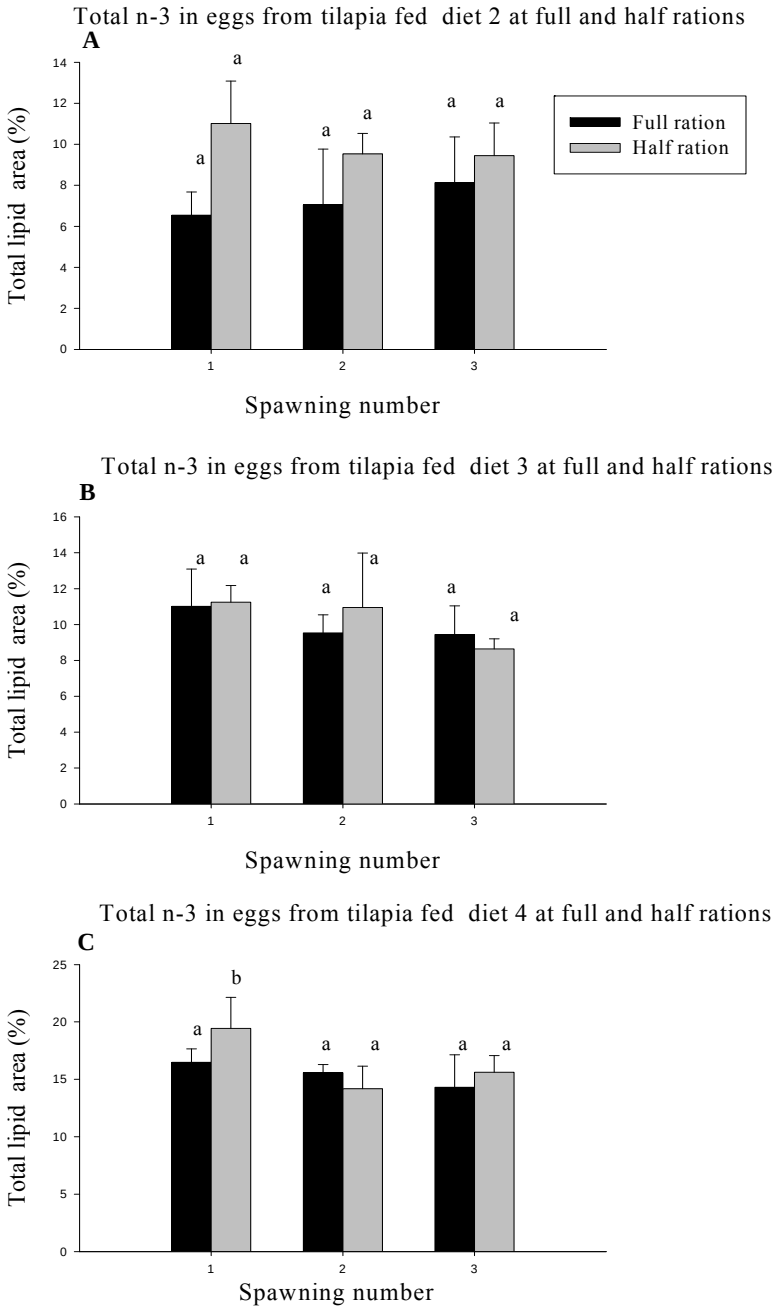


Figure 4.11 Total n-3 fatty acid in eggs originating from *O. niloticus* fed different diets at full and half ration. Data presented as %, mean ± SD, n=3. In each graph, bars with different letters between rations but, within spawning are significantly different ($P<0.05$).

4.4.3.8 Total polyunsaturated fatty acids (PUFA)

The total PUFA in eggs from fish fed diet 2 ranged between 20.7-21.5 %, while in eggs derived from the fish fed diet 3 ranged between 21.2-22.6%, at full and half

ration over three spawnings (Figure 4.12 A and B). The total PUFA in eggs from fish fed a full ration was not significantly different ($p>0.05$) from eggs of fish fed a half ration of diets 2 and 3. Nevertheless, total PUFA in eggs from fish fed diet 4 ranged between 24.4-32.0%, for full and half ration over three consecutive spawnings. Total PUFA in eggs from fish fed at full ration, the first spawning was significantly ($P<0.05$) lower than in eggs from half ration fish, but no significant differences ($P>0.05$) were observed between the two ratins at the second and third spawnings (Figure 4.12 C).

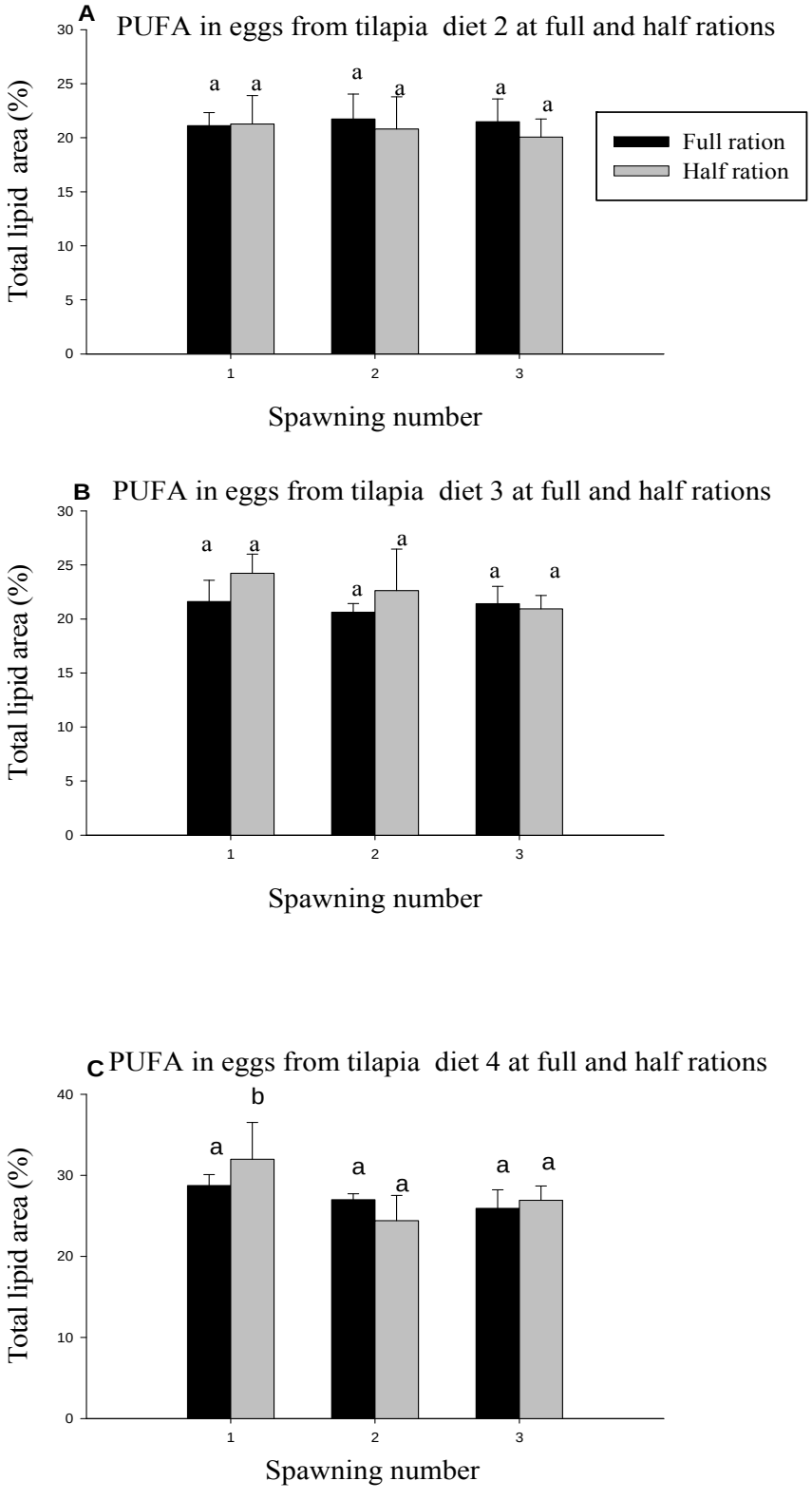


Figure 4.12 Total PUFA in eggs originating from *O. niloticus* fed different diets at full and half ration. Data represented as %, mean ± SD, n=3. In each graph, bars with different letters between the rations but within the spawning are significantly different ($P<0.05$).

4.4.3.9 Total n-3/n-6 ratio

The n-3/n-6 fatty acid ratio in eggs from fish fed diet 2 ranged between 0.48-0.52, while in eggs from fish diet 3 ranged between 0.8-0.9 with diet 3, and between 1.4-1.5 with diet 4, for full and half rations over three spawnings. However, feed ration level had no significant ($p>0.05$) effect on total n-3/n-6 ratio over three consecutive spawnings (Figure 4.13).

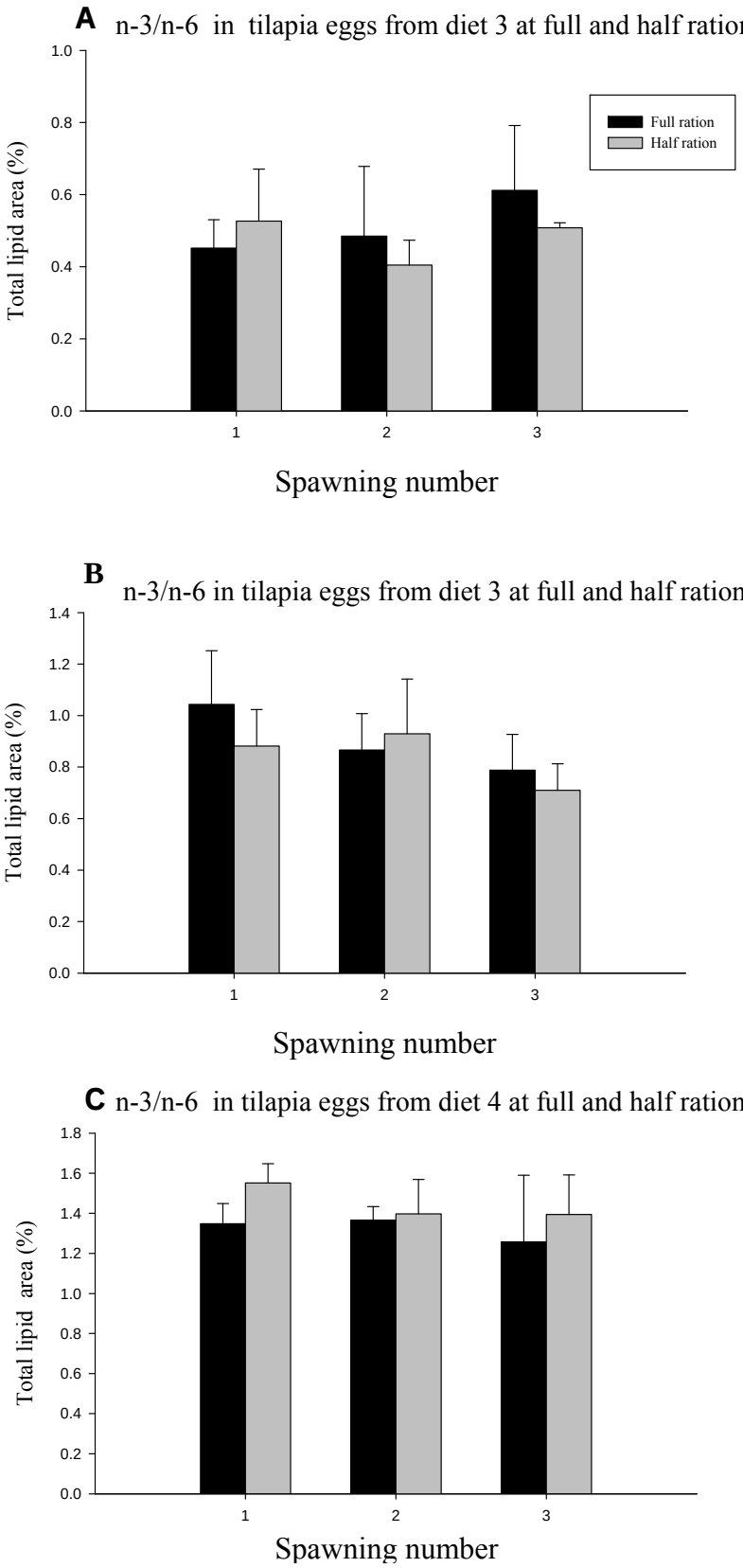


Figure 4.13 n-3: n-6 ratio in eggs originating from *O. niloticus* fed different diets at full and half ration. Data represented as %, mean ± SD, n=3. In each graph, bars between the rations but within the spawnings are not significantly different (P>0.05).

Table 4.3 Fatty acid composition in eggs originating from *O. niloticus* fed diet 2 at full and half ration (data presented as % of total lipid area, mean ± SD, n=3)

Fatty acids	Full ration			Half ration		
	Spawning number			Spawning number		
	1	2	3	1	2	3
14:0	1.7±0.3	2.2±0.3	2.1±0.3	2.0±0.2	1.6±0.3	2.5±0.1
15:0	0.2±0	0.2±0	0.2±0	0.2±0.1	0.2±0.0	0.2±0.1
16:0	26.3±1	25.7±1.4	26.4±0.9	26.2±1.5	26.2±1.0	25.6±0.9
18:00	7.3±3	8.0±0.9	7.2±0.4	7.2±0.2	7.5±0.7	6.6±0.3
20:0	0.2±0	0.2±0.1	0.2±0	0.2±0.0	0.2±0.0	0.2±0.0
22:00	1.0±0.6	0.6±0.6	0.5±0.7	0.3±0.2	0.4±0.3	0.3±0.2
Total saturated	36.7±2.4	36.9±1.8	36.3±1.1	36.2±1.6	35.8±1.5	35.3±0.8
16:1n-9	1.8±0.2	1.6±0.1	1.8±0.2	1.4±0.5	1.6±0.3	1.9±0.2
16:1n-7	3.8±0.3	4.8±0.3	4.8±0.4	5.5±0.7	4.4±0.6	60±.0.2
18:1n-9	31.6±1.1	29.0±1.1	30.5±2.3	30.5±3	33.2±2.3	31.3±1.5
18:1n-7	2.5±0.3	3.0±0.2	2.7±0.3	2.5±0.2	2.2±0.8	3.1±0.5
20:1n-9	1.8±0.3	2.2±0.2	1.8±0.2	2.2±0.7	1.6±0.3	2.0±0.1
20:1n-7	0.2±0.1	0.2±0	0.2±0.1	0.2±0.1	0.2±0.0	0.2±0.0
22:01	0.4±0.1	0.5±0.1	0.3±0.1	0.9±0.2	0.2±0.1	0.4±0.0
24:1n-9	0.1±0	0.1±0.1	0.2±0.1	0.1±0.0	0.1±0.0	0.1±0.0
Total MUFA	42.1±1.5	41.3±1.3	42.2±1.8	42.6±1.2	43.3±2.8	44.6±1
18:2n-6	4.6±0.3	5.5±0.2	4.3±1.5	5.3±0.1	4.9±0.3	4.5±.0.3
18:3n-6	0.7±0.1	0.7±0.3	1.4±1.8	0.9±0.1	0.9±0.1	1.0±0.1
20:2n-6	1.4±0.2	1.1±0.1	1.1±0.1	1.1±0.2	1.3±0.1	1.3±0.2
20:3n-6	1.2±0.1	1.3±0.1	1.2±0.2	1.4±0.1	1.±40.1	1.3±0.1
20:4n-6 (ARA)	3.5±0.1	3.4±0.3	3.0±0.2	2.9±0.3	3.3±0.2	3.0±0.4
22:4n-6	1.0±0.1	1.1±0.1	0.9±0.1	0.8±0.1	0.9±0.2	0.8±0.1
22:5n-6	2.1±0.1	1.7±0.2	1.4±0.2	1.5±0.2	2.2±1.9	1.5±0.3
Total n-6 PUFA	14.6±0.5	14.7±0.8	13.3±0.4	13.9±0.5	14.8±2.0	13.3±1.1
18:3n-3	0.2±0.2	0.2±0	0.2±0.1	0.2±0.1	0.2±0.0	0.2±0.1
18:4n-3	0.1±0.1	0.1±0	0.1±0	0.1±0.0	0.1±0.0	0.1±0.1
20:3n-3	ND	ND	ND	ND	ND	ND
20:4n-3	0.1±0	0.1±0	0.6±1.3	0.1±0.0	0.0±0.0	0.1±0.0
20:5n-3 (EPA)	0.1±0.1	0.1±0.1	0.2±0	0.2±0.1	0.1±0.0	0.2±0.1
22:5n-3	0.4±0.2	1.0±0.2	0.7±0.1	0.7±0.3	0.5±0.1	0.7±0.1
22:6n-3 (DHA)	5.7±0.9	5.5±2.8	6.3±1.1	6.2±1.7	5.2±1.3	5.7±0.5
Total n-3 PUFA	6.5±1.1	7.0±2.7	8.1±2.23	7.4±2.2	6.0±1.3	6.8±0.6
Total PUFA	21.1±1.2	21.7±2.3	21.5±2.1	21.3±2.6	20.8±3.0	20.1±1.7
ARA/EPA	28.9±10.9	37.3±30.8	16.6±3.5	23.5±16.3	37.1±11.6	28.0±27.3
DHA /EPA	48.4±20.2	54.7±64.9	34.5±6.3	51.6±47.6	60.2±27.2	52.8±49.5
n-3/n-6	0.5±0.1	0.5±0.2	0.6±0.2	0.5±0.1	0.4±0.1	0.5±0.0

ND=not detected

MUFA= monounsaturated fatty acid

Table 4.4 Fatty acid composition in eggs originating from *O. niloticus* fed diet 3 at full and half ration (data presented as % of total lipid area, mean ± SD, n=3)

Fatty acids	Full ration			Half ration		
	Spawning number			Spawning number		
	1	2	3	1	2	3
14:0	2.1±0.2	1.9±0.6	2.0±0.2	1.5±0.2	2.5±0.9	2.0±0.3
15:0	0.2±0	0.3±0	0.2±0	0.2±0	0.2±0	0.2±0.1
16:0	25.2±2.3	26.9±2.7	25.8±0.9	25.1±2.3	24.3±1.5	26.4±1.3
18:00	7.5±0.7	7.1±0.6	7.7±0.4	7.4±0.6	7.7±0.8	7.7±0.2
20:0	0.2±0	0.2±0	0.2±0	0.2±0.1	0.2±0	0.3±0.1
22:00	0.7±0.9	0.1±0.1	0.3±0.2	0.3±0.2	0.3±0.3	0.2±0.2
Total saturated	35.8±2.3	36.6±1.7	36.2±1.4	34.7±2.7	35.2±1.7	36.8±1.7
16:1n-9	1.7±0.1	1.8±0.2	1.6±0.3	1.7±0.5	1.7±0.2	1.6±0.1
16:1n-7	4.8±0.8	4.7±0.8	5.1±0.3	4.7±0.4	5.7±1.5	5.0±0.3
18:1n-9	30.6±1.1	31.8±1.3	30.2±0.5	29.6±2.4	28.6±6.1	30.4±1.8
18:1n-7	2.8±0.3	2.0±1.3	2.8±0.2	2.4±1.1	3.3±1.2	2.7±0.2
20:1n-9	1.9±0.3	1.8±0.3	2.0±0.2	1.9±0.1	2.6±0.6	1.8±0.1
20:1n-7	0.2±0	0.2±0	0.2±0	0.2±0	0.3±0.1	0.2±0
22:01	0.5±0.2	0.4±0	0.4±0.1	0.3±0.1	0.6±0.3	0.3±0
24:1n-9	0.2±0	0.1±0	0.1±0.1	0.2±0.1	0.2±0.1	0.1±0.1
Total MUFA	42.6±1.1	42.8±1.4	42.4±0.5	41.0±1.9	42.2±2.8	42.2±1.4
18:2n-6	5.0±0.1	5.2±0.2	4.9±0.1	5.3±0.7	5.5±0.7	4.3±2.1
18:3n-6	0.6±0.3	0.9±0	0.6±0.4	0.7±0.3	0.7±0.1	0.8±0.1
20:2n-6	0.7±0.1	0.7±0	0.9±0.1	1.0±0.3	0.8±0.1	1.2±0.3
20:3n-6	0.9±0.4	1.1±0.2	1.1±0.1	1.2±0.2	1.0±0.1	1.2±0.1
20:4n-6 (ARA)	2.2±0.2	1.9±0.9	2.7±0.2	2.9±0.5	2.2±0.3	3.0±0.5
22:4n-6	0.6±0.1	0.6±0.1	0.7±0.1	0.8±0.2	0.8±0.115	0.8±0.1
22:5n-6	0.6±0.1	0.7±0.1	0.9±0.1	1.1±0.2	0.8±0.2	1.0±0.4
Total n-6 PUFA	10.6±0.4	11.1±0.7	12.0±0.4	13.0±1.9	11.7±1.2	12.3±1.2
18:3n-3	0.2±0	0.2±0	0.2±0	0.3±0	0.3±0.1	0.2±0
18:4n-3	0.2±0.1	0.1±0	0.1±0	0.1±0	0.1±0.1	0.1±0
20:3n-3	ND	ND	ND	ND	ND	ND
20:4n-3	0.8±1.2	0.1±0	0.1±0	0.1±0	0.1±0.1	0.1±0
20:5n-3 (EPA)	0.3±0.1	0.3±0	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.4
22:5n-3	1.1±0.3	0.9±0.2	0.9±0.2	1.0±0.1	1.1±0.5	0.7±0.1
22:6n-3 (DHA)	8.9±1.8	8.0±0.7	8.0±1.3	9.7±0.8	9.1±2.3	7.3±0.6
Total n-3 PUFA	11.0±2.1	9.5±1	9.4±1.6	11.2±0.9	11.0±3	8.6±0.6
Total PUFA	7.6±1.8	8.0±3.9	32.8±52.6	34.7±46	13.2±6.4	21.6±17.1
ARA/EPA	29.2±3.2	32.5±2.7	33.4±4.3	97.8±99	51.0±28.8	55.2±47.1
DHA /EPA	1.0±0.2	0.9±0.1	0.8±0.1	0.9±0.1	0.9±0.2	0.7±0.1
n-3/n-6	21.6±2	20.6±0.8	21.4±1.6	24.2±1.8	22.6±3.8	20.9±1.3

ND=not detected

MUFA= monounsaturated fatty acid

Table 4.5 Fatty acid composition in eggs originating from *O. niloticus* fed diet 4 at full and half ration (data presented as % of total lipid area, mean ± SD, n=3)

Fatty acids	Full ration			Half ration		
	Spawning number			Spawning number		
	1	2	3	1	2	3
14:0	4.1±0.4	4.5±0.3	3.6±0.5	3.2±0.3	3.7±0.5	3.5 ±0.5
15:0	0.2±0	0.2±0	0.2±0	0.3±0.1	0.2±0	0.2±0
16:0	22.0±1.4	22.7±0.7	23.4±1.9	21.4±0.8	22.7±3.3	22.7±1
18:00	7.8±1.2	8.7±0.8	7.9±0.6	7.2±1.6	9.2±1.5	9.5±1
20:0	0.2±0	0.2±0	0.2±0.1	0.2±0.1	0.2±0	0.2±0
22:00	0.5±0.6	0.3±0.2	0.3±0.2	0.3±0.4	0.3±0.3	0.2±0.1
Total saturated	34.6±2.0	36.5±1.3	35.5±1.6	32.5±2.4	36.3±5.3	36.3±1.9
16:1n-9	0.5±0.3	0.4±0.3	1.2±0.2	0.7±0.3	0.6±0.5	1.0±0.2
16:1n-7	7.0±1	7.7±1.1	6.7±0.8	6.3±0.7	7.0±1.2	6.7±0.9
18:1n-9	20.5±1.1	19.3±1	23.0±1.9	20.2±2.4	20.8±3	22.0±0.7
18:1n-7	3.8±0.5	4.0±0.3	3.7±0.5	3.5±0.1	3.7±0.6	3.8±0.1
20:1n-9	3.3±1	3.5±0.4	2.7±1.1	3.5±0.2	2.5±0.4	2.6±0.3
20:1n-7	0.3±0.1	0.3±0	0.3±0	0.3±0	0.2±0	0.2±0
22:01	1.1±0.3	1.1±0.2	0.9±0.3	1.0±0.3	0.7±0.2	0.8±0.1
24:1n-9	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0.1	0.1±0
Total MUFA	36.6±2.5	36.5±1.0	38.6±1.9	35.5±2.4	39.3±7.6	36.8±0.8
18:2n-6	7.0±0.6	5.8±0.4	5.6±0.3	7.6±2.1	5.9±0.7	6.4±0.9
18:3n-6	0.6±0.1	0.5±0.1	0.7±0.1	0.5±0.1	0.4±0.1	0.4±0.2
20:2n-6	0.6±0.1	0.7±0.2	0.8±0.2	0.6±0.1	0.6±0.1	0.6±0.1
20:3n-6	1.0±0.2	1.1±0.1	1.1±0.2	0.9±0.1	1.0±0.2	1.0±0
20:4n-6 (ARA)	2.0±0.4	2.2±0.2	2.2±0.4	2.0±0.2	1.7±0.5	1.8±0.6
22:4n-6	0.6±0.1	0.6±0.1	0.6±0.1	0.5±0.1	0.5±0.2	0.5±0.1
22:5n-6	0.4±0.1	0.5±0.1	0.6±0.3	0.4±0.1	0.4±0.1	0.4±0.1
Total n-6 PUFA	12.2±0.5	11.4±0.2	11.6±1.2	12.6±1.9	10.2±1.4	11.3±1
18:3n-3	0.5±0	0.4±0.1	0.4±0.1	0.5±0.2	0.3±0.1	0.4±0.1
18:4n-3	0.3±0	0.2±0	0.2±0.1	0.2±0	0.2±0	0.2±0
20:3n-3	0.1±0	ND	0.1±0	0.1±0	0.5±0.7	0.1±0
20:4n-3	0.2±0	0.2±0	0.1±0.1	0.2±0.1	0.1±0	0.2±0
20:5n-3 (EPA)	0.5±0.2	0.4±0.2	0.6±0.5	0.6±0.2	0.4±0.1	0.4±0.2
22:5n-3	2.1±0.2	1.8±0.3	1.5±0.4	2.6±0.9	1.8±0.2	1.9±0.2
22:6n-3 (DHA)	12.9±1	12.5±0.8	11.5±2	15.1±1.4	11.0±1.9	12.5±1.3
Total n-3 PUFA	16.5±1.2	15.6±0.7	14.3±2.8	19.4±2.7	14.2±2	15.6±1.5
Total PUFA	28.7±1.4	27.0±0.7	25.9±2.3	32.0±4.5	24.4±3.1	26.9±1.8
ARA/EPA	9.4±16.1	22.9±44.6	5.9±3.7	3.8±1.6	3.9±1.2	19.1±35.8
DHA /EPA	59.1±98.4	134.1±264	27.4±9.1	27.3±6.8	24.7±3.5	130.3±243
n-3/n-6	1.3±0.1	1.4±0.1	1.3±0.3	1.6±0.1	1.4±0.2	1.4±0.2

ND= not detected

MUFA= monounsaturated fatty acid

4.4.4 Effect of dietary lipid sources and ration size on fatty acid composition of eggs in serial spawnings

The major fatty acids which are vital for growth and, particularly, for reproductive performance were analysed in order to find out the interaction between diets, rations and spawnings by GLM three-way ANOVA using SPSS. If the interactions were found significant, the data were then analysed using Minitab version 15 to discriminate the significant levels of fatty acids between the diets, rations and spawnings. The relevant data were then compared to each other (*e. g.* full ration of diet to full ration or half to half ration). To facilitate comprehension, the interaction results were abbreviated as follows: Diet 2, 3 and 4 (D 2, 3 and 4), Ration 1 (full ration) R2 (half ration) spawning 1, 2, and 3 (Sp 1, 2 and 3) and versus (vs). However, three-way ANOVA showed that most interactions between diets, rations and spawnings were not significant or the significant levels varied between the inactions.

4.4.4.1 Saturated fatty acid (SFA)

The SFA in eggs from fish fed diet 2 ranged between 35.8-36.4%, between 35.6-36.2% with diet 3, and between 35.0 -35.3 with diet 4%, for full and half rations in three spawnings. SFA decreased slightly in eggs from fish fed half rations diet treatments, however, differences between diets, rations and spawnings in eggs from fish fed diets 2, 3 and 4 were not significant ($P>0.05$).

4.4.4.2 Monounsaturated fatty acid (MUFA)

The mean MUFA content in eggs from broodstock fed diet 2 ranged between 41.3± 1.3 - 44.6±1.0%, whilst in eggs from fish fed diet 3, it ranged between 42.4±0.5 -

41.1±1.9%, and in eggs from fish fed diet 4 ranged between 36.5 and 35.5%, at full and half ration, respectively over three spawnings. There were no significant differences ($P>0.05$) in mean percentage MUFA in eggs from fish fed full ration of diet 2 vs those from fish fed half ration of diet 3 and full ration of diet 4 over three spawnings. The differences in mean percentage of MUFA were also not significant in eggs from fish fed diet 2 at half ration compared to those fed diet 3 at half ration, over three spawnings. On the other hand, the MUFA in eggs from fish D2R1at Sp1 and 3 with those compared from fish fed D4R2 at Sp 1 and 3 were significantly different ($P<0.05$) but there was no significant difference ($P>0.05$) at Sp2 between the two diets. Also, there were no significant differences observed in eggs from fish fed D2 R2 in Sp1 and 3 compared with those from fish fed D4 R2 at the same spawnings (Sp1 and 3) while at Sp 2 there was a significant difference ($p<0.05$) between these two diets. MUFA in eggs from fish fed D3 R1 at Sp 1 and 2 vs those from fish fed D4 R1 at Sp1 and 2 was not significantly different but at Sp 3 was significantly different ($p<0.05$) between two diets. In eggs from fish fed D3 R2 at Sp 1 and 2 vs those from fish fed D4 R2 at the same spawnings there was a significant difference ($p<0.05$), but not at Sp3. Eggs from fish fed D3 R1 at Sp1 and 3 compared to those from fish fed D4 R2 at Sp1 and 3 were significantly different ($p<0.05$) but at Sp2 there was no significantly different ($p>0.05$) between two diets, (see Appendix Table 7.1).

4.4.4.3 Arachidonic acid (ARA)

The ARA in eggs from tilapia fed experimental diets and their significant interaction levels between diets, rations and spawnings are shown in Appendix Table 7.2. Three-way ANOVA showed that the ARA in eggs originating from fish fed D2 R1 at Sp2 and 3 and those from fish fed diet D3 R1 at the same spawnings

were not significantly different ($p>0.05$), but there was a significant diference ($P<0.05$) at Sp1. The ARA levels in eggs originating from fish fed D2 R1 at Sp1, 2 and 3 ranged between 3.5, 3.4 and 3% while in eggs from fish fed D4 R1 at Sp1, 2 and 3, it ranged between 2, 2.2 and 2.2%. However, the level of ARA in eggs from fish fed diet 2 at full ration was significantly ($P<0.05$) higher than in eggs from fish fed diet 3 at full ration over the three spawnings. Nevertheless, the ARA in eggs from fish fed D2 R2 at Sp 1 and 3 compared with those from fish fed D3 R2 at the same spawnings were not significantly different, but there was a significant difference ($p<0.05$) observed at Sp2 between two diets. ARA levels in egs originatinf from fish fed a half ration of diet 2 were significantly greater than those from fish fed a half ration of diet 4 over three spawnings. The ARA in eggs from fish fed diet 3 at full ration ranged between 2.3-3 %, and in eggs from fish fed diet 4 at full ration ranged between 2 .2-2.5%, and they were not significantly different between the two diets over three spawnings. The mean values of ARA in eggs from fish fed D3 R2 at Sp 1, 2 and 3 vs those from fish fed D4 R2 at Sp1, 2 and 3 was significantly greater than those from fish fed a half ration of diet 4 over three spawnings, with the exception of Sp2 which there was no significant between the two diets. Likewise, there were no significant differences ($P>0.05$) observed in ARA content of eggs from fish fed D2 R1 at Sp1, 2 and 3 vs those from fish fed D3 R2 at the same spawnins. The ARA in eggs from fish fed D2 R1 at Sp1, 2 and 3 compared with those from fish fed D4 R2 at Sp1, 2 and 3 was not significant difference ($p>0.05$) between the first and second spawnings at a full ration of diet 2 vs first and second spawnings of diet 4, but a significant difference was observed at the third spawning between two diets. The ARA levels in eggs originating from fish fed D3 R1 at Sp1, 2 and 3 was 2.2, 1.9 and 2.7%, while in eggs from fish fed

D4 R1 at the same spawnings was 2, 1.7 and 1.8%, over three spawnings; as a result, ARA in eggs from fish fed D3 R1 at Sp1 and 2 compared with those from fish fed D4 R2 at Sp1 and 2 was no significantly different, but a significant difference ($p<0.05$) was observed at Sp3 between the two diets (see Appendix Table 7.2)

4.4.4.4 Total n-6 fatty acids

Total n-6 fatty acid in eggs ranged between 13.3-14.8% and 10.6-13%, from fish fed full ration of diet 2 and 3, respectively, over the three spawnings. The total n-6 levels in eggs from fish fed diet 2 at full ration was significantly greater than in eggs from fish fed diet 3 at full ration over three spawnings, with the exception of Sp3 which there was no significant difference ($P>0.05$). On the other hand, total n-6 in eggs from fish fed D2 R2 at Sp2 was significantly greater than those from fish fed D3 R2 at Sp2, but there were no significant differences ($P>0.05$) observed in eggs from fish fed D2 R2 at Sp1 and 3 compared with those from fish fed D3 R2 at the same spawnings. The total n-6 levels in eggs from fish fed diet 4 at both full and half ration was significantly ($P<0.05$) greater than in eggs from fish fed diet 2 over three spawnings, with the exception of Sp3 where there was not significant difference ($P>0.05$) between the two diets. Total n-6 levels in eggs from fish fed a full ration of diet 3 vs those from fish fed a full ration of diet 4 and also in eggs from fish fed a half ration of diet 3 compared with those from fish fed half ration of diet 4 were not significantly different ($P>0.05$) over the three spawnings (see Appendix Table 7.3).

4.4.4.5 EPA

The comprehensive data on EPA fatty acid level in eggs from fish fed different diets and feeding regimes over three consecutive spawnings is shown in Appendix Table 7.4. The EPA ranged between 0.1-0.3% in the eggs from fish fed 2 and diet 3 at both full and half rations over the three spawnings. There were no significant differences ($P>0.05$) observed in EPA between the eggs from fish fed full and half rations of diet 2 compared with those from fish fed at full and half rations of diet 3 over the three spawnings. The EPA ranged between 0.25-0.3%, in eggs from fish fed D3 R1 at Sp1, 2 and 3, between 0.4 - 0.6%, in eggs from brooders fed D4 R1at Sp1, 2 and 3, and between 0.2 - 0.3 in eggs from fish fed D3 at R2 in Sp1, 2 and 3 over the three spawnings. The EPA in eggs from fish fed full and half ration of diet 3 compared with those from fish fed full and half ration of diet 4 were not significantly different ($P>0.05$) over three spawnings. The EPA inclusion also in eggs from fish fed at full ration of diet 2 vs those from fish fed at half ration of diet 3 were not significantly different ($P>0.05$) over three spawnings. However, the mean values of EPA in eggs from fish fed D2 R1 at Sp1 and 2 vs those from fish fed D4 R1 at the same spawning were not significantly different, but a significant difference was observed at at Sp3. The EPA levels in eggs from fish fed D2 R2 at Sp1 and 2 vs D4R2 at the same spawnings were significantly different ($P<0.05$) but there was no significant difference observed at Sp3 between the two diets. A significant difference in EPA level was also observed in eggs from fish fed D2 R1at Sp1 vs those from fish fed D4 R2 at Sp1, but no significant differences were observed at Sp2 and3 between the two diets (see Appendix Table 7.4).

4.4.4.6 DHA

The DHA level in eggs from fish fed diet 2 and 3 ranged between 5.2-6.3% and 7.3-9.7%, respectively at both full and half ration over three spawnings (Table 4.4 and Table 4.5). Tthe DHA levels in eggs from fish fed the expeimnat diets at full and hand half rations over three spawnings were nalaysed using three-way ANOVA, some significant were occurred when diets, rations and spawning were comapered each ther but the siginicant differnces were inconsistent the comparhisive resuts presented in Appendix Table 7.5.

4.4.4.7 Total n-3 fatty acids

The total n-3 in eggs from fish fed different dietary lipid sources and the comprehensive interactions between diets, rations and spawnings was analysed by three-way ANOVA as shown in Appendix Table 7.6.

Total n-3 in eggs from fish fed diet 2 ranged between 6-8.1%, and in eggs from fish fed diet 3 between 8.6-11%, for full and half rations, respectively, over three spawnings. Three-way ANOVA revealed that total n-3 was not significantly different ($P>0.05$) in eggs from fish fed D2 R1 at Sp2 and 3 compared with those from fish fed D3 R1 at the same spawnings, but total n-3 at Sp1 was significantly different ($P<0.05$) between the two diets. Conversely, comparing the total n-3 between half ration groups, the level in eggs from fish fed D2 R2 at Sp1 and 2 vs those from fish fed D3 R2 at Sp1 and 2 was significantly different ($p<0.05$), but not at Sp3 between the two diets.

Total n-3 in eggs from fish fed diet 2 ranged between 6-8.1%, and in eggs from fish fed diet 4 between 14.3-19.4% for full and half rations over three spawnings (see Table 4.3 and Table 4.5). Notably, total n-3 in eggs from fish fed at full and half

rations of diet 4 was significantly greater than in those from fish fed full and half ration diet 2 when total n-3 was compared between diets, rations and spawnings. Nevertheless, total n-3 in eggs from fish fed at full and half rations of diet 3 compared with those from fish fed at full and half rations of diet 4 over three spawnings was significantly different ($P<0.05$) (see Appendix Table 7.6).

4.4.4.8 Total Polyunsaturated Fatty Acids (PUFA)

Total PUFA in eggs from fish fed different dietary lipid sources and the interactions between diets, rations and spawnings were analysed by three-way ANOVA, the comprehensive results presentd in Appendix Table 7.7. Three-way ANOVA revealed that few sginfant differences observed when PUFA levels in egss from fish fed the experimental dites at either full or half rations over three spawnings, but the differnces were inconsistent.

4.4.4.9 The n-3: n-6 ratio

The n-3: n-6 ratio in eggs from fish fed different dietary lipid sources was analysed by three-way ANOVA. The ratio of n-3 to n-6 in eggs between diets, rations and spawnings are shown in Appendix Table 7.8. The samw reusts were also found when n-3/ n-6 ratio in eggs from fish fed the experimenta diets and ratins over three sapwnins. Howevr due to inconsistent differs, the resuts were not interprtated.

4.4.4.10 ARA: EPA ratio

The ARA/EPA ratios in eggs from fish fed the experimental diets were compared between their diets, rations and spawnings by three-way ANOVA. The results are shown in Appendix Table 7.9 . There were no significant differences ($P>0.05$)

observed in ARA: EPA ratio in eggs from fish fed diets 2, 3 and 4 at full and half ration over the three spawnings.

4.4.5 Total lipid and fatty acid composition of experimental diets

Total lipid and fatty acid composition of the experimental diets, including a commercial diet as a control, were analysed to compare the lipid and fatty acid profiles between the diets and the eggs from broodstock fed the experimental diets. The total lipid and fatty acid composition of the three experimental diets and the control diets are shown in Table 2.5 and 2.6 in Chapter 2. The total lipid levels in the diet were around 10% for diets 1, 2 and 3 and 7% for diet 4 (control). Overall, fatty acid composition of diet 1 (cod liver oil), diet 2 (palm oil), diet 3 (palm and cod liver oil 9:1 ratio) and diet 4 as control had a range of monounsaturated fatty acids (39-43.9%), particularly of the *n*-9, ARA, EPA and DHA series, which dominated in the control diet; whilst the total saturated fatty acids were the major fatty acid class (41.2 and 45.5%) for diets 2 and 3, respectively. The level of palmitic acid (16:0) was 35.6 and 38.1% for diets 2 and 3, respectively, amounting to approximately a 3 fold increase over the 16.8 and 14.0% found in diets 1 and 4. The ARA level in diets 2 and 3 were not detected moreover the EPA was significantly lower in those diets, as a result ARA: EPA ratio intended zero for diets 2, 3 and 0.1 for diets 1 and 4, respectively. Diets 1 and 4 were characterised by very large amounts of DHA (9.9 and 6.3% for diets 1 and 4, respectively) compared with diets 2 and 3. DHA was not detected in diet 2 and ahd a levl of only 0.9% for diet 3. Total polyunsaturated fatty acids (PUFA) dominated in diet 1 and 4 (34.8 and 35.0% for diets 1 and 4, respectively) whilst PUFA in diets 2 and 3 was approximately half that of diets 1 and 4 (14.8 and 13.5%).

4.5 Comparison of fatty acids between broodstock diets and eggs

The major fatty acids of eggs from fish fed their respective diets and rations were grouped as full and half rations and compared with the fatty acid levels of the experimental diets. Prior to grouping, due to a few significant but inconsistent differences between spawnings, data from the three spawnings of each group were pooled.

4.5.1 Total saturated fatty acids (TSFA)

The total saturated fatty acids in the diets ranged between 21.1-45.5%, whilst in the eggs from fish fed their respective diets at full and half rations they ranged between 35.2-36.7%. Figure 4.14 shows that, although the level of total fatty acids in the diets varied, the level of TSFA in eggs remained constant.

4.5.2 Arachidonic acid (ARA)

The ARA levels ranged between 2.1-3.3% and 1.8-3.1% in eggs from fish fed their respective diets at full and half rations, respectively. ARA was not detected in diets 2 and 3 and was at only 0.4% in diet 4. Figure 4.15 shows that the ARA level increased in eggs from fish fed diet 2, and then tend to decrease in those from fed diets 3 and 4, at both full and half rations. However, the ARA in eggs was significantly higher than in the diets.

4.5.3 EPA

The EPA level ranged between 0.1-0.5% in eggs from fish fed the experimental diets at full and half rations, while in the diets the level ranged between 0.2-5.6%. The EPA level was increased slightly in eggs from fish fed diets 3 and 4. However,

although the EPA level in the diets was highly varied, in the eggs it was unchanged (see Figure 4.16).

4.5.4 DHA

The DHA level in eggs from fish fed their respective diets ranged between 5.7-12.7%. It was not detected in diet 2 but was 0.9 and 6.3% for diets 3 and 4, respectively. Despite the fact that the DHA level in diet 2 was not detected and was low in diet 3, the level of DHA was increased markedly in the eggs from fish fed the diets at both full and half rations (Figure 4.17).

4.5.5 Total n-6 PUFA

The total n-6 level was 13.7, 10.6 and 17.6% for diets 2, 3 and 4, respectively, whilst the level in eggs from fish fed the diets ranged between 11.2-14.2% at both full and half rations. Figure 4.18 shows that the total n-6 level in eggs was slightly higher than in their respective diets, with the exception of eggs from fish fed diet 4, which it was approximately 3% lower.

4.5.6 Total n-3 PUFA

The total n-3 level in diets ranged between 0.9-16.5% whilst in the eggs, it ranged between 7.2-15.5% and 6.7-16.5% for full and half rations, respectively. Figure 4.19 showed that the n-3 PUFA in the eggs increased with increasing dietary n-3 PUFA in the diets.

4.5.7 Total poly unsaturated fatty acids (PUFA)

The total PUFA levels in the diets ranged between 13.5-35% whilst in eggs from fish fed their respective diets, it ranged between 20.7-27.5% at both full and half rations. The total PUFA level increased in the eggs from fish fed diets 2 and 3 and also tended to increase in those fed diet 4. However, Figure 4.20 shows that, although total PUFA levels in diet 4 were higher, in eggs it was considerably lower than in the diet.

4.5.8 Total n-3:n-6 ratio

The n-3:n-6 ratio ranged between 0.1-0.9 in diets, whilst it was 0.5-1.4 in the eggs from fish fed their respective diets at full and half ration. The n-3:n-6 ratio increased in the eggs as the ratio was increased in the diets (Figure 4.21).

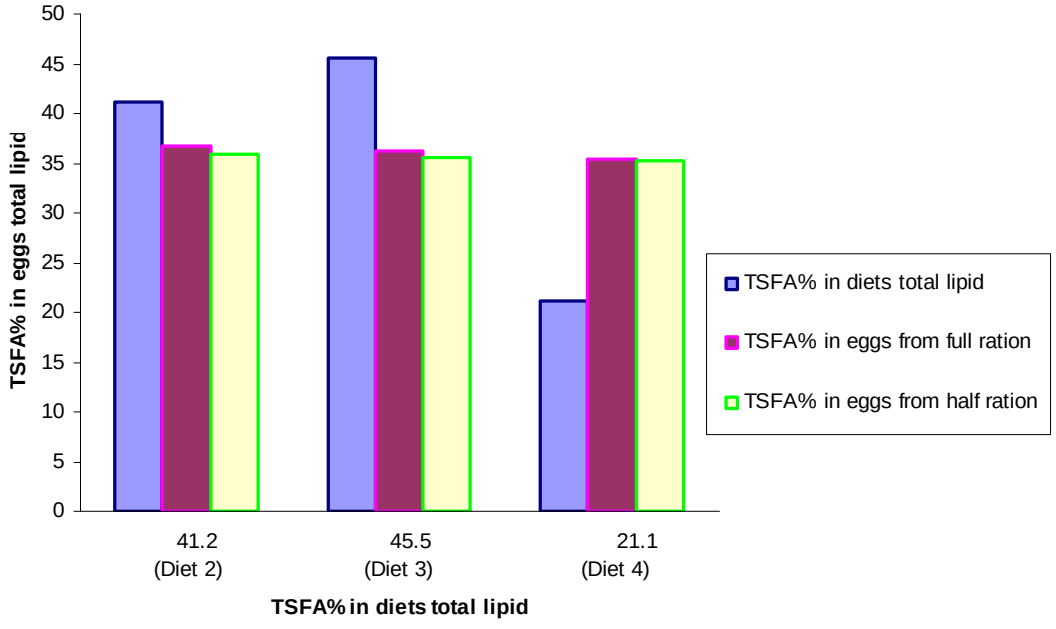


Figure 4.14 Total saturated fatty acid (TSFA) levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

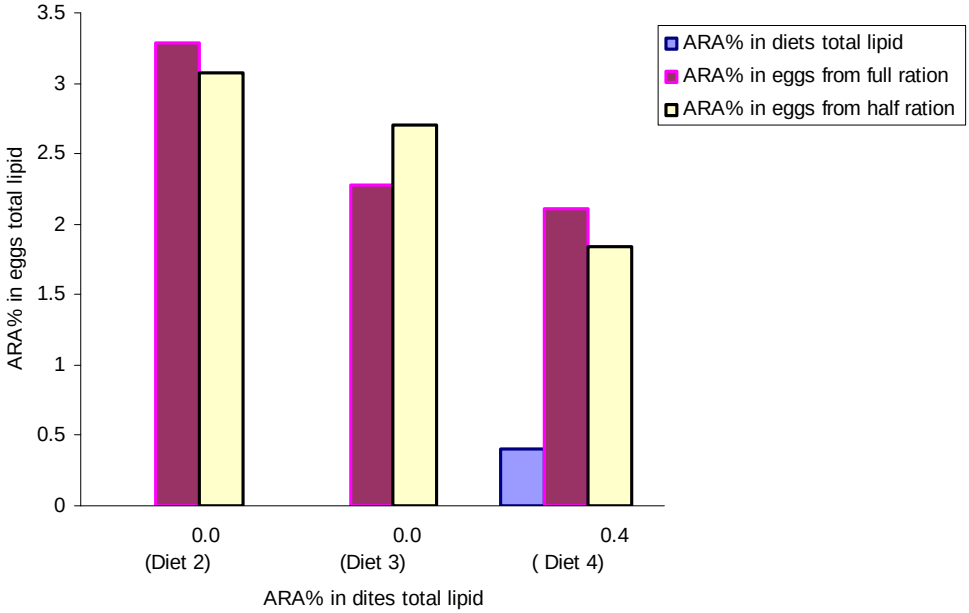


Figure 4.15 Arachidonic acid (ARA) levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

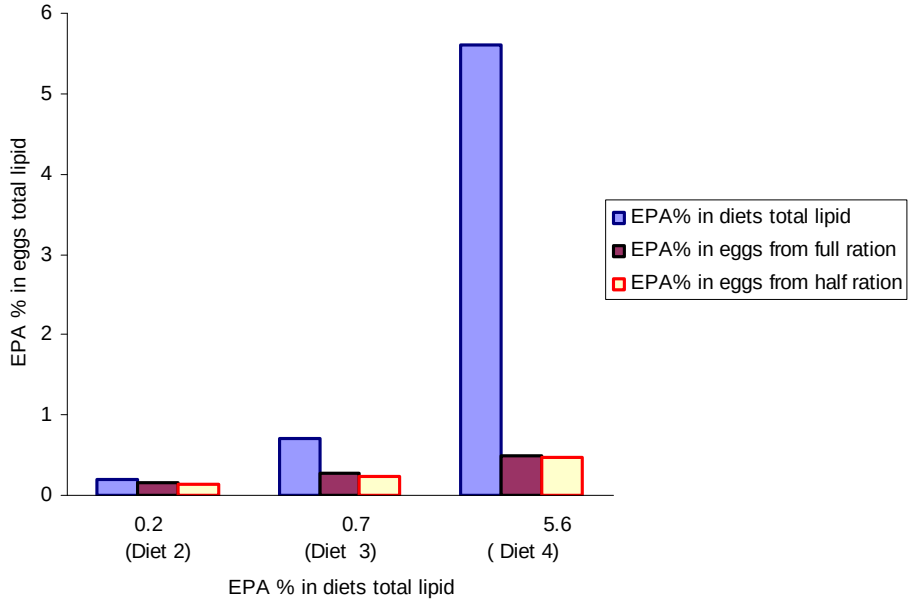


Figure 4.16 EPA levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

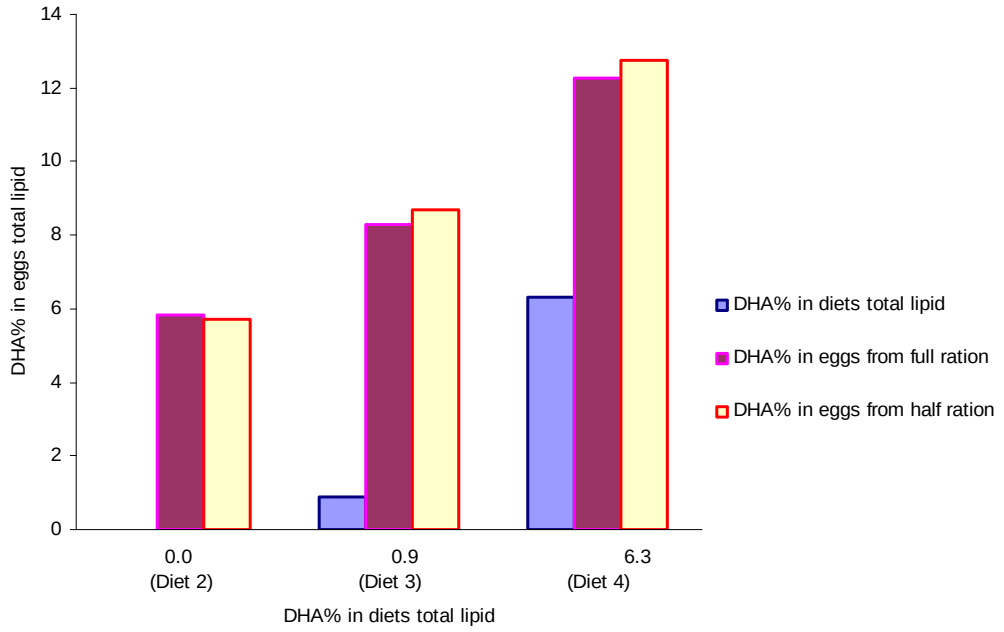


Figure 4.17 DHA levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

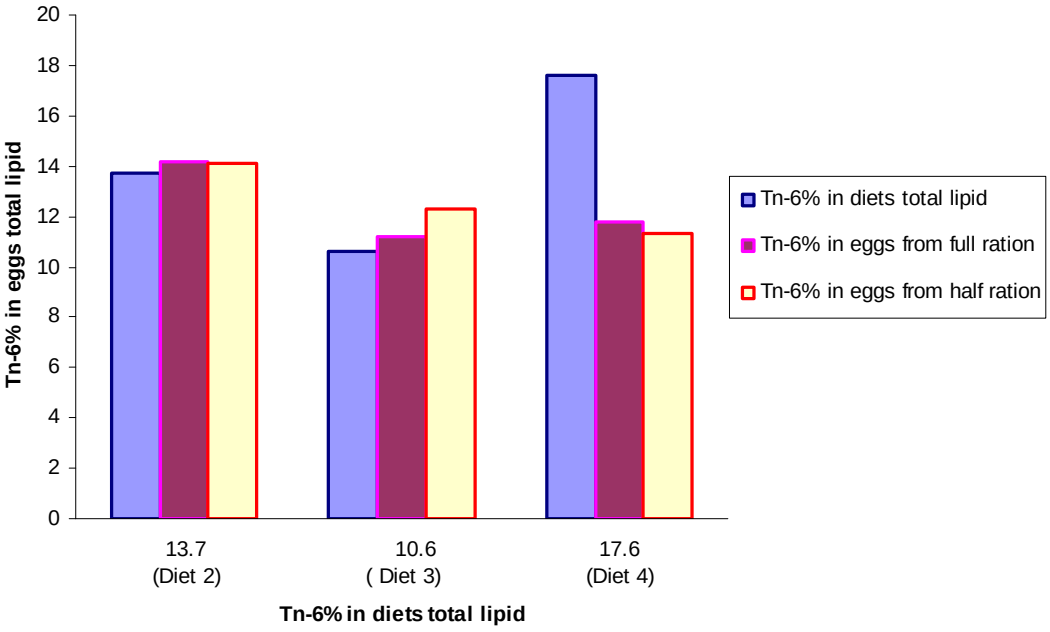


Figure 4.18 Total (T) n-6 levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

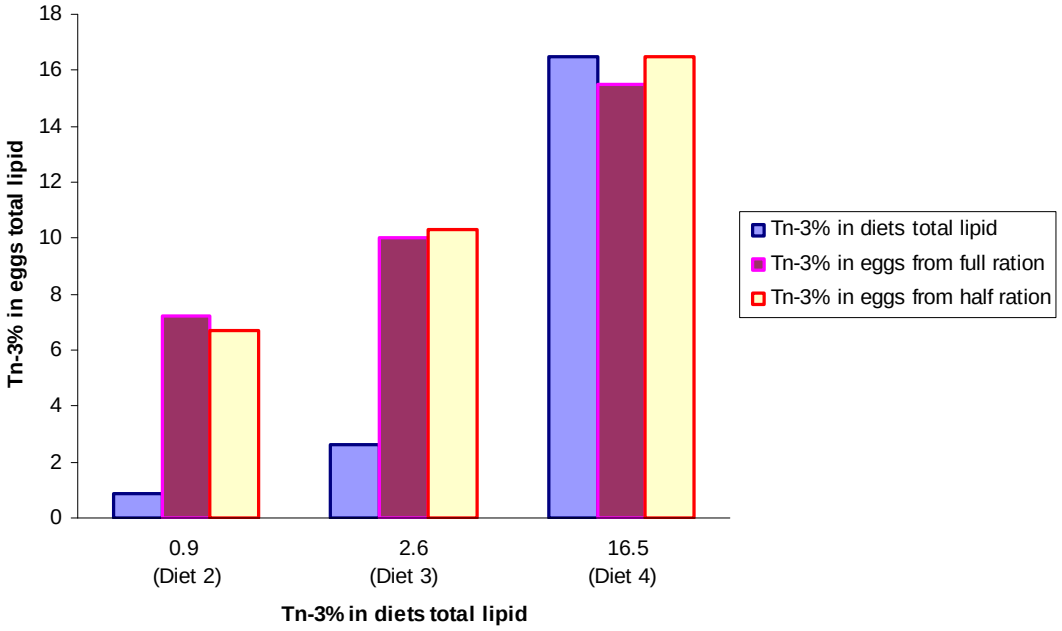


Figure 4.19 Total (T) n-3 levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

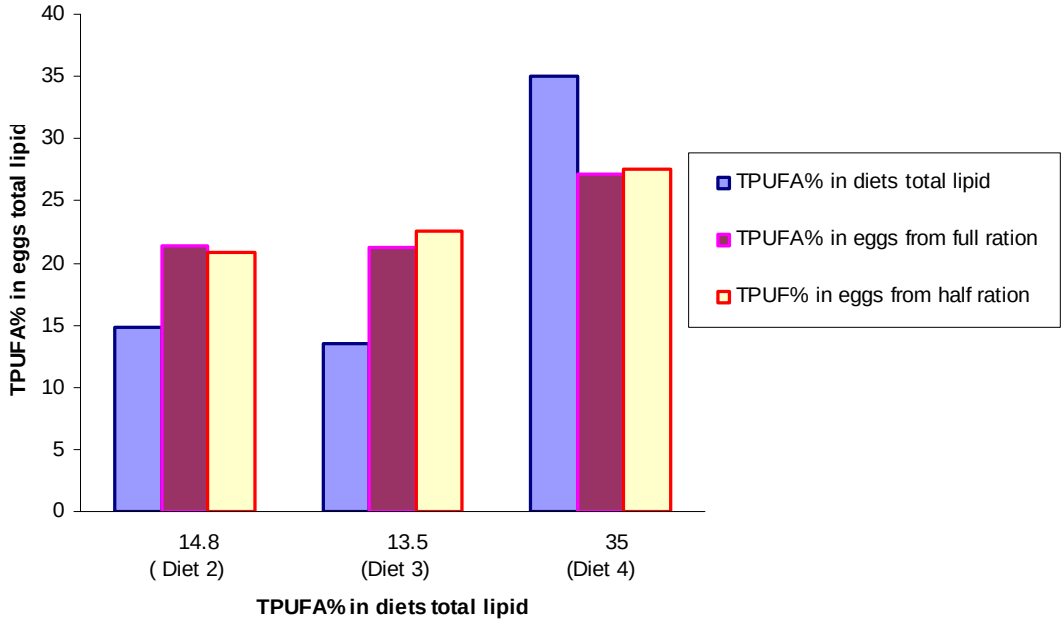


Figure 4.20 Total (T) PUFA levels in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

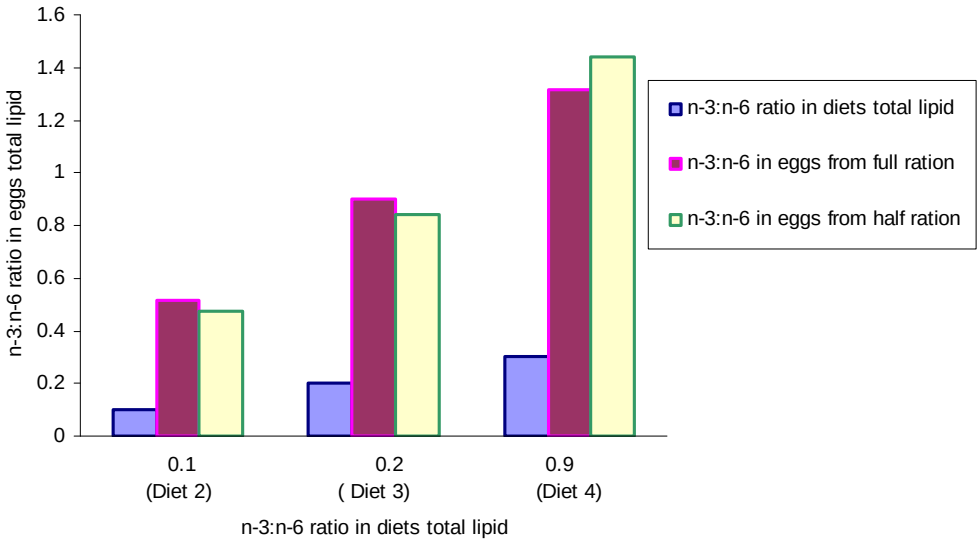


Figure 4.21 n-3:n-6 ratios in three diets and in eggs originating from *O. niloticus* fed these diets at either full or half ration. Data presented as percentage total lipid.

4.6 Discussion

To determine eggs quality, in the present study, eggs originating from fish fed their respective diets and rations during their entre life cycle; from the onset of exogenous feeding until spawning (for a period of 18 months).

Broodstock nutrition and genetics appear to be major factors in egg quality (Kjesbu and Holm, 1994). Broodstock nutrition is also vital in the production of high-quality eggs and larvae, and dietary lipid and fatty acid compositions are known to be important factors in determining the success of the developing embryos and larvae (Hu *et al.,* 2009; Izquierdo *et al.,* 2001b; Salze *et al.,* 2005; Tandler *et al.,* 1995). The biochemical composition of fish eggs is related to spawning performance and egg quality, as egg composition must satisfy embryonic nutritional needs for development and growth (Craik, 1985; Harel *et al.,* 1994; Lu and Takeuchi, 2004; Sandnes *et al.,* 1984). In fish, lipids are important nutrients for successful embryonic development (Boulekbache, 1981; Castell *et al.,* 2004; Sargent, 1995; Terner, 1979). Particular interest has focused on the role that the essential fatty acids (EFA), particularly docosahexaenoic acid (DHA: 22:6(*n*−3) and eicosapentaenoic acid (EPA: 20:5*n*−3), play in egg and larval development (Bell *et al.,* 1997; Bruce *et al.,* 1993, 1999; Castell *et al.,* 2004; Czesny *et al.,* 2000; Estevez *et al.,* 1999; Gunasekera *et al.,* 1995; Hu *et al.,* 2009; Ramos *et al.,* 1993; Sargent, 1995; Thrush *et al.,* 1994). Recently, attention has partly shifted to include arachidonic acid (ARA: 20:4*n*−6), mainly because of its role in eicosanoid production (Abayasekara and Wathes, 1999; Bell *et al.,* 1996; Farndale *et al.,* 1999). This is also due to the involvement of eicosanoids in a range of physiological

functions, including reproduction and egg development (Abayasekara and Wathes, 1999).

One of the principal objectives of the present study was to evaluate the effect of different dietary lipid sources on egg quality in terms of lipids and fatty acid composition, with the goal of replacing fish oil with palm oil in tilapia feed. Fishmeal which is commonly used as a protein source for aquafeed, contains up to 9% fish oil (De Boer and Bickel, 1988). Therefore, to avoid the effects of fish oil, soy-protein concentrate was used as the protein source for experimental diets.

Previous studies in other species have shown that palm oil can be used to replace fish oil with no negative effect on growth performance (Al-Owafeir and Belal, 1996; Bell *et al.,* 2002; Bell and Sargent, 2003; Kanazawa *et al.,* 1980; Legendre *et al.,* 1995; Ng *et al.,* 2000; Ochang *et al.,* 2007a; Ochang *et al.,* 2007b; Tortensen *et al.,* 2000; Varghese and Oommen, 2000). Limited information, however, is available on the effect of palm oil on tilapia reproductive performance, particularly the effect of lipid and fatty acid composition of tilapia eggs. The present study is the first attempt to investigate the effect of dietary lipid sources on reproductive performance of tilapia which were fed the same diet and ration levels throughout their entire life cycle.

4.6.1 Effect of diet and ration on egg quality in terms of total lipid

Lu and Takeuchi (2004) noted that female tilapia *O. niloticus* appear to produce a constant egg composition (as diet quality and body reserves allow). Similar results were found in the present study, although the total lipid in eggs was higher than in the diets, which could be due to selective accumulation of lipid in eggs. The total

lipid levels were found similar in eggs originating from all diet groups at either full or half rations over three spawnings. Only the total lipid between one spawning was different. The reason for this difference is unknown but it could be due to an analytical error or the variation in biochemical composition of individual fish (see Table 4.1). A similar result was found by Craik and Harvey (1984b) in Atlantic salmon (*Salmo salar*) who demonstrated a significant variation of biochemical composition of eggs from the same broodstock, fed the same diet.

This study showed that different dietary lipid sources and also the ration size (3% and 1.5% BW) had no significant bearing on the total lipid quantity in the eggs. Therefore, palm oil could replace by fish oil in diets with no negative effect in terms of egg lipid quantity. This is in accordance with observations by Lu and Takeuchi (2004).

In general, fish fed diet 1 in this study had poor egg quality. However, the growth gain was lower and the mortality was high (see section **3.2.1**). These results agree with several authors Santiago and Reyes (1993), Kanazawa *et al.* (1980), Ng *et al.* (2001) and Ng (2004).

4.6.2 Effect of diet and ration on egg quality in terms of fatty acid composition

The fatty acid composition of eggs in this study fits the general freshwater species fatty acid profile, with saturated fatty acid (SFA), mainly palmitic acid (16:0) predominating, which is important as an energy source; and it is also structurally important in the sn-1 position of membrane phospholipids (Sargent, 1995) , setric acid (18:0) and, with range of carbon chain lengths C_{14} to C_{24} can be found in minor amounts. Oleic acid (18:1n-9) was the main monounsaturated fatty acid (monoene)

and confers fluidity to the membranes when inserted in the sn-1 position of phosphatidyl ethanolamine. It also supplies energy for embryos (Dey *et al.,* 1993) and is the most abundant onounsaturated fatty acid, followed by palmitoleic acid (16:1n-7) and the vaccenic acid (18:1n-7). This is the general pattern reported for the fatty acid compositions of the eggs from many fish species (Kaitaranta and Linko, 1984; Sargent, 1995; Sargent *et al.,* 2002; Tocher and Sargent, 1984a; Wiegand, 1996). In addition, linoleic acid (18:2n-6) is also an abundant fatty acid in tilapia eggs (Bell *et al.,* 1997; Bruce *et al.,* 1999; Linars and Henderson, 1991; Lu and Takeuchi, 2004). In the present study, the major fatty acids in eggs originating from Nile tilapia fed different dietary lipid sources at full or half ration over three spawnings were similar to those found the previous studies (Kaitaranta and Linko, 1984; Lu, 2003; Lu and Takeuchi, 2004; Sargent *et al.,* 2002).

No significant differences were observed in the fatty acid composition, particularly ARA, total n-6 PUFA, EPA, DHA, n-3 PUFA, and n-3/n-6 ratio in eggs from fish fed diets 2 , 3 and 4 at either full or half rations over their three spawnings, with the exception of the first spawning of diet 3 for n-6, the first spawning of diet 4 for DHA, and the first spawning of diet 4 for n-3 , at full and half rations. As these differences were only related to a few first spawnings, they could be due to an analytical error or due to the individual fish fatty acid variation associated with the first spawning in fish. Overall, the present study showed that the egg quality in terms of fatty acid composition was similar at either full or half ration. This is accordance with Lu and Takeuchi, (2004), Lu (2003) and Takeuchi *et al.* (2002).

Generally, it is accepted that freshwater fish require essentiality for 18:3n - 3 and 18:2n - 6 (see Figure 4.2), whereas marine fish require substantial levels of PUFAs

such as DHA and EPA and EPA and ARA (Bell *et al.,* 1997, 2002; Bruce *et al.,* 1999; Castell *et al.,* 2004; Henderson and Tocher, 1987; Linars and Henderson, 1991; Lu and Takeuchi, 2004; NRC, 1993; Santiago and Reyes, 1993; Sargent *et al.,* 2003; Tocher *et al.,* 2002). It is evident that *O. niloticus*, like all tilapiine fish, differs from other warm water fish with regard to fatty acid utilisation during food deprivation. This is possibly a reflection of the differences in the essential fatty acid requirements of the group, when compared with other warm water species in that it has a greater requirement for n - 6 fatty acids (De Silva *et al.,* 1997; Kanazawa *et al.,* 1980; Stickney and Hardy, 1989). The n - 6 fatty acid requirement in tilapias can be met with either 18:2n - 6 or 20:4n - 6 and both of these fatty acids were observed to be conserved during starvation. On the other hand, the requirement for n - 3 fatty acids in tilapias is known to be considerably lower than in other warm water fish (Stickney and Hardy, 1989). The overall n - 3 fatty acid requirement can be met from high molecular fatty acids such as 20:5n - 3 and 22:6n - 3. The tendency to conserve 22:6n - 3 in tilapia during food deprivation may be a reflection of this. As observed in studies of other freshwater species, this study noted that *O. niloticus* is able to synthesis DHA, EPA and ARA from linolenic and linoleic acids and accumulate it in the eggs. The present study also clearly indicates that the ARA in the eggs was high while in the diets, it was not detectable (Figure 4.15). On the other hand the EPA level in the eggs was almost constant despite the levels being higher in the diets. These results are in agreement with Pickova *et al.* (1999, 2006) except for DHA level which was contradictory to the results found by these authors. DHA level varied in eggs from fish fed different dietary lipid sources and was considerably influencing by dietary fatty acids (Figure 4.10). This could be due to the ability of Nile tilapia to preferentially convert DHA from linolenic acid.

Studies on the effect of dietary lipid sources on serial spawning of tilapia are limited. This study suggested that palm oil can be replaced by fish oil with no negative effect on egg quality in terms of fatty acid quantity of the eggs. These results are in accordance with several authors who reported that palm oil can be replaced by fish oil with no negative effect on growth performance (Al-Owafeir and Belal, 1996; Bell and Sargent, 2003; Kanazawa *et al.,* 1980; Legendre *et al.,* 1995; Ng *et al.,* 2000, 2003, 2004, 2006; Ochang *et al.,* 2007a; Ochang *et al.,* 2007b; Tortensen *et al.,* 2000; Varghese and Oommen, 2000).

4.6.3 Interactions between egg fatty acids of broodstock fed different dietary lipid and ration and their spawnings

To find out the interaction between diets, rations and spawning frequency data were analysed by three-way ANOVA. The results are summarised and the relevant data of diets, rations and spawnings (*e.g.* comparing the egg fatty acids from full ration group vs full ration, half ration vs full ratio, half ration vs half ration and half ration vs full ration between the diets over the three spawnings) were compared with each other.

The present study showed that overall there were no significant interactions between diets, rations and spawnings in fatty acid composition of eggs. Some significant interactions were found but the significance levels were inconsistent. The reason for these significant levels is unknown, but could be due to analytical error or the variation in fatty acid composition of individual fish.

4.6.3.1 Saturated fatty acids (SFA)

No interaction was found in SFA levels in eggs originating from fish fed the experimental diets (2, 3 and 4) at full or half ration over their three spawnings.

4.6.3.2 Monounsaturated fatty acids (MUFA)

There were no significant interactions were found in MUFA levels in eggs from fish fed diets 2, 3 and 4 at full ration vs half ration over their three spawnings. However, some significant differences were found between diets, rations and spawnings vs at full or half rations. These could be due to analytical error or the fatty acid variation of individual fish eggs. The details are shown in Appendix Table 7.1.

4.6.3.3 Arachidonic acid (ARA)

There were no significant differences in ARA content in eggs originating from fish fed a full ration of diet 2 vs full and half rations of diet 3 over their three successive spawnings, and similar results were obtained when comparing the ARA level of eggs from broodstock fed half ration of diet 2 vs those fed full and half rations of diet 3. Similar results were observed with ARA in eggs from fish fed a full ration of diet 2 vs a full ration of diet 4, and eggs originating from fish fed a full ration of diet 3 vs eggs from fish fed a full and half rations of diet 4. A significant diference was only found in eggs from fish fed diet 2 at half ration compared with those fed diet 4 at half ration over the three spawnings. Overall, the majority of fatty acids (mainly EPA DHA, n-3 PUFA and n-6 PUFA) should a similar trend to ARA between diets and rations and spawnings. Similar results were reported by several authors (Lu and Takeuchi, 2004; Lu, 2003; Takeuchi *et al.,* 1983, 2002; Sandnes, 1984; Craik, 1985; Harel *et al.,* 1994).

The results of the present study showed that egg quality, in terms of fatty acid composition, was not compromised as a result of using of palm oil in diets. The fatty acid profiles of tilapia eggs correspond to published data and the observed FA profile was similar to those recorded for tilapia eggs collected from different dietary regimes (El-Sayed *et al.,* 2005; Lu, 2003; Lu and Takeuchi, 2004; Takeuchi *et al.,* 1983, 2002).

4.6.3.4 Impact of diets on the fatty acid composition of eggs

Broodstock diets have a significant influence on the fatty acids of tissues in many species. A correlation has been demonstrated between the increase of C_{20} and C_{22} PUFAs in the tissues of *Mesodesma mactroids* and food abundance (De Moreno *et al.,* 1980), as well as in the mollusc, *Mytilus platensis* d'Orbigny (De Moreno *et al.,* 1980). Increases of 16:0 and 16:1 acids, which can be easily synthesised *de novo* by the mollusc, occur when food is less abundant (De Moreno *et al.,* 1980). Soudant *et al.* (1997) demonstrated that dietary fatty acids are one of the main nutrients, which significantly affect fish reproduction (specifically the fatty acid composition and the quality of eggs). The spawning of Nile tilapia increases when fed soybean oil rich in n-6 fatty acids (Soudant *et al.,* 1997). Some animals have developed a mechanism to retain the proportions of DHA, EPA and ARA in the lipids of reproductive organs, even under deficient conditions (Soudant *et al.,* 1997). The present study found a similar result with DHA and ARA which were higher in the eggs even they were either not detected or were lower in the diets.

Overall, the present study showed the fatty acid compositions of eggs from each diet group reflected from the levels found in the diets, *i.e.* higher monoene and lower PUFA in the diets, suggesting that dietary fatty acids were selectively incorporated

into eggs. Therefore, it was obvious that the differences of fatty acids in eggs for all diet groups were similar at either full or half rations. This concurs with previous findings (Bell *et al.,* 2002, 2003; Tveiten *et al.,* 2004), who demonstrated that, although dietary fatty acid correlated to FA deposited in flesh, specific FA were selectively utilised or retained in the flesh.

Although present, these differences are known less pronounced. However, immediately obvious are the high levels of DHA in the eggs regardless of the levels found in the diets (Figure 4.17). This selectivity for DHA meant that the difference observed between the DHA: EPA ratio in the diets (0.0, 1.3 and 1.1% for diets 2, 3 and 4, respectively) were increased in the eggs from fish fed the experimental diets. The significant differences in the AA: EPA ratio between the diets (0.0, 0.0 and 0.1 for diets 2, 3 and 4) were maintained in the resulting eggs (28.5, 19.7 and 10.8% for diets 2, 3 and 4, respectively. The high level of ARA in the eggs of *O. niloticus* was independent of levels found in the diets. The ARA in egg from fish fed diets 2, 3 and 4 contained 3.3, 2.3 and 2.1% which compared with a dietary supply of 0, 0 and 0.4% in the same diets. Overall, the present study showed that DHA, EPA and ARA content was high in the eggs while they were not detected or only at very low levels in the diets. However, the results show that, tilapia, in accordance with other freshwater fish, have the ability to convert linolenic acid (18:3n-3) into EPA and DHA and linoleic acid (18:2n-6) to ARA by the enzymatic sets outlined in Figure 4.3. This result agrees with (Bruce *et al.,* 1999; Furuita *et al.,* 2007). To summarise, the present study has shown that fish egg fatty acid composition reflects the fatty acid composition of the diets, although specific fatty acids were selectively utilised or retained in the eggs. In addition, there was no significant difference in total lipid

in the eggs between groups of fish fed experimental or control diets, either at full or half rations.

4.7 Conclusion

Dietary lipid source (palm oil) had no significant effect on egg quality in terms of lipid and fatty acid compositions. In conclusion, this study suggests that under controled conditions, palm oil can be used to substitute fish oil for tilapia feed. This alternative oil can be used to reduce the seed production cost.

Chapter 5 - Effect of dietary lipid sources on ovarian recrudescence and associated reproductive endocrinology in female *O. niloticus*

5.1 Introduction

According to Policansky (1983) and Mihelakakis (2001), optimal maturation should occur amongst fish who have access to abundant food and stable conditions for development. Similarly, the bioenergetic balance of farmed fish has been described by Brett and Groves (1979). These authors indicated that food consumed by farmed fish represents their sole source of energy, and that growth is the net outcome of a series of behavioural and physiological processes. These authors also noted that the net useful energy is used in part to maintain basal metabolism and the differences between net energy and that spent on the basal metabolism is surplus energy available for activity as well as somatic and gonadal growth.

Lipids provide two main functions during gonadal development in fish; firstly to meet the energy demands of reproduction, and secondly, to be deposited in the oocytes in the form of vitellogenin (VTG). These lipids are either derived from dietary fatty acids, mobilised from fatty acid reserves, or may be synthesised *de novo* (Chatzifotis *et al.,* 2004; Wiegand, 1996). There is a close correlation between the fatty acid composition of the broodstock diet and the egg, *e.g.* rainbow trout (*Oncorhynchus mykiss)* (Corraze *et al.,* 1993a; Henderson and Sargent, 1984), sea bass (*Dicentrarchus labrax*) (Bruce *et al.,* 1999) and halibut (*Hippoglossus hippoglossus*) (Sargent *et al.,* 1999). Henderson *et al.* (1984) studied changes in the lipid composition of capelin (*Mallotus villosus*) which accumulated 10 to 20 % of their body weight in the form of lipid during gonadal development. They also

determined that over 70% of the lipid content of the muscle was mobilised during ovarian recrudescence or rematuration, and of this, 38% was deposited in the ovary and the rest catabolised to provide metabolic energy for gonadal growth. Similar energy costs are associated with gonadal development in plaice (*Pleuronectes platessa*) and perch (*Perca fluviatilis*) (Wiegand, 1996). In Atlantic cod (*Gadus morhua*), several studies (Black and Love, 1986; Dahle *et al.,* 2003; Dambergs, 1964; Eliassen and Vahl, 1982; Jangaard *et al.,* 1967; Kjesbu, 1996; Lambert and Dutil, 1997) have indicated that liver lipids and muscle protein are the main available energy sources, and hepatosomatic index (HSI) is a good indicator of liver energy content (Lambert and Dutil, 1997). Furthermore, lipid stores affect age of puberty in Chinook salmon (*Oncorhynchus tshawytscha*) (Shearer and Swanson, 2000).

This chapter aims to investigate the effect of different dietary lipid sources and two rations on the dynamics of ovarian development and concurrent reproductive endocrinology in *Oreochromis niloticus.* Despite research focusing on tilapia reproductive biology (Bogomolnaya and Yaron, 1984; Coward, 1997; Coward and Bromage, 2000; Smith and Haley, 1987, 1988; Srisakultiew, 1993; Tacon *et al.,* 1996, 2000), little is known of the effect of nutritional status on reproductive endocrinology or association with oocyte growth in the mouth-breeding *O. niloticus*.

Under natural conditions (with the exception of equatorial stocks) tilapias are seasonal breeders (Hussein, 1984; Mckaye, 1984) and breeding seasons are timed so they coincide with optimal food availability for developing fry. In contrast, captive tilapia may spawn throughout the year as long as environmental conditions remain

suitable (Brummett, 1995; Fishelson, 1966; Lowe-McConnell, 1959). Spawning frequencies of multiple-spawning individual tilapia broodstock exhibit great variability according to several variables including fish size, temperature, degree of parental care, stocking density, sex ratio, food ration and dietary protein and lipid levels. The mechanisms controlling such variability in spawning frequency in tilapias remains unclear (Mires, 1982) and need to be investigated more thoroughly, preferably in controlled environmental conditions. As a consequence, broodstock spawning patterns often exhibit asynchrony; a significant problem for farm managers (Jalabert and Zohar, 1982).

Although the process of ovarian development is similar amongst teleosts, the duration required for each reproductive cycle shows species-specific patterns. Since tilapia species have evolved complex courtship and spawning behaviour, it seems likely that the length of a spawning cycle would not only reflect fish species and fish size but also take into account the degree of parental care (Srisakultiew, 1993). Mechanisms underlying such variability remain unclear and highlight the need for a greater understanding of the dynamics of ovarian growth and its endocrine control in such species. This is particularly important in the case of the mouth-breeding *O. niloticus,* where not only is research into ovarian growth lacking but where spawning cycles also exhibit great variability. Furthermore, detailed knowledge of the hormonal factors involved in the control of ovarian development and their secretion during different phases of ovarian growth may allow artificial manipulation of spawning cycles (for example, via the administration of exogenous hormones) and this in turn, could prove important in the management of farm stocks.

Three main patterns of oocyte development have been described in teleosts: synchronous (*e.g.* eels and Pacific salmon), group-synchronous (*e.g.* rainbow trout) and asynchronous (*e.g.* tilapia) (Billard, 1992; Billard and Breton, 1978; Pankhurst, 1998; Sun and Pankhurst, 2004; Wallace *et al.,* 1987; Wallace and Selman, 1981). Discrete stages of ovarian development are synchronised by an inter-related series of internal (endogenous) and external (exogenous) stimuli; exogenous factors such as photoperiod and food availability are perceived by the brain and stimulate various endocrine pathways to respond in an appropriate fashion (Dodd and Sumpter, 1984; Fontaine, 1976; Fontainhas-Fernandes *et al.,* 2000; Nagahama *et al.,* 1995; Weltzien *et al.,* 2004; Yaron *et al.,* 1983, 2001, 2003; Yaron, 1995; Young *et al.,* 1983). Modulation of spawning cycles in teleosts occur via changing levels of hormones from the "hypothalamus-pituitary-gonadal axis" (HPG) timed by exogenous cues from the environment (Bromage and Cumaranatunga, 1988) (see Figure 5.1). The fish brain receives external stimuli *e.g.* light or food (see Chapter 1 review) as translations to enhance endocrine response (Bromage *et al.,* 2001). In brief, major hormones involved include hypothalamic gonadotropin releasing hormone (GnRH), gonadotropin release inhibitory factor (GnRIH), pituitary gonadotropin(s) (GTH I and GTH II, whose functions are now known to be similar to those of follicle- stimulating hormone (FSH) and luteinising hormone (LH) in higher vertebrates respectively), sex steroids (*e.g.* 17β-oestradiol (E2), oestrone (E1) and testosterone (T)), progestagens and prostaglandins. Studies on hormones and their inter-related cycles serve as sensitive indicators in the determination of gonadal development.

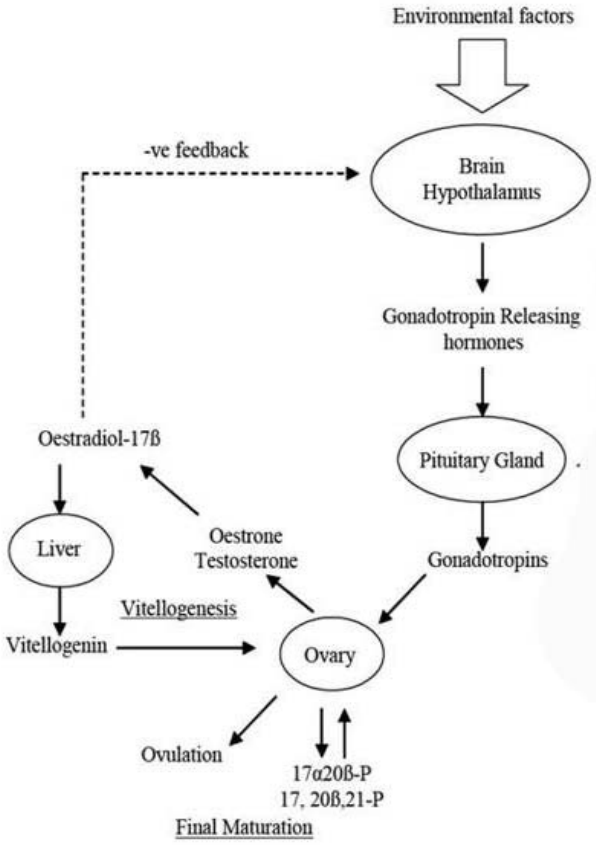


Figure 5.1 Outline of the neuroendocrine basis for the HPG axis in teleosts (Bromage, 1988).

Most studies on ovarian development and associated endocrine control have been undertaken in salmonid species *e.g.* (Breton *et al.,* 1972, 1983; Bromage and Cumaranatunga, 1988; Bromage *et al.,* 2001; Crim *et al.,* 1973; Nagahama, 1994; Nagahama *et al.,* 1995; Sumpter *et al.,* 1984; Swanson *et al.,* 2003). And, carp (*Cyprinus carpio*) (Billard and Breton, 1978; Santos *et al.,* 1986; Smith and Walker, 2004), goldfish (*Carassius auratus*) (Kobayashi *et al.,* 1986, 1988, 1989) and Atlantic cod (*Gadus morhua*) (Dahle *et al.,* 2003). However, relatively few studies have been conducted using tilapias (Bogomolnaya and Yaron, 1984; Coward and Bromage, 1998, 2001; Smith and Haley, 1988), and even fewer on commercially important species such as *O. niloticus*.

Studies on carp, goldfish and various salmonids have shown that, in general, levels of E2 and E1 increase during the early part of the reproductive cycle, peak during the most active period of vitellogenesis and then fall, remaining at low levels until the next spawning phase (Bromage *et al.,* 1982; Bromage and Cumaranatunga, 1988; Coward, 1997; Elliott *et al.,* 1984; Kobayashi *et al.,* 1986, 1988; Norberg and Björnsson, 1989; Weltzien *et al.,* 2004; Whitehead *et al.,* 1983). Although the primary role of oestrogens (E1 and E2) is in the stimulation of VTG from the liver, a possible role in the metabolism of fat was suggested by Bromage and Cumaranatunga (1988).

5.1.1 Role of steroid hormones in vitellogenesis and ovarian recruitment

In ovaries, two somatic cell layers of the follicle appear to play separate roles in steroid biosynthesis (Devlin and Nagahama, 2002; Hoar and Nagahama, 1978; Nagahama *et al.,* 1982). The ovarian thecal layer produce testosterone and other precursor androgens, whereas the granulosa layer does not synthesise steroids *de novo*, but is capable of converting testosterone to oestrogen via the enzyme aromatase (Nagahama, 1997) (see Figure 5.2). However, sex steroid hormones are important regulators of sexual maturation; 17β –oestradiol (E2) controls synthesis of vitellogenin (Mommsen and Walsh, 1988; Tyler *et al.,* 2000). A series of studies revealed that during the vitellogenic phase, the steroidogenic steps, beginning with cholesterol side-chain cleavage and culminating in the formation of androgens (mainly testosterone), take place in the theca layer cells and are controlled by gonadotropin. Testosterone diffuses into the granulosa to be aromatised to oestradiol- 17β (Arukwe, 2006; Kagawa *et al.,* 1982; Nagahama *et al.,* 1996) (Figure 5.1). A similar two-cell model was described for oocyte final maturation.

All steroidogenic steps culminating in the formation of 17α-hydroxyprogesterone occur in the thecal cells; this steroid diffuses into granulosa cells to be converted into 17α, 20β -dihydroxy-4-pregnen-3-One (17, 20-P), and then to the maturation-inducing hormone (MIH) in many fish species (Mita *et al.,* 1987; Redding and Patiño, 2006; Young *et al.,* 2005).

In the ovarian follicles of mature females, testosterone (T) is produced in the theca layer under stimulation by gonadotropin-I (GtH- I), and is then converted to E2 in the presence of aromatase in the granulosa layer (Figure 5.2). Testosterone (T) increases during the early part of the cycle (along with E1 and E2) but peaks after the oestrogens. However, the function of T in ovarian growth and maturation remains unclear (Nagahama, 1984, 1994; Nagahama *et al.,* 1996; Nagahama and Yamashita, 2008). In goldfish (*Carassius auratus*), T peaks at ovulation (Kobayashi *et al.,* 1986; Kobayashi *et al.,* 1988; Kobayashi *et al.,* 1989) and in rainbow trout (*oncorhyncu mykiss*) peaks approximately 8 days prior to ovulation and falls thereafter (Scott and Sumpter, 1983).

In the multiple-spawning Japanese sardine (*Sardinops melanositicus*), E2 was found to be relatively high during vitellogenesis but decreased during final oocyte maturation. Oestradiol-17β, however, rose immediately after spawning (Matsuyama *et al.,* 1988; Matsuyama *et al.,* 1991). It was also found at high levels during vitellogenesis in other multiple-spawners *e.g.* sea bass (*Dicentrarchus labrax*) (Matsuyama *et al.,* 1991; Prat *et al.,* 1990). In the sea bass (*Dicentrarchus labrax*), plasma VTG and the number of vitellogenic oocytes both peaked in December at a time when E2 levels attained high values. During the spawning period (January to March), the number of vitellogenic oocytes and plasma E2 remained elevated,

though plasma VTG levels fell by approximately one half (Prat *et al.,* 1990). A similar scenario was observed in the spotted sea trout(*Cynoscion nebulosus*), which begins spawning in late spring, with each individual spawning several times during a two month period (Prat *et al.,* 1996). Prior to the spawning period, levels of plasma E2, T and VTG are elevated but decline during the spawning (Matsuyama *et al.,* 1988, 1991; Prat *et al.,* 1996).

In the rainbow trout (*Oncorhynchus mykiss)*, GTH I was shown to increase during early vitellogenesis, fall to a basal level shortly before ovulation then rise once again at ovulation. GTH II however, remained undetectable throughout most of the reproductive cycle, but rose immediately preceding ovulation (Nagahama and Yamashita, 2008; Prat *et al.,* 1996). Similarly, in the spotted sea trout final maturation and ovulation was also initiated by a surge in GTH I and GTH II where they appeared to prime follicle-enclosed oocytes so that they become competent and underwent final maturation in response to the maturation-inducing steroid (MIS) (Nagahama and Yamashita, 2008; Prat *et al.,* 1996).

Several other sex steroids known as prostaglandins increase just before ovulation; dihydrogestrone 17α-hydroprogestrone (17αOH-P) and 17α-hydroxy-20β-dihydroprogestrone (17αβ-P also known as 17α-hydroxy-4-pregnen-3-one) are thought to be the most important (Young *et al.,* 2005). In rainbow trout (*Oncorhynchus mykiss)*, levels of 17αβ-P peaked sharply just prior to ovulation (Scott *et al.,* 1990; Scott and Sumpter, 1983). 17αβ-P was found at very low levels throughout ovarian growth in carp (*Cyprinus carpio*) (Rothbard and Yaron, 1992).

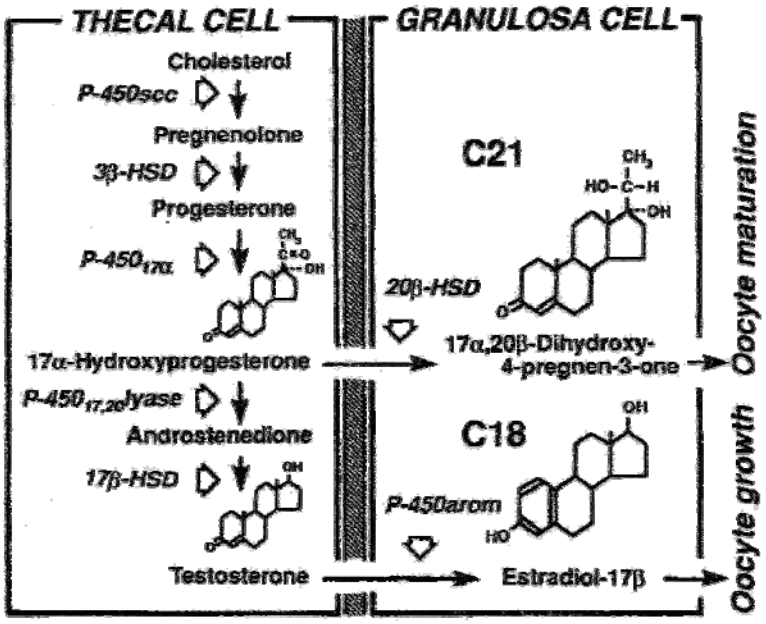


Figure 5.2 Pathway of steroid biosynthesis in the ovarian follicle of salmonids during oocyte growth and maturation, showing the relative contribution of thecal and granulosa cell layers in the production of estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP). P-450scc, cholesterol side-chain cleavage cytochrome P-450: 3 β-HSD, 17 β-hydroxysteroid dehydrogenase-isomerase; P-450$_{17\alpha}$, P-450 17α-hydroxylase; P-450 $_{17,20}$ lyase, P-450 17,20 lyase; 17 β-HSD, 17 β-hydroxy-steroid deyhdrogenase; P-450arom, aromatase cytochrome P-450; 20β-HSD, 20β-hydroxysteroid dehydrogenase. Source (Nagahama *et al.*, 1996)

In multiple-spawning tilapia, the profiles of reproductive hormones are not as well defined as those in salmonid species such as rainbow trout (*Oncorhynchus mykiss)*. The profile of E2 and T during the spawning cycle of tilapia remains unclear and, unlike salmonids, can show several peaks prior to spawning (Smith and Haley, 1988). Early work in *O. mossambicus* found increasing levels of E2 coincided with an increasing gonadosomatic index (GSI). When E2 levels fell, progesterone levels rose (Cornish *et al.,* 1997; Cornish, 1998). In *O. niloticus*, Rothbard *et al.* (1991) monitored hormonal profiles throughout a 7-day phase of the breeding cycle (from quiescence through courtship and eventually to mouth-brooding). In their study testosterone and specifically E2 increased gradually during acquisition of nuptial colouring and pairing but decreased during spawning and mouth-brooding. The

peak in GTH was found to occur after peaks of E2 and T had receded, suggesting an association between GTH and final oocyte maturation. In individually cycling *O. niloticus*, levels of E2 were found to increase from day 1 after spawning, peak on day 5 and fall on day 10, suggesting that vitellogenesis occurred at day 5 but was completed by day 10 post-spawning. This is concomitant with maximum proportions of stage 6 (late vitellogenic) oocytes. On the basis of these observations, Srisakultiew (1993) suggested that the *O. niloticus* ovary is fully mature just 10 days after spawning.

A major disparity between the life history and strategies of salmonids and tilapia species is the extent of parental behaviour extended to eggs and young. Tilapia expend considerable amounts of energy in parental care, especially in the case of mouth-brooding species. Parental care strategies have been shown to influence levels of sex steroids in tilapia. In a study of *O. mossambicus*, Smith and Haley (1988) found differences in the hormonal profiles of mouth-brooding and non mouth-brooding fish. The authors suggested that the high level of T in mouth-brooding females may serve to retard oocyte growth until brooding ceases and that high E2 levels may serve in the protection of oocytes from atresia.

Rana (1988) also reported that parental care in mouth-brooding tilapia after spawning may affect the dynamics of ovarian development in the ensuing reproductive cycle. In non-mouthbrooding *O. mossambicus* ovarian development occurred over a longer period than in mouthbrooders (Smith and Haley, 1987). It is clear therefore that the pattern of ovarian development in tilapia and its associated endocrine control is not as well defined as in other teleosts such as rainbow trout or carp. Levels of circulating reproductive hormones not only depend upon the phase

of ovarian development but also reflect behavioural phases such as courtship or parental care. It is known that ovarian dynamics, growth and associated hormonal profiles in mouth-brooding tilapia depending on their their food status. A detailed knowledge of this area of physiology may allow artificial manipulation of the spawning cycle in these fish *e.g.* via the administration of exogenous hormones (Jalabert and Zohar, 1982).

5.1.2 Plasma calcium as precursor of vitellogenin

Calcium ions are incorporated into the post-translational modification of the vitellogenin molecule prior to its release from the liver (Tata, 1978). As a result, serum calcium levels rise during vitellogenesis; an increase that is directly correlated to blood vitellogenin concentration (Elliott *et al.,* 1984). Calcium ions have been found to be incorporated into the post-translational modification of the vitellogenin molecule prior to its release from the liver (Tata, 1978). Moreover, studies have shown plasma calcium levels rise during exogenous vitellogenesis (de Vlaming, 1983; Elliott *et al.,* 1984); a rise shown to be directly correlated to blood vitellogenin concentration (Elliott *et al.,* 1984). Thus, plasma calcium analysis has been widely adopted as an indicator of serum VTG secretion (Elliott *et al.,* 1984). This is primarily due to the strong correlation between serum calcium levels and serum VTG levels and the ease with which the former can be measured.

5.1.3 Aims

The aims of this study were to:

- Investigate the effect of dietary lipid sources on oocyte recruitment.
- Investigate the effect of feed ration size on oocyte recruitment.

- Determine the effect of dietary lipid source on sex steroid hormones during oocyte recruitment.

5.2 Materials and methods

5.2.1 Fish Supply

Fifty-six female *O. niloticus* were taken from broodstock reared under experimental conditions at the Tropical Aquarium, Institute of Aquaculture, University of Stirling. The broodstock were fed exclusively on experimental diets and solely according to their specific feeding regime throughout their entire life cycle from the onset of exogenous feeding to the termination of this study.

5.2.2 Diets and rations

In the present study, fish were fed their respective diets and rations according to the experimental design (see section 3.2.2).

5.2.3 Experimental system

Female fish from the previous experiment (spawning periodicity, egg and larval quality, see Chapter 3) were left undisrupted in the same glass aquarium until spawning activity occur occurred; spawning was allowed to proceed until the egg clutch was seen in the mouth. Immediately after completion of spawning, post-spawned fish were removed from their respective aquaria, anaesthetised, weighed, measured and blood sampled (section **2.17**). Plasma was obtained by centrifugation (section **2.17**). Blood samples taken from fish immediately after spawning were referred to as day 0 post-spawning. Fish were sacrificed and weighed (to the nearest 0.01g) and gonad and liver were dissected and weighed (to the nearest 0.001 g), to

determine the GSI and HSI respectively (section **2.7**). Small transverse sections from mid-way along, the ovary were taken and fixed in Bouin's fluid, embedded in wax, sectioned at 5µm and stained in haematoxylin and eosin as detailed in sections **2.8** and **2.8.1**. The volume fraction of each stage of oocyte was categorised according to the classification scheme of Bromage and Cumaranatunga (1988), Coward and Bromage (1998) and Srisakultiew (1993). Blood, gonad and liver samples were taken from each fish on days 3 and 6 days post-spawning from each treatment. Plasma E2 and T levels were measured by radioimmunoassay (RIA) according to the method of Duston and Bromage (1987) as detailed in section 2.23. Total plasma calcium was measured using an atomic absorption spectrophotometer as detailed in section 2.22. Analyses of endocrine control of ovarian growth were by measurement of the sex steroids E2 and T according to the procedure detailed in section 2.23.

5.2.4 Analyses of ovarian histology by stereology and estimation of ovarian volume fraction

The present study use stereology to derive the quantitative parameters from histologically prepared ovarian tissue; known as ovarian volume fraction. Stereology is a three-dimensional reconstruction of a two-dimensional histological slide to estimate volume fraction of developed stage of oocytes (Coward and Bromage, 2002; Newman *et al.,* 2007; Weibel, 1979; Weibel and Gomez, 1962). Stereological techniques developed to date are based upon the 'Delesse Principle' (Delesse, 1847). Stereology systems require that a test system (*e.g.* grids, points, lines) be placed over the photograph of a section cut through a specimen with a view to estimating geometrical parameters such as volume, surface area, length and total curvature. According to this, a cuboial structure is placed into an x, y and z

coordinate system and sliced parallel to the x, z plane into thin slices of thickness d_y.

Total sectional area (Ast) of each histological slide and sectional object area (Aso) of interest thus contains a certain volume of sectional object (Vso) and the total volume of section (Vst) which must be equal to the profile area multiplied with the slice thickness as demonstrated in equations 1 and 2 below:

$$V_{so} = A_{so} \times d_y \quad (1)$$

$$V_{st} = A_{st} \times d_y \quad (2)$$

By taking the sum of the total section volume (V_{st}) and total sectional object volume (V_{so}) one can obtain the total volume of the cuboid and object. Total object ($\sum V_{so}$) is then divided by total section volume ($\sum$Vst) (see equation 3) to calculate volume density.

$$\frac{\sum V_{vo}}{\sum V_{st}} = \frac{V_{so}}{V_{st}} = V_{vo} \quad (3)$$

By replacing V_{st} by the products of area multiplied by thickness (equation (1) and (2) one can perform the following calculation:

$$\frac{\sum A_{so} \times dy}{\sum A_{st} \times dy} = \frac{(dy \sum A_{so}}{(dy \sum A_{st})} = \frac{A_{so}}{A_{st}} = A_{ao} \quad (4)$$

Since slide thickness is constant it can be cancelled from the calculations. The ratio of the sum of profile areas [total sectional area (A_{st}) and Sectional object area (A_{so})] is the area density of profiles on the section A_{ao} . It is also apparent that equations (3) and (4) are equal to each other and thus it true to say that:

$$V_{vo} = A_{ao} \quad (5)$$

A_{ao} can be estimated by the use of the ratio of counted (P_{po}) whereby a random grid point procedure is utilised to measure the area fraction occupied by objects of interest (DPS_o) within a total sample area (DPS_t) of histological section such that:

$$P_{po} = \frac{P_{so}}{P_{st}} \quad (6)$$

Finally, it can thus be concluded that:

$$V_{vo} = A_{ao} = P_p o \quad (7)$$

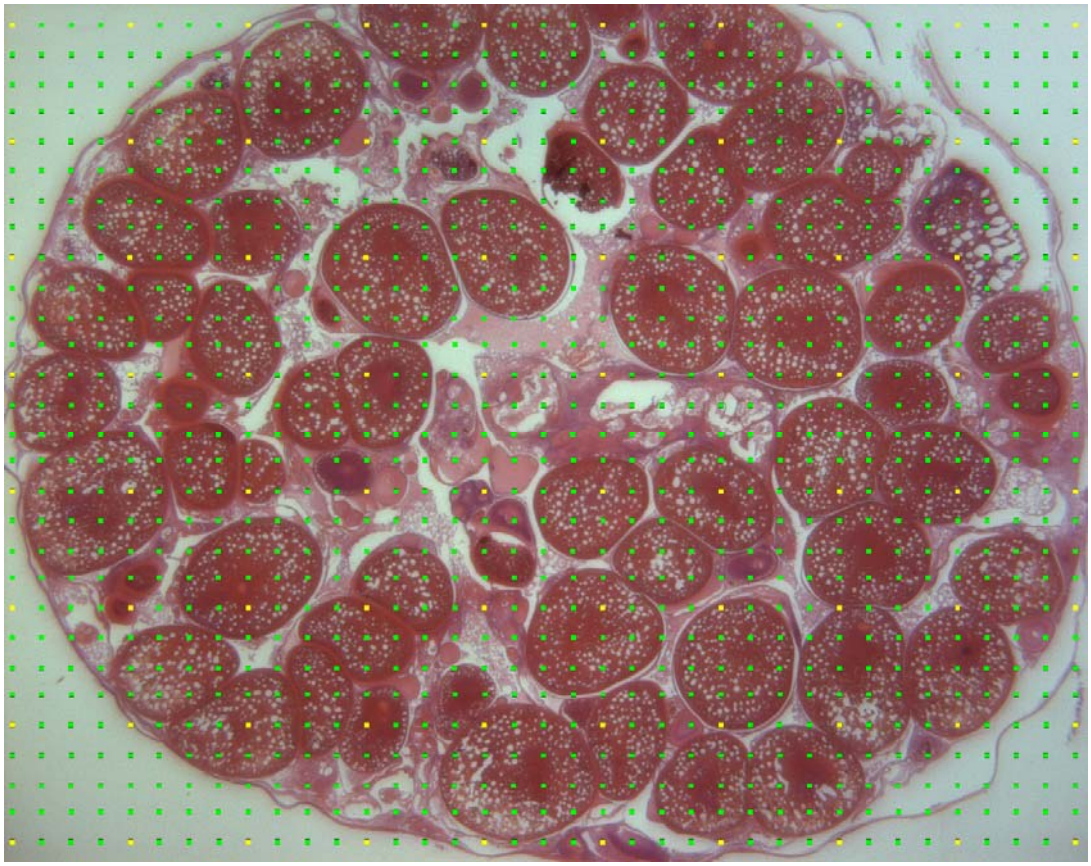
This means that the 3-dimensional volume occupied by features in tissue can be estimated accurately from their area in 2-dimentional slices and that this can in turn be estimated by counting the number of intersections of each object of interest with points on a grid overlying the tissue slices. This method can therefore be applied to quantify the numbers or proportions of different oocyte developmental stages in histological sections prepared from experimental fish ovaries.

5.2.5 Calculation of ovarian volume fraction from histologically prepared ovarian tissue.

A histological estimation technique incorporating the point-counting methodology was used to measure the oocyte area occupied by stage "X" oocytes (Asx) on histological preparations of the ovaries from experimental animals. Stage "X" is classified as stage 2, 3, 4, 5, 6/7, atretic or post-ovulatory according to the oocyte stage classification of *O. niloticus* (Srisakultiew, 1993) and *T. zillii* (Coward and Bromage, 1999b).

Histological sections were prepared as detailed in section **2.9**. Four sections were cut from each ovarian sample and stained in either H&E (2.10.1) or polychrome stains (section 2.10.2). Slides were examined under an Olympus BH-2 binocular microscope (Olympus Optical Co. Ltd, U.K.) connected to a Zeiss AxioCam MRc digital camera and images captured using Zeiss Axiovision software (see section **2.21**). The total area of a single captured field was calculated using a calibrated 1mm slide graticule (x1 objective lens and x 2.5 mm trinocular lens insert).

Because the oocyte stages displayed a wide range of sizes, it was difficult to produce a grid with a sampling interval (distance between sampling points) that was appropriate for all oocytes. Thus an attempt was made to use a so-called "double square lattice test system", which employs two sampling scales in a single grid. The grid employed (shown in Figure 5.3) was based on the D64 test system (Weibel, 1979), which incorporates a lattice ratio (q^2) of 16. This grid was scaled to cover the captured ovary images (1300x1030 pixels), with small and large sampling intervals scaled such that the square of the distance between points (for large distance = D^2 and small distance = d^2) was greater than the mean largest or smallest oocyte stage sectional area for large and small inter-point distances, respectively. This lattice had a total of 1073 small distance (d) points and 80 large distance (D) points. The frame width was 8D and height was 9D.



Total number of grid points on the field =893

Ovary volume fractions (V_{vx}) calculated as follows:

V_v stage 2 oocytes = 38/893×100= 4.3%

V_v stage 3 oocytes = 32/893×100=3.6%

V_v stage 4 oocytes = 18/893×100=1.8%

V_v atretic oocytes = 95/893×100= 10.6%

V_v stage 6/7 oocytes = 578/893×100= 64.7%

V_v atretic oocytes = 0/893×100= 0%

Figure 5.3 Demonstration of the estimation of volume fraction (V_{vx}) of various oocyte developmental stages in a unit volume of *O. niloticus* ovary. Note that for demonstration purposes only, the total number of grid points in the field in the above figure is 893.

Image processing and grid overlay were carried out using a dedicated macro written for the Zeiss KS300 image analysis system, which also allowed counting of the number of points (large and small distance) overlying the ovary section in a given field (since ovaries differed in size, they did not always fill the full image field at the selected magnification). From the image with a grid overlay, the number of

points overlaying each of the different oocyte stages present in a given ovary section was directly counted by the researcher. Whilst it was found that the small sampling distances gave appropriate estimates of the area occupied for smaller stage oocytes, the larger distances gave too few samples of large stage oocytes per ovary section and thus inaccurately estimated occupied area. Thus for all oocyte stages, only small distance points were finally employed, this giving reasonable estimates of all oocyte stages present.

The mean fraction (volume fraction) of stage "X" oocytes ($A_{ax}=V_{vx}$) on each of four histological sections was calculated as detailed below:

Firstly captured images of ovary sections were processed as follows:

1) The field image containing the ovary was segmented to outline the ovary section only.

2) Objects smaller than the ovary (image noise) were removed from the image.

3) Holes in the segmented ovary image were filled to give a complete tissue area.

4) The ovary image was overlaid with the sampling grid.

5) The ovary image was used to "cookie cut" the grid image in order to leave only grid points which overlaid the ovary directly.

6) The total number of points overlaid by the entire ovary section (Ats) was automatically counted by the image analysis software.

7) The number of points overlying each oocyte stage in a given section (Asx) was then counted manually by the researcher from saved colour field images with overlaid grids.

The area fraction occupied by each stage of ovarian development was computed as detailed in equation (8):

Aaxp= volume fraction (vv) of stage x oocytes.

$$A_{axp} = \frac{A_{sx}}{A_{ts}} \qquad (8)$$

A_{axp} = area fraction of oocyte stage x

A_{sx}= area occupied with number of points in stage x

A_{ts}= total point on the monitor

Oocyte volume fractions were transformed using an arcsine transformation prior to statistical analysis.

Data were analysed by three-way ANOVA followed by Bonferroni/Dunn multiple comparisons test where appropriate, using either SPSS V15 for Microsoft Windows or Minitab version 15. Total plasma calcium and volume fraction data were arcsine transformed where necessary. One-way ANOVA was then used to compare the volume fractions calculated for each oocyte stage between diets, rations and days post-spawning.

5.3 Results

A total of 56 females were used for all diet treatments at full and half ration for this experiment. In diet 1 containing cod liver oil (CO) only one fish was sampled due to high mortality and poor egg quality from the previous experiment.Due to insufficient data, this fish fed was discarded from the analysis. The analysis was run using diet 2 containing palm oil (PO), diet 3 containing palm cod liver oil 9:1 ratio (PO & CO) and diet 4, a commercial trout feed (Skretting UK), containing fish meal and fish oil.

5.3.1 Profile of E2, T, total calcium and E2/T ratio

Mean E2, T, total calcium and E2/T ratio were analysed by GLM three-way ANOVA using SPSS. Data is shown in Table 5.2. Three or two-way ANOVA showed that no significant interactions were observed between diet, ration and day post-spawning on the E2 profile. However, one-way ANOVA showed that the mean

E2 level was significantly different ($P<0.05$) according to day post-spawning for all treatments (Table 5.2). The mean E2 (refer to Figure 5.4) rose from 12.6 to 44.2 ng/ml for day 0 and day 6 post-spawning. Plasma E2 level showed significant increases ($P<0.05$) over successive time sampling points; 12.6 (day 0) 35.6 (day 3) 44.2 ng/ml (day 6) post-spawning.

Table 5.1 Effect of dietary lipid source and ration size on the profile of steroid hormones and plasma calcium at 0, 3 and 6 days post-spawning in *O. niloticus.* Values are given as mean ± SEM.

Treatments			Parameters					
Diets	Rations	DPS	Calcium (mg%)	Oestradiol (ng/ml)	Testosterone (ng/ml)	E2/T	HIS (%)	GSI (%)
2 (PO)	Full ration	0	23.5±1.0	14,2±3.6	5.0±1.8	3.6±0.7	2.0±0.2	4.3±3.0
		3	25.5±2.6	29.1±12.4	13.4±0.3	2.2±1.0	1.8±0.2	2.4±0.1
		6	44.0±1.9	52.7±14.3	26.7±6.1	2±0.1	1.8±0.3	3.6±0.6
	Half ration	0	23.8±0.6	10.5±2.8	2.9±1.1	4.o±0.5	1.9±0.1	2.6±0.4
		3	29.9±2.8	45.1±19.3	14.7±0.6	3.1±1.4	1.9±0.1	2.8±0.2
		6	44.0±1.7	47.7±3.5	28.1±8.2	2.1±0.4	2.1±0.2	4.2±0.4
3 (P&CO)	Full ration	0	21.9±2.0	9.0±3.6	4.1±3.4	4.8±3.1	1.6±1.0	2.4±0.2
		3	27.2±3.3	38.2±10.0	14.9±1.6	2.5±0.4	2.5±0.2	3.2±0.7
		6	44.5±3.1	40.6±3.9	31.5±1.1	1.3±0.2	2.3±1.0	8.3±2.0
	Half ration	0	21.7±1.4	6.5±0.6	2.4±1.0	4.3±1.2	1.8±0.1	3.0±0.6
		3	22.5±1.2	25.7±4.6	10.5±2.2	2.5±0.2	2.0±0.2	4.6±2.6
		6	45.9±1.5	43.3±4.9	35.2±5.3	1.3±0.1	2.2±0.2	6.7±1.1
4 (control)	Full ration	0	19.1±1.8	10.1±4.6	1.1±0.4	10.0±3.5	2.1±0.1	2.3±0.5
		3	27.0±0.8	31.2±7.3	13.3±5.1	2.6±0.3	2.6±0.5	3.7±0.6
		6	44.9±0.6	46.1±5.5	61.3±8.5	0.8±0.2	2.9±0.8	4.2±0.8
	Half ration	0	21.0±2.4	27.9±4.3	10.2±1.4	2.7±0.1	3.1±0.1	3.6±0.4
		3	24.4±0.1	41.9±0.5	17.7±0.5	2.4±0.1	2.0±0.1	2.4±0.1
		6	45.5±0.7	38.3±6.4	57.1±3.9	0.7±0.1	2.5±0.2	4.3±0.8

DPS= day post-spawnining

Table 5.2 Significant values for GLM analysis of effect of dietary lipid sources and rations on the profile of steroid hormones and plasma calcium at 0, 3 and 6 days post-spawning in *O. niloticus*

Source of variation	d.f.	Calcium (mg %)	Oestradiol (E2) (ng/ml)	Testosterone (T) (ng/ml)	E2/T ratio	HSI	GSI
Diet levels	2	0.354	0.412	0.0001*	0.827	0.0001*	0.183
Ration levels	1	0.879	0.663	0.691	0.211	0.834	0.964
Day post-spawning (DPS)	2	0.0001*	0.0001*	0.0001*	0.0001*	0.229	0.014*
Diet × Ration	2	0.449	0.544	0.741	0.102	0.704	0.973
Diet × DPS	2	0.335	0.786	0.0001*	0.255	0.142	0.222
Ration × DPS	4	0.713	0.668	0.944	0.120	0.058	0.950
Diet × Ration × DPS	4	0.287	0.369	0.516	0.153	0.059	0.562

*P value significant at 0.05 level.

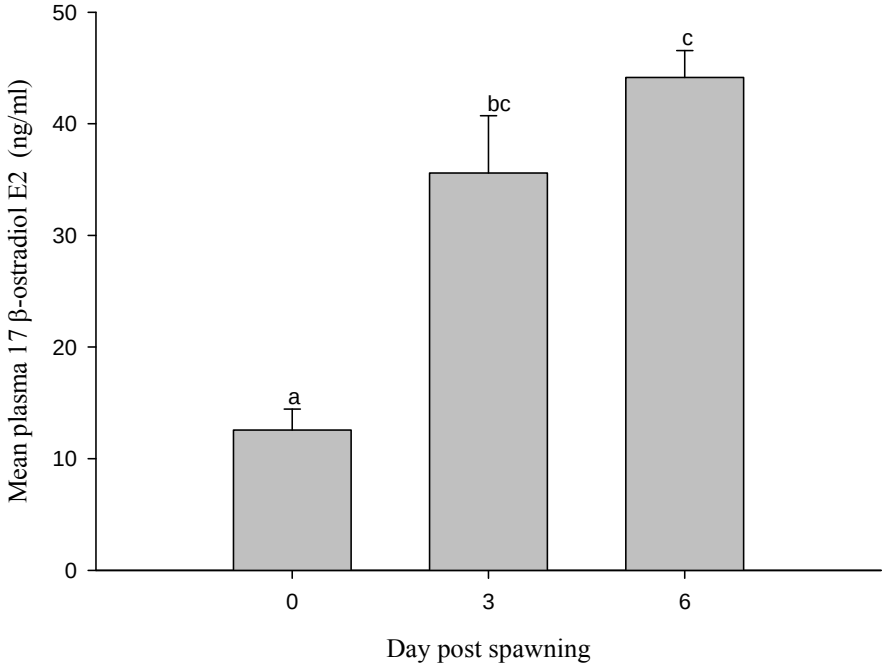


Figure 5.4 Mean E2 (ng/ml) profile in *O. niloticus* over 0, 3 and 6 days after spawning (day 0=day of spawning). Non significant data from diet and ration were pooled. Data between columns with different letters are significantly different ($P<0.05$, n=2).

5.3.2 Total calcium

Mean total plasma calcium was analysed between diets, rations and days post-spawning (Table 5.2). There were no significant interactions ($P>0.05$) for total plasma calcium of fish fed different diets or rations over the 3 sampling points (Table 5.2). One-way ANOVA revealed that a significant difference ($P<0.05$) only occurred on day post-spawning of entire treatments. The highest levels of calcium were detected at day 6 (44.9±0.57 mg %) and the lowest level at day 0 (22.9±0.63 mg %) post-spawning (Figure 5.5).

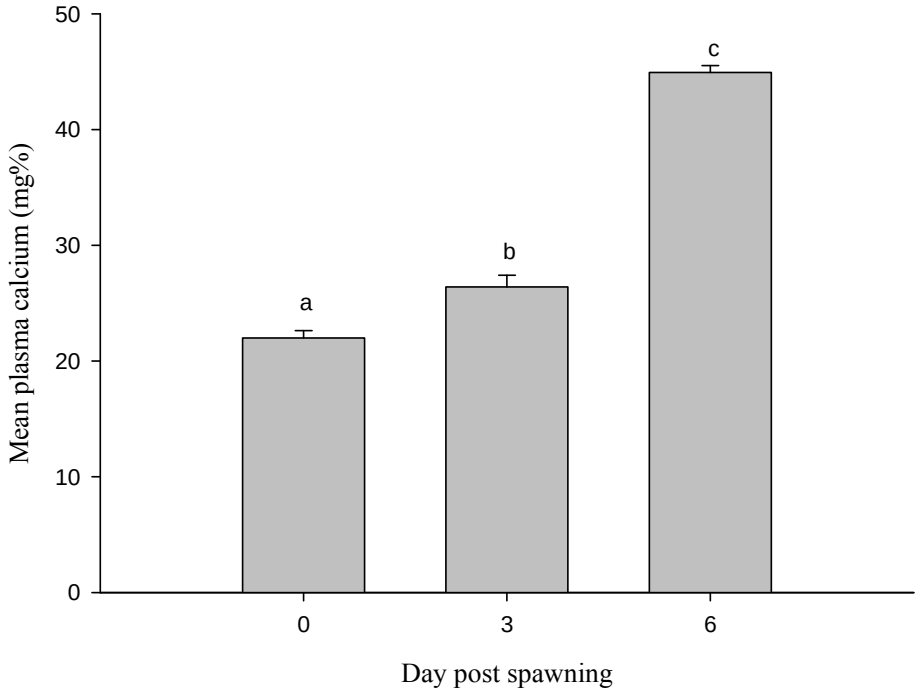


Figure 5.5 Mean plasma calcium levels in *O. niloticus* over 0, 3 and 6 days after spawning (day 0=day of spawning). Data between columns with different letters are significantly different (P<0.05, n=2). Non significant data from diets and rations were pooled.

5.3.3 Testosterone

Three-way ANOVA showed that there were no significant interactions (P>0.05) in mean testosterone levels of females fed diets 2, 3 and 4 at full or half ration over three post-spawning sampling points. However, a significant interaction (P<0.05) was found between diets and days post-spawning by two-way ANOVA (see Table 5.2). To find the differences in T level between diets and days post-spawning, data were analysed using Minitab with the results shown in Table 5.3. There were significant differences in T level between the diets for each day sampling period as well as between days post-spawning (Figure 5.6). Immediately after spawning the T level was low (3.9±1.03, 2.9±1.04 and 5.6±2.1 ng/ml) for fish fed diets 2, 3 and 4,

respectively, on day 0 post-spawning. Plasma T levels then rose significantly ($P<0.05$) to peak on day 6 after spawning (27.7±5.4, 34.1±3.7 and 58.5±3.4 ng/ml) for fish fed diets 2, 3 and 4, respectively.

Table 5.3 Interactions between diet and day post-spawning on plasma testosterone (T) of *O. niloticus*. The diets are: diet 2 containing palm oil; diet 3 containing palm and cod liver oil (9:1); and diet 4 a control diet. The diet days-post spawning (Diet DPS) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) differences in plasma T levels.

		Diets	2	2	2	3	3	3	4	4	4
		DPS	0	3	6	0	3	6	0	3	6
Diets	DPS										
2	0		x								
2	3		0.2878 (ns)	x							
2	6		0.0001(s)	0.1065 (ns)	x						
3	0		1 (ns)	0.2986 (ns)	0.0001 (s)	x		`			
3	3		0.5 181 (ns)	1 (ns)	0.0657 (ns)	0.5113 (ns)	x				
3	6		0.0001 (s)	0.0029 (s)	0.9145 (ns)	0.0001 (s)	0.0018 (s)	x			
4	0		1 (ns)	0.615 (ns)	0.0005 (s)	0.9997 (ns)	0.8246 (ns)	0.0001 (s)	x		
4	3		0.1743 (ns)	1 (ns)	0.2376 (ns)	0.1865 (ns)	0.9995 (ns)	0.0103 (s)	0.4381 (ns)	x	
4	6		0.0001 (s)	0.00001(s)	0.0001 (s)	0.0001 (s)	0.0001 (s)	0.0001 (s)	0.0001 (s)	0.0001 (s)	x

*P value significant at 0.05 level.

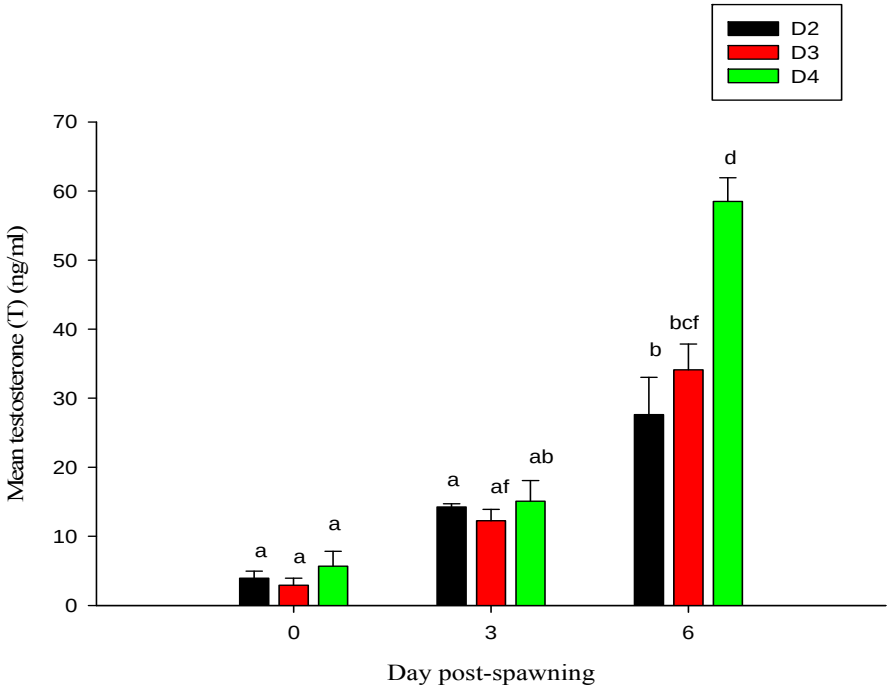


Figure 5.6 Comparison of plasma testosterone of *O. niloticus* fed different dietary lipid sources (D2 palm oil , D3 palm & cod liver oil 9:1 ratio and D4 control commercial trout diet) over 3 time periods after spawning (day 0=day of spawning). Each column different letters denote significant differences ($P<0.05$). Data are means± SEM, (n=2).

5.3.4 E2/T ratio

There were no significant interactions for E2/T ratios of fish fed different diets or rations with respect to day post-spawning for all treatments. However, a significant difference ($P<0.05$) was observed by one-way ANOVA in E2/T profile on days post-spawning for all treatments. The mean plasma E2/T ratio was found to be highest immediately after spawning (day 0) and fell steadily thereafter until day 6 (Figure 5.7).

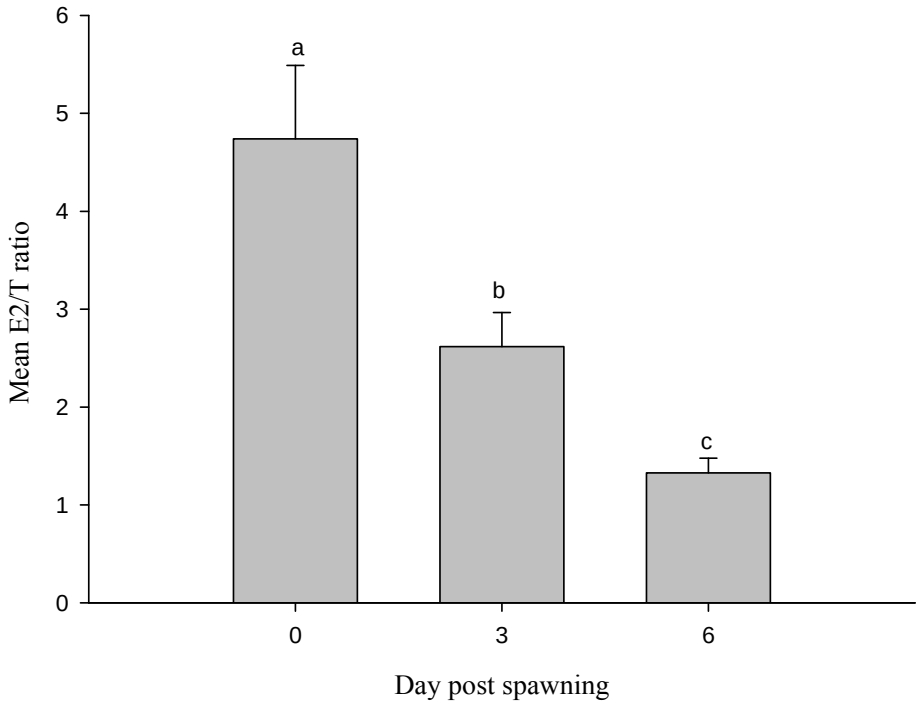


Figure 5.7 Mean 17β-oestradiol/testosterone ratio profile in tilapia over 0, 3 and 6 days after spawning (day 0=day of spawning). Data between columns with different letters are significantly different (P<0.05, n=2). Non significant data from diet and ration were pooled.

5.3.5 Gonadosomatic index (GSI)

No significant interactions were found for GSI in diets, ration or day post-spawning (Table 5.2). However, a significant difference ($p<0.05$) occurred only for days post-spawning for entire treatments by one-way ANOVA. Mean GSI immediately after spawning was 3.1± 0.56% and rose steadily to 5.2 ± 0.5% by day 6. Mean GSI increased significantly between time sampling points from days 0 and 6 post-spawning but between days 0 and 3 was not significantly different ($p>0.05$) (Figure 5.8).

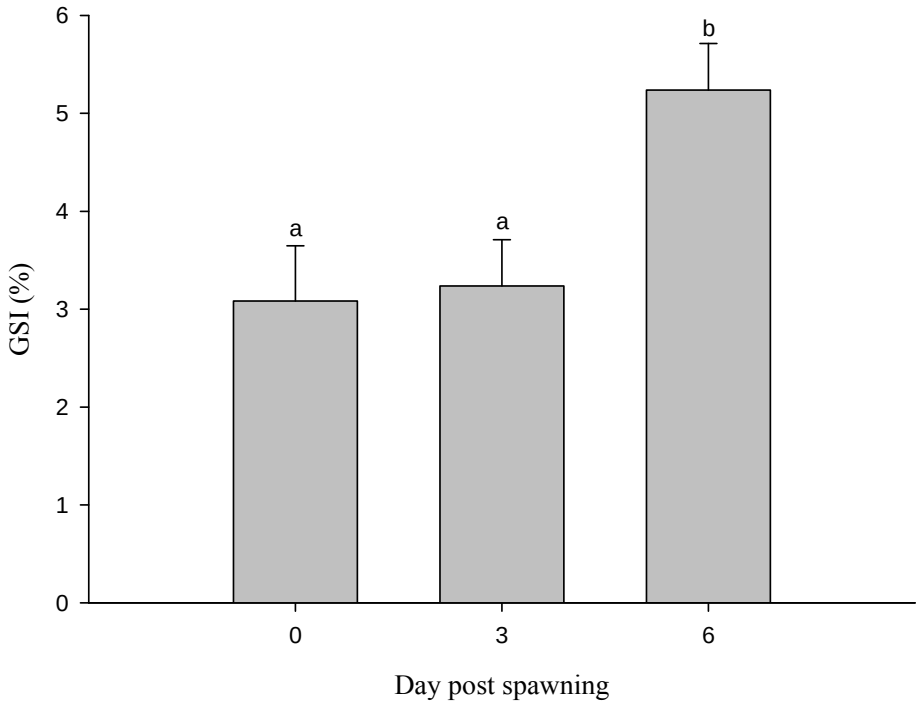


Figure 5.8 The mean GSI in *O. niloticus* over 0, 3 and 6 days post-spawning (day 0=day of spawning).Data between columns with different letters are significantly different ($P<0.05$, n=2). Non significant data from diet and ration were pooled. Data represented as mean ±SEM.

5.3.6 Hepatosomatic index (HSI)

Three and two-way ANOVA revealed that diets, rations or days post-spawning had no significant effect on HSI (Table 5.2). A significant difference ($P<0.05$) only occurred when HSI was compared between diets by one-way ANOVA on entire treatments. The mean HSI ranged from 2.1 ±0.01 to 2.3±0.12% for all diet treatments. One-way ANOVA revealed that the HSI from fish fed diet 4 was significantly greater than in those fed diets 2 and 3, but that fish fed diet 2 and 3 were not significantly different ($P>0.05$) (Figure 5.9).

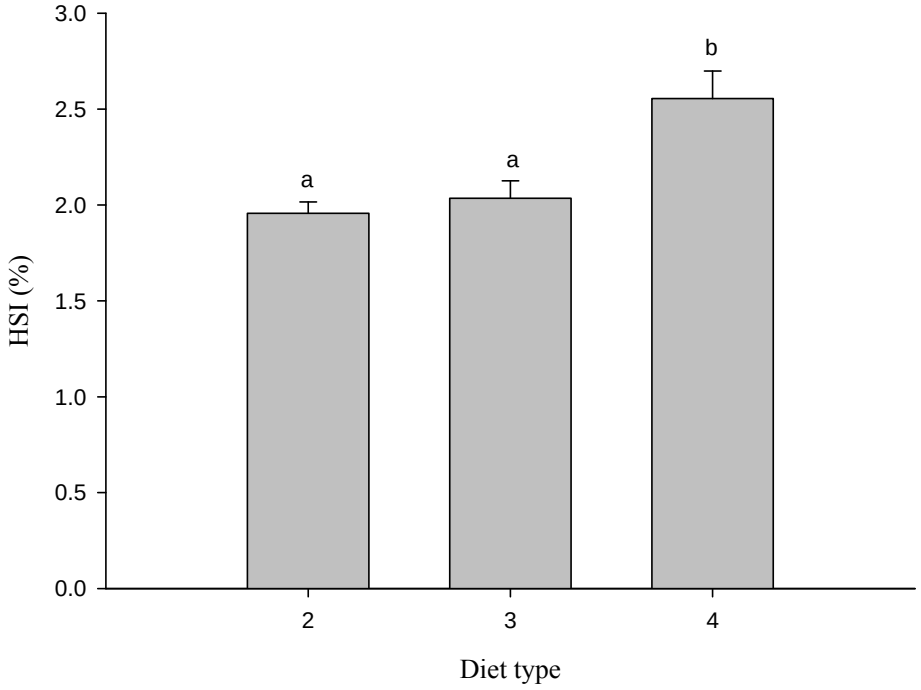


Figure 5.9 Mean HSI in *O. niloticus* fed different dietary lipid sources. Data between columns with different letter is significantly different ($P<0.05$). Data are mean ± SEM, n=2). Diet 2, palm oil, 3 mixed palm and cod liver oil 9:1 ratio; and 4 commercial trout diet as a control. Non significant data from ration and day post-spawning were pooled.

Table 5.4 Mean steroid hormones, plasma calcium, GSI and HSI among time sampling points for *O. niloticus.*

Day post- spawning	calcium	T	E2	E2/T	GSI	HSI
0	22.0 ±0.63^{a}	4.1±0.80^{a}	12.6±1.88^{a}	4.7±0.75^{a}	3.1±0.56^{a}	2.1±0.11^{a}
3	26.4 ±1.02^{b}	13.9±1.05^{b}	35.6±5.13^{bc}	2.6±0.35^{b}	3.2±0.47^{a}	2.1±0.12^{a}
6	44.9±0.60^{c}	39.8±3.83^{c}	44.2±2.41^{c}	1.3±0.15^{c}	5.2±0.48^{b}	2.3±0.12^{b}

Columns with different superscripts are significantly different (P<0.05). Non significant data from diets and rations were pooled. Data are mean ±SEM, n=6.

5.3.7 Volume fractions (VF) of oocyte stages using stereological analysis

In order to measure the area occupied by the various stages of oocytes, histological slides were digitally captured (section 2.21) and the captured image was analysed utilising stereological techniques via image analysis software (section 2.21). The volume fraction of each stage from fish fed successive diets rations over time sampling points were calculated and recorded individually.

The mean percentage values of volume fractions of each stage from fish fed different diets and rations at 0, 3 and 6 days post-spawning which is shown in (Table 5.5) were analysed using three- way ANOVA and the results are presented in Table 5.6.

Table 5.5 Effect of dietary lipid sources and rations (%) on oocyte volume fraction at 0, 3 and 6 days post-spawning in *O. niloticus*

Treatments			Parameters					
Diets	Rations	DPS	St 2	St 3	St 4	St 5	St 6/7	Atresia& spent tissue
2 (PO)	Full ration	0	1.8±0.1	9.2±0.3	6.2±0.3	11.5±0.6	6.0±2.0	43.4±2.7
		3	3.5±0.3	4.5±0.3	5.7±0.9	7.4±0.7	14.8±8.7	20.1±0.6
		6	3.9±0.7	4.4±0.3	7.5±0.7	4.5±0.2	58.3±3.0	0.0±0.0
	Half ration	0	2.5±0.2	9.1±0.3	6.5±0.7	10.4±1.1	10.9±3.7	42.6±3.7
		3	3.7±0.1	4.3±0.2	7.9±0.5	6.6±0.3	9.5±2.7	18.7±0.9
		6	3.8±0.2	4.8±0.2	6.0±0.3	4.1±0.2	58.1±0.5	0.5±0.2
3 (P&CO)	Full ration	0	2.7±0.2	5.3±0.2	5.4±0.4	8.9±0.6	7.6±1.9	45.8±4.2
		3	4.5±0.5	3.7±0.1	7.7±0.5	6.1±0.3	6.5±4.3	18.2±0.8
		6	2.6±0.1	7.0±1.6	6.5±1.1	5.1±1.1	56.8±3.5	2.3±2.3
	Half ration	0	2.6±0.2	8.3±0.2	6.3±0.3	10.6±0.4	3.9±1.8	47.6±1.6
		3	4.1±0.2	5.1±0.4	6.4±0.5	5.3±0.3	4.7±2.0	19.8±0.5
		6	3.8±0.2	4.7±0.3	6.4±0.5	4.7±0.2	57.5±0.9	1.4±0.7
4 (control)	Full ration	0	2.1±0.1	7.9±0.3	6.1±0.5	11.0±0.5	3.4±1.9	48.3±2.3
		3	3.7±0.2	4.3±0.1	6.6±0.8	5.4±0.3	5.2±2.1	21.1±1.4
		6	4.2±0.3	4.2±0.2	4.5±0.8	5.1±0.4	53.2±1.9	3.1±1.6
	Half ration	0	2.1±0.1	7.5±0.6	5.9±0.5	10.4±0.6	3.5±1.1	48.2±1.7
		3	4.8±0.7	6.3±1.1	6.5±0.6	5.4±0.4	4.9±2.8	16.8±1.5
		6	3.9±0.2	4.2±0.2	6.1±0.4	4.4±0.3	56.4±1.1	3.3±1.3

Data are presented as mean % of ovary occupied ± SEM, n=6, and the analysis results and their significance levels are presented in Table 5.6.

Table 5.6 Significance values for GLM analysis of effect of diets and rations on the oocyte volume fraction at 0, 3 and 6 days post-spawning in *O. niloticus.*

Source of variation	d.f.	St 2	St3	St 4	St5	St6/7	Atresia
Diet Levels	2	0.262	0.344	0.195	0.158	0.003	0.077
Ration levels	1	0.132	0.058	0.532	0.222	0.827	0.723
Day post-spawning (DPS)	2	0.0001	0.0001	0.192	0.0001	0.0001	0.0001
Diet × Ration	2	0.993	0.476	0.786	0.417	0.728	0.694
Diet × DPS	2	0.913	0.005	0.931	0.732	0.497	0.778
Ration × DPS	4	0.024	0.0001	0.528	0.082	0.720	0.409
Diet × ration × DPS	4	0.025	0.0001	0.022	0.295	0.470	0.858

$P<0.05$ = significant and $p>0.05$ = not significant.

5.3.7.1 Oocyte development during ovarian recrudescence

The females were sacrificed and ovaries were dissected immediately after spawning on day 0, or days 3 and 6 after spawning. The ovaries contained various sizes and stages of oocytes. The composition of these ovaries, however, varied depending on number of days post-spawning (DPS). The ovaries at day 0 post-spawning contained a mean of 10.6% of stage 5 and 46.4% of atretic oocytes. Data from atretic oocytes and spent ovarian tissues (residual tissues) were pooled, with a total mean of 26 and 20% for atretic and spent tissue, respectively. Oocyte stages 2, 3 and 4 accounted for 16.5% of the ovaries. At 0 DPS the atretic and stage 5 oocyte volume fractions were found significantly greater then stages 2, 3 and 4 oocyte (Table 5.7). By 3 and 6 DPS, however, volume fractions of stage 5 and atretic oocytes decreased from 5.9 and 19.3% to 4.4 and 1.8%, respectively. By 6 DPS, stage 6 oocytes occupied the greater part of the ovary when compared with volume fraction of oocytes. The volume fraction of oocytes increased from 5.2 to 56.9%

from 0 to 6 DPS, respectively. Table 5.7 shows that the volume fraction of stage 6 oocytes at day 6 after spawning were significantly greater than at days 0 and 3 after spawning, but days 0 and 3 DPS were not significantly different ($P>0.05$).

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Table 5.7 Volume fractions (%±SEM) determined using stereological analysis in *O.niloticus* over 0, 3 and 6 days post-spawning (0 day is day of spawning). Data between columns showing different letters are significantly different ($P<0.05$, n=6). Non significant data from diets and rations were pooled.

Day post-spawning	Oocyte developmental stages					
	S2	S3	S4	S5	S6/7	Atretic oocytes
0	2.3±0.09[a]	8.1±0.20[a]	6.1±0.20[a]	10.6±0.28[a]	5.2±0.90[a]	[*]46.4±0.98[a]
3	4.0±0.10[b]	4.5±0.13[b]	7.1±0.27[a]	5.9±0.16[b]	6.7±1.24[a]	19.3±0.43[b]
6	3.9±0.10[b]	4.6±0.12[b]	6.0±0.21[a]	4.4±0.12[b]	56.9±0.49[b]	1.8±0.43[c]

* 26± 0.58% atretic oocytes and 20±0.49% spent ovarian tissue.

5.4 Discussion

The general aim of the studies undertaken here was to examine the effect of dietary lipid source on the dynamics of ovarian recrudescence and associated changes in sex steroid levels in the Nile tilapia, *O. niloticus*. Little is known about this area of reproductive physiology despite the prominence of tilapias in world aquaculture. More detailed knowledge of this area would aid the development of techniques that might be used to artificially manipulate the spawning cycle of this species and reduce feed costs. Manipulation of the spawning cycle is of particular importance considering the wide variability observed in spawning periodicity (see Chapter 3) and the asynchronous nature of spawning cycles in tilapia (Jalabert and Zohar, 1982).

Generally, in most studies on broodstock nutrition in fish, emphasis is based on growth performance (Furuita *et al.,* 2003; Luo *et al.,* 2005). Only in very few broodstock nutrition studies has there been any attempt to investigate ovarian recrudescence or rematuration (Burton, 1994; Cerdà *et al.,* 1994b; Washburn *et al.,* 1990). These studies have been mainly conducted on annual spawners and therefore ovarian recrudescence was studied at monthly intervals through a spawning cycle. *O. niloticus*, on the other hand, spawns several times a year and the breeding period does not show a marked seasonality (Brummett, 1995), and, as such, all the females are unlikely to be in the same stage of oocyte development at a given time. In nature, tilapiine fish are known to breed throughout the year, with minor peaks of activity occurring in relation to environmental factors such as rainfall patterns (Brummett, 1995) (see Chapter 1 for review). To our knowledge, seasonal reproductive activity under experimental conditions has not been reported for tilapiine fish. Gunasekera and Lam (1997) reported that broodstock dietary protein levels influence oocyte recrudescence of adult *O.*

niloticus. However, these results are dependent on the nutritional history of the fish prior to sampling. When *O. niloticus* females were maintained on diets of different protein content for 2 months no marked differences were found in the type of oocytes present in the ovary (Gunasekera and Lam, 1997). However, differences in oocyte growth were more marked when females were maintained on diets of different protein content for 4 months.

In the present study, in order to overcome these problems and to investigate the precise effect of dietary lipids on oocyte recrudescence, fish were fed the experimental diets and rations from the onset of exogenous feeding to termination of spawning. Protein and oil levels of experimental diets were also kept at a constant level (40 and 10%, respectively). Ovarian recrudescence was recorded, starting from immediately after spawning (day 0 post-spawning) and continuing at 3 and 6 days post-spawning, for individual females. In this manner it was thought that dietary effects on ovarian rematuration could be more objectively evaluated, since in all probability oocyte development in all the females would have been at a comparable stage within the sampling groups.

In the past, changing levels of total plasma calcium have been used as an indicator of VTG secretion into the serum; calcium being used in the post-translational modification of VTG (Elliott *et al.,* 1984). This method has often been used to indirectly measure changes in VTG concentration in preference to a specific radioimmunoassay, largely due to the high correlation between serum calcium levels and serum VTG concentration and the ease with which calcium levels can be determined. This technique has been shown to be effective both in salmonids (*e.g.* (Elliott *et al.,* 1984; Norberg and Björnsson, 1989; Whitehead *et al.,* 1987), and in *Oreochromis* species (Hussain *et al.,*

1995; Srisakultiew, 1993), in cod (*Gadus morhua*) (Weltzien *et al.,* 2004) and in *Tilapia zillii* (Coward and Bromage, 2002). The present study showed that time had a significant effect on total plasma calcium which increased from 29.9 to 44.9 mg% (mg/100ml) from 0 to 6 day post-spawning, respectively. However, the levels of total plasma calcium from fish fed diets 2, 3 and 4 at full and half rations over their respective time sampling points (0, 3 and 6 day post-spawning) were similar. The histological study of oocyte stages, suggests that oocytes are recruited to the vitellogenic stage by day 6 after spawning. A similar result was found by Srisakultiew (1993) and Tyler *et al.* (1990).

The results of the present study demonstrated that the E2 profiles of *O. niloticus* did not change significantly between time for females fed diets 2, 3 and 4 at either full or half ration. However, E2 levels between the sampling-points were significantly different and the E2 profiles at time sampling points undergoing ovarian recrudescence consistently exhibited a low level on day 0 post-spawning and peaked by day 6 post-spawning, showing that E2 profiles are significantly increased during oocyte recrudescence. Testosterone levels were found to vary quite widely amongst individuals at the same time sampling-point. The T level markedly increased over time sampling-points for fish fed diets 2, 3 and 4. The plasma T levels also exhibited an interaction between diets and time sampling points for fish fed experimental diets. However, the most significant changes in T level appeared between days post-spawning during oocyte recruitment; it increased noticeably from 4.1ng/ml immediately after spawning (0 day post-spawning) and reached 39.8ng/ml by day 6 post-spawning.

The present study showed that both E2 and T increased immediately after spawning and peaked by day 6 after spawning. This concurs with the findings of Srisakultiew (1993)

and Yaron *et al.* (1983). Since hepatic VTG production is under the control of E2, and to a lesser extent E1 (Van Bohemen *et al.,* 1982; Wallace *et al.,* 1987), this suggests that developing oocytes were recruited into vitellogenesis as early as 2 days post-spawning and that the phase of vitellogenic growth had been largely completed by 10 days post-spawning. The profiles of E2 observed in the present study and by Srisakultiew (1993) are generally similar to those reported for annual spawners such as rainbow trout (*Oncorhynchus mykiss)* (Bromage and Cumaranatunga, 1988; Scott and Sumpter, 1983), goldfish (Kobayashi *et al.,* 1986, 1988, 1989) and Japanese sardine (Matsuyama *et al.,* 1994). These observations also agree with the findings of Smith and Haley (1988) in non-brooding *O. mossambicus*; E2, T and progesterone all exhibited post-spawning peaks (though somewhat later at day 10) that fell by day 15 but increased again towards the end of the cycle. The case of actively mouth-brooding *O. mossambicus* was somewhat different however; two early-cycle peaks of E2 were observed. It was claimed that the first peak (day 10) 'protected' oocytes from atresia during mouth-brooding behaviour (Sundaraj and Goswami, 1968) and that the second peak (day 25) corresponded to the period of vitellogenic growth (Smith and Haley, 1988).

That parental care can influence levels of reproductive hormones is well documented; for example, Rothbard *et al.* (1991) found maximal levels of E2 occurring in *O. niloticus* during pair-bonding behaviour. As explained earlier, the precise role of T in ovarian recrudescence is unclear. T has been used as an indicator of spawning in some fish (Fitzpatrick *et al.,* 1987; Redding and Patiño, 2006), and T of thecal origin is known to be converted to E2 in the granulosa via the "two cell type model" (Kagawa *et al.,* 1982; Nagahama *et al.,* 1995; Redding and Patiño, 2006; Reinecke *et al.,* 2006). It is now commonly considered that T can stimulate GTH synthesis (Crim *et al.,* 1973, 1981; Crim and Evans, 1979; Gielen and Goos, 1984), and stimulate yolk globule formation

(Crim and Evans, 1979). In the present study, E2/T ratio changed significantly between the time sampling points (0, 3 and 6 days post-spawning). The peak E2/T ratio was found at 0 day post-spawning which contradicts the findings of Yaron *et al.* (1983) who reported that E2/T to be maximal in *O. niloticus* on day 4 post-spawning. The reason for this difference is unknown. However, the ratio of E2/T was not significantly different between fish fed different dietary lipid sources at either full or half ration. Smith and Haley (1988) recorded high levels of T in *O. mossambicus* during mouth-brooding but found levels to drop when brooding behaviour ceased, and suggested that T may have retarded oocyte growth until parental care had ceased. Similarly, Bogomolnaya and Yaron (1984) reported high levels of T during mouth-brooding in *O. aureus*. Peak levels of T were found in goldfish, for example, at ovulation (Kobayashi *et al.,* 1986, 1988, 1989; Mennigen *et al.,* 2008). In rainbow trout (*Oncorhynchus mykiss)*, T was found to peak 8 days prior to ovulation (Scott and Sumpter, 1983). It is widely thought that T is involved in oocyte recruitment and ovulation. The present study showed that during oocyte recruitment the T level was markedly increased and reached a peak at day 6 post-spawning.

The present study revealed that oocyte recruitment of fish fed experimental diets 2 and 3 was similar to the control diet 4 at either full or half rations across time sampling points (0, 3 and 6 days post-spawning). However, some changes occurred at stage 2, 3 and 4 for all diet groups across the time sampling points. This difference could be due to variability of oocytes, differences in individual fish or due to the history of spawning frequency in fish. A similar result was found by Srisakultiew (1993) who reported that composition varied according to spawning history; for example, volume fraction (VF) of atretic oocytes after spawning was found to be far less on day 1 in the second spawning cycle than on day 1 in the first spawning cycle.

In the present study, a significant effect on oocyte recruitment occurred only between the day sampling-points, and immediately after spawning the ovary contained a mixture of all oocyte developmental stages (from stage 2 – stage 6/7 and atresia). The highest percentage of the volume fraction was occupied by atretic oocytes plus spent ovarian tissue (26 and 20% for atretic oocytes and spent tissue of ovaries, respectively) and the lowest proportion was occupied by stage 2 oocytes, which was around 2.3 % by day 0 (immediately) after spawning. The atretic stage then decreased to 19 and 1.8 % by days 3 and 6 post-spawning, respectively. The vitellogenic stage 6/7 oocytes occupied about 60% of VF by day 6 after spawning. It is clear that in *O. niloticus* oocyte recrudescence could be completed by day 6 post-spawning. These observations broadly agree with Srisakultiew (1993), who reported that in *O. niloticus,* proportions of stage 4 and stage 5 oocytes remained relatively constant over time except during the first three or four days after spawning, where proportions were elevated. As the proportion of stage 4 and 5 oocytes declined (by day 3) in parallel with dramatic increases in both E2 and T, the proportion of stage 6/7 oocytes began to peak. Significant increases in the VF of stage 6/7 were observed between days 0, 3 and 6, these changes being reflected in GSI which increased gradually from day 0 to day 3 post-spawning but increased rapidly between days 3 and 6. This suggests that very soon after spawning a batch of pre-vitellogenic oocytes enter stages 4 and 5, progressing into stage 6/7 extremely quickly. At 6 days post-spawning, the ovary was dominated by stage 6/7 oocytes occupying up to 60% of the ovary. Srisakultiew (1993) reported a similar result in *O. niloticus*, but the author revealed that VF of stage 6/7 oocytes can reach up to 70% within 10 days of spawning. Dadzie and Wangila (1980) also observed that vitellogenic oocytes appear in the *T. zilii* ovary soon after spawning. A new batch of vitellogenic oocytes were found to be

already present in the ovary at the time of spawning in *O. niloticus* by Tacon *et al.* (1996), Hyder (1970) and Peters (1983).

In the present study, GSI in *O. niloticus* reached maximal values by day 6 post-spawning and was highly correlated to the VF of stage 6/7 oocytes. The estimates of GSI in mouth-brooding tilapia increased 4.6 - 10.2% as reported by Peters (1983), 7.0 - 8.2% by Srisakultiew (1993) and 6% by Tacon (1996). GSI is often used as an indicator of the state of maturity in many teleost species and can reach up to 30% in some salmonid species *e.g.* rainbow trout (Tyler *et al.,* 1990). In the case of serial spawners however, GSI greatly underestimates reproductive investment (Wootton, 1979). Up to 20% of the ovaries from immediately post-spawned *O. niloticus* (*i.e.* day 0) was occupied by stage 6/7 oocytes that for one reason or another had not ovulated. Residual eggs were also seen in the post-spawned ovary of *O. niloticus* by Avarindan and Padmanabhan (1972). Peters (1983) also noted the presence of residual eggs and claimed that residual eggs swiftly progress into atresia; 'mechanical' effects were said to determine whether eggs ovulated or not, though the precise meaning of this statement was not clarified. In *Oreochromis* species, Peters (1983) found a number of residual eggs remaining in the ovary after spawning and indicated that these advanced oocytes are likely to become atretic.

In *O. niloticus*, Srisakultiew (1993) found that up to 40% of the ovary 1 day after spawning could be occupied by atretic oocytes. In the present study, atretic oocytes and spent ovarian tissue occupied 46% of the ovary, with values of 26 and 20% for atretic oocytes and spent tissue of ovaries, respectively at 0 day post-spawning. The process of atresia is known to affect any stage of oocyte development and occurs in all vertebrate groups (Brambell, 1956; Bromage and Cumaranatunga, 1988; Byskova, 1987; Saidapur

and Nadkarni, 1976). Incidence of atresia is believed to be influenced by age, oocyte developmental stage, dietary status and hormonal status (Bromage and Cumaranatunga, 1988). These authors reported that atresia plays an important role in ovarian development, particularly in the determination of fecundity. It has also been suggested that atresia is more common in fish where recruitment into vitellogenesis occurs continually, for example in the case of indeterminate spawners (Tyler, 1996). Peters (1983) reported that both captive and wild tilapia exhibit a marked tendency to reabsorb ripe eggs.

The precise function of atresia in fish and the factors regulating its occurrence remain unclear, though it is believed to be involved in the initiation of follicular growth and the selection of follicles for ovulation (Tyler *et al.,* 2000) and steroidogenesis (*e.g.* Saidapur and Nadkarni, 1976) and is also thought to be an important determinant of fecundity (Bromage *et al.,* 1992; Springate and Bromage, 1985). In the present study, atretic oocytes appeared as atretic oocyte plus post-ovulatory follicles (POFs). The POFs were only found in the *O. niloticus* ovary on day 0 post-spawning and were reduced by day 3. A similar result was reported by Srisakultiew (1993) who found that POFs occupied up to 23% of the ovary on day 1 falling to 10% and 0% by days 5 and 10, respectively. Several authors have claimed that POFs exhibit a steroidogenic function (Bara, 1965; Kagawa *et al.,* 1981; Kouwenhoven *et al.,* 1978; Lang, 1981; Nagahama *et al.,* 1976; Smith and Haley, 1987), but the precise role and potential significance to ensuing spawning cycles remains unclear. Atretic oocytes may also exhibit steroidogenic activity (*e.g.* Saidapur and Nadkarni, 1976). The presence of atretic oocytes and POFs during the ovarian cycle of *O. niloticus* may therefore contribute to ovarian steroidogenesis, particularly during key periods. For example, in the 6 days following spawning, pre-vitellogenic oocytes were rapidly recruited into active vitellogenesis

coincident with dramatic increases in circulating E2 and T. There are numerous sites within the ovary where steroids have been shown to be produced, *e.g.* granulosa cells, special thecal cells (STCs), POFs, atretic oocytes, interstitial gland cells (Kagawa *et al.,* 1982; Lam *et al.,* 1978; Lang, 1981; Yaron, 1971) and, more recently, steroid producing cells (SPCs) (Nakamura and Nagahama, 1993). Steroid producing cells were found to appear coincident with gonadal differentiation and later invaded the thecal layer as oocytes began production of E2 and became vitellogenic (Nakamura and Nagahama, 1993).

Morphology of the follicular layer was also found to change as the ovary developed; theca, granulosa and zona radiata were all found to increase in size between day 0 and day 6 post-spawning. These results agree with Coward (1997) who found that the follicular layer of developing stage 6/7 oocytes in *T. zillii* was extremely well developed as early as day 4 post-spawning, coincident with peak levels of E2 and T. Synthesis of steroids by granulosa cells has been found to occur coincident with several major intra-cellular changes. Such activity during oocyte maturation provides further evidence that the granulosa is involved in the production of the maturation inducing steroid (MIS). Testosterone is produced in the theca and converted to E2 in the granulosa via an enzyme known as aromatase (Kagawa *et al.,* 1982; Nagahama *et al.,* 1996; Scott and Baynes, 1982) (Figure 5.2). Further evidence supports the hypothesis that during ovarian maturation, 17a-OH-P is also produced by the theca and converted into 17a-20ß-P (widely believed to be the MIS) in the granulosa (Kawauchi *et al.,* 1989; Nagahama, 1985; Nagahama *et al.,* 1995, 1996). In the present study, a significant increase was observed in both E2 and T levels during ovarian recruitment.

In summary, levels of E2 and T peaked in *O. niloticus* by 6 days after spawning suggesting that vitellogenesis began as early as day 3 post-spawning and is almost completed by day 6. However, by day 6, the ovary was dominated by stage 6/7 oocytes and GSI peaked. This suggests that vitellogenesis is rapid and vitellogenic oocytes are recruited into the vitellogenic growth phase immediately after spawning and completed as early as day 6 post-spawning. It is also evident from the present study that *O. niloticus* females will maintain their ovarian recrudescence at half ration as well as full ration. HSI did not reduce/ change during oocyte recrudescence and remained stable on days 0, 3 and 6 post-spawning, in agreement with other species such as common dentex (*Dentex dentex*) (Chatzifotis *et al.,* 2004).

Gonadal recrudescence is the recruitment of oocytes after the act of spawning. Most fish species reproduce more than once in their life-time and as such recrudescence is an important aspect of broodstock management. The effect of temperature on gonadal recrudescence has been investigated in blue tilapia by Terkatin-Shimony *et al*. (1980), African catfish (*Clarias lazeru*) by Richter *et al.* (1982) and Indian catfish (*Heteropneustes ,fossilis*) by Saxena and Sandhu (1994). The dietary effects on ovarian recrudescence have been studied on two temperate species: Burton (1994) on winter flounder (*Pleuronectes americanus*) and Cerd´a *et al.* (1994b) on the European sea bass (*Dicentrarchus labrax*).

Overall, it is evident that the palm oil diet is comparable with the control diet and fish oil can be replaced with palm oil with no negative effects on steroid hormones and oocyte recrudescence. It is also evident that no negative effect was observed on steroid hormones profile and oocyte recrudescence when fish were fed a half ration. Therefore, half ration feed should increase profitability of seed production.

Chapter 6 - General discussion

Potential for expansion of tilapia production to contribute to the predicted shortfall in fish supplies is promising (FAO, 2004).

According to Fitzsimmons (2008) production figures indicate that in 2006, global tilapia production reached 2,369,966 tonnes, which was mere 4.6% of total aquaculture production. Clearly, the further development and intensification of tilapia culture not only shows great promise but will become a necessity within the next decade and it is vital that culture operations strive to meet increasing consumer demand. Fish feed, cost and availability of fish feed ingredients are vital factors for sustainable aquaculture, particularly for *O. niloticus* to compete with other species. In the light of increasing costs optimisation of hatchery efficiency will be paramount importance if production is to be maximised and maintained. Broodfish productivity and related costs remain the most significant constraints to commercial production and knowledge of the factors affecting brood stock productivity such fish oil feeds is of immense importance if tilapia culture is to be developed effectively. The replacement of fish oil in the diet with palm oil could reduce the production costs.

Fish oil is produced from small, marine pelagic fish and represents a finite fishery resource. Marine fish oils are traditionally used as the main dietary lipid source in many commercial fish feeds providing energy and essential fatty acids. It is also commonly used to coat the extruded pellets to improve palatability and appearance of feed. Due to the rapid development of global aquaculture there have been parallel technological advances with the fish feed manufacturing sector which is now able incorporate high level of dietary oil into their products to produce an energy dense pelleted feed. Over the past decade, global fish oil production has reached a plateau and is not expected to

increase beyond current levels. According to estimates, aquafeeds currently use approximately 88.5% of the global supply of FO as a lipid source (Tacon and Metian, 2008). The global use and demand for FO by various aquaculture species has been estimated by Tacon and Metian (2008), who predicted that the future consumption of fish oil will hover at approximately 94.5% of global supplies by 2012. Others, however, predict that aquaculture will consume up to 98% of the global FO supply by the year 2010 (Pike and Barlow, 2003). . In addition, it is becoming increasingly expensive and difficult to obtain fish oil in many tropical countries practicing aquaculture because FO production is localised to specific regions of the temperate world.

In the production of tilapia feeds, manufactures are constantly faced with the need to reduce feed costs to match fluctuating, and at times low, ex-farm prices of tilapia. Whenever there is an increase in the cost of imported feed ingredients, this greatly cuts into the profit margin of local tilapia growers. Since fish feeds can account from 45 to 85% of the farm prices of tilapia (Ng and Chong, 2004), there is currently great interest to reduce feed costs by using locally available or alternative feed ingredients. Therefore there is, at present, a great urgency within the aquafeed industry to evaluate alternatives to fish oil. The aquaculture industry cannot continue to rely on finite stocks of marine pelagic fish as a fish oil supply in order to sustain its rapid development. Several vegetable oils have been shown to be potential replacements for fish oil in fish diets, either by partial or total substitution.

One potential substitute for fish oil in aquafeeds is palm oil, which is currently is the world's most important oil crop producing 24.9% of total vegetable oils and fast surpassing soybean at 23.9% (ISTA Mielke Gmbh, 2007). Total world production of crude palm oil (CPO) stands at about 38 million tonnes (Soh *et al.,* 2009). Nevertheless,

in addition to its low cost and high availability, palm oil also has many additional advantages over other vegetable oils when used in aquafeed formulations *e.g.* the saturated fatty acid (SFA) (*i.e.* 16:0) and MUFA (18:1n-9) abundance in palm oil for energy production in the mitochondrial system of fish (Henderson and Sargent, 1985). The incorporation of this palm oil in the diets of tilapia will greatly contribute to reducing the impact of rising feed costs in the culture of this species in many tropical and subtropical countries (Ng *et al.,* 2006).

Previous studies have demonstrated the potential and advantages of palm oil over other vegetable oils when used in diets for Atlantic salmon (*Salmo salar*) (Bell *et al.,* 2002; Ng *et al.,* 2004; Regost *et al.,* 2003; Tortensen *et al.,* 2000), rainbow trout (Fonseca-Madrigal *et al.,* 2005) , tilapia (*Oreochromis* spp.) (Ng *et al.,* 2004, 2006; Ng and Low, 2005; Ochang *et al.,* 2007b) and various catfish (Legendre *et al.,* 1995; Ng *et al.,* 2001, 2003, 2004). These authors found that the use of CPO in the diets of these fish elicited growth and feed utilization efficiency comparable with fish fed equivalent levels of dietary marine fish oils. Previous studies also demonstrated that palm oil can be used as dietary lipid source with no negative effect on fish growth. However, limited information is available on the effect of lipid sources on tilapia reproductive performance. The present study is the first attempt to investigate the effect of dietary lipid sources on the reproductive performance of tilapia fed solely their respective experimental diets for their entire life cycle.

The aims of the present study were to determine the effects of different lipid sources in the experimental diets on egg quality, specifically egg biochemical composition, in order to evaluate palm oil as a potential substitute for fish oil in the diet of *O. niloticus*.

Spawning periodicity, fecundity and egg size

Previous studies have reported that fecundity and fish size often exhibit marked variation around a fitted curve. This was attributed partly to the effects of broodstock age and egg size and partly to the effects of genetic and environmental factors on fecundity (Bagenal, 1957; Bromage *et al.,* 1992; El-Sayed *et al.,* 2005; Wootton, 1979). Several studies have also found that fecundity may be modified by broodstock nutrition *e.g.* (Allen and Wootton, 1982; Bagenal, 1969; Bromage and Jones, 1991; Bromage *et al.,* 1992; Townshend and Wootton, 1984, 1985). Fecundity in rainbow trout, for example, is reduced significantly in females maintained at a half ration during the early part of ovarian recrudescence and fed a full ration thereafter (Bromage and Jones, 1991), suggesting that fecundity is determined early in the reproductive cycle.

The present study demonstrated that palm oil at a inclusion level of 10% and fed at 1.5 and 3% of BW had no significant bearing on overall egg and larval quality *i.e.* mean fecundity, relative fecundity, mean egg wet and dry weight, mean total egg volume, mean egg diameter and larval weight and length between groups of fish fed diets 2,3 and 4 either at full or at half rations (Chapter 3). The mean total fecundity (number of eggs/ fish) of *O. niloticus* was found to range from 760 -780 for fish fed diet 2 , 800-880 for fish fed diet 3 and 640-690 for fish fed diet 4 at full and half ration, respectively, over three spawnings. As expected, present estimates of total fecundity were greater than those given for the same species *e.g.* 242 – 351 (El-Sayed *et al.,* 2005; El-Sayed and Kawanna, 2007) and was in the range of 309 – 1158 (Rana, 1986). This could be due to constant environmental conditions and the same age of the fish which had been used for the experiments. It may be that half ration (1.5%BWday^{-1}) is sufficient for reproductive performance. It is suggested that investment in reproduction

had remained remarkably consistent despite very large differences in food availability throughout their life cycle. Similar results were found in *Tilapia zillii* by Coward (1997). However, reducing the food ration would increase the profitability of seed production.

The most important features of tilapia reproductive biology are the plasticity of age and size at first sexual maturity (Rana and Macintosh, 1988). This was particularly evident with *O. niloticus* in the present study where the ISI increased with increase in overall fish size. The mean ISI averaged 14, 19 and 24 days for fish fed palm oil diet (diet 2), mixed palm and cod liver oil diet (9:1) ratio (diet 3) and control diet (diet 4), respectively. However, mean ISI is based solely on completed reproductive cycles and therefore underestimates true spawning periodicity. The best index of spawning periodicity was considered to be mean days elapsed between two consecutive spawnings in a female (see Chapter 3). As with ISI, the mean days elapsed between two consecutive spawns increased in fish fed the control diet and some fish from this group did not spawn in 98 days. The reason for non-spawning fish could be due to fish size which was larger than other groups. The female needs more space than expected, or genetic differences between females. The shortest spawning cycles observed in the present study were just 7 days for fish fed diet 2 (palm oil), considerably shorter than previous reports (*e.g.* 25 days by El-Sayed and Kawanna (2007). Interestingly and of great significance to the hatchery manager, is the fact that considerable variation in spawning intervals was observed within a given maternal size class. Tacon *et al.* (1996) and Coward and Bromage (1999) also observed variability in ISI in aquarium-held *O. niloticus* and suggested that this was probably due to genetic differences between females and from individual responses to a variety of factors including social status and conspecific stimulation.

Biochemical egg quality assessment in *O. niloticus*

Generally, broodstock nutrition is vital to produce high-quality eggs and larvae and lipid and fatty acid compositions of diets are known as important factors in determining egg quality (Izquierdo *et al.,* 2001a; Salze *et al.,* 2005; Sink and Lochmann, 2008; Tandler *et al.,* 1995). The potential of an egg to produce viable fry can be determined by biochemical parameters such as lipid content and fatty acid composition. More specifically, egg lipids have several critical roles in the development of fish embryos. Good egg and larval quality are vital for tilapia aquaculture development; particularly for tilapia that currently is the second most popular species in world aquaculture after carp (Fitzsimmons, 2006).

In the present study, no significant difference was observed in egg lipid levels when broodstock fed palm oil, palm and cod liver oil or a control diets at either full or half rations. Other authors had also observed unchanging lipid levels in muscles when *O. niloticus* was fed with various dietary lipids (Chou and Shiau, 1996; Fitzsimmons *et al.,* 1997; Ng and Chong, 2004).

Changes in dietary formulations resulted in significant changes in the fish egg fatty acid composition. However, the present study has shown that fish egg fatty acid composition reflects the fatty acid composition of the diet, although specific FA were selectively utilized or retained in the eggs. This result is in agreement with previous work (Abi-Ayad *et al.,* 2004; Bell *et al.,* 1997; Henderson and Sargent, 1984; Lu and Takeuchi, 2004; Mourente *et al.,* 2005; Richard *et al.,* 2006; Takeuchi *et al.,* 2002).

Despite the fact that the palm oil diet did not contain the fatty acids, such DHA, EPA and ARA, they were however, observed in the eggs of fish fed the palm oil diet. This result is in agreement with other works who reported freshwater fish species are able to

produce DHA, EPA and ARA from linolenic and linoleic acid, respectively, with the presence of Δ5-desaturatase enzyme (Bruce *et al.,* 1999; Henderson and Sargent, 1984; Olsen *et al.,* 1990).

Stereological analysis of ovarian histology and radioimmunoassay of sex steroid levels provided unique information concerning the dynamic of ovarian recrudescence in this species. The present study demonstrated that fish fed different dietary lipid sources at two ration levels (full and half ration) had no significant interaction with oocyte recrudescence. This study also revealed that oocyte recrudescence exhibited changes between time sampling-points and was associated with steroid levels. Briefly, plasma E2 and T levels peaked by day 6 post-spawning and were significantly correlated to the VF of stage 6/7 oocytes, suggesting that vitellogenic growth began as early as day 2 or 3 post-spawning. By day 6 after spawning, the ovary was dominated by stage 6/7 oocytes occupying 60-65% of the ovary. GSI was significantly correlated to the volume fraction (VF) of stage 6/7 oocytes, and was observed to increase (as oocytes matured) to reach maximal levels of 5.0 - 5.5% by day 6 post-spawning. It is suggested that pre-vitellogenic oocytes are recruited into vitellogenic growth immediately after spawning and complete vitellogenesis can occur as early as day 6 post-spawning. Similar results were found in *O. niloticus* by Srisakultiew (1993) who suggested that pre-vitellogenic oocytes are recruited into vitellogenic growth very soon after spawning and complete vitellogenesis as early as day 8 post-spawning.

Vitellogenesis is arguably the most important phase of oocyte growth and in rainbow trout, for example, accounts for up to 95% of egg volume (Coward and Bromage, 2000; Tyler *et al.,* 1990). In teleosts, as in other oviparous vertebrates, VTG is sequestered by receptor-mediated endocytosis involving a specific VTG receptor located on the oocyte

membrane (Chan *et al.,* 1991; Lancaster and Tyler, 1994; Tyler and Lancaster, 1993). Hormones and growth factors regulating the VTG receptor are therefore likely to play critical roles in oocyte growth. A greater understanding of vitellogenesis and oocyte growth in fish is likely to come from studies involving the developmental expression of the VTG receptor using specific probes designed to the receptor cDNA sequence. Such studies are already well underway in the rainbow trout (Tyler, 1996).

As a direct result, there is increasing pressure to reduce hatchery running costs. This is particularly relevant for feed costs and this issue has been addressed through the development of practical diets incorporating vegetable oil, as previously mentioned, inclusion of plant ingredients (*e.g.* De Silva *et al.,* 1991), by the evaluation of alternative lipid and protein sources other than fish oil and fishmeal (Gunasekera *et al.,* 1995; Santiago *et al.,* 1983, 1985; Wee and Tuan, 1988) or by restriction of ration size (Macintosh and De Silva, 1984; Mironova, 1977).

In the present study, *O. niloticus* was rationed at two levels (full and half) (see section in Chapter 3) from first-feeding and throughout the entire life cycle. Despite reducing the ration by half, no significant differences were detected in total fecundity, egg diameter or total egg volume once differences in fish size had been partitioned using ANCOVA. The fact that no differences were found in terms of GSI, or in circulating levels of E2 and T, further suggests that despite differences in ration size throughout the life cycle periods investment in reproduction remained relatively consistent. Under conditions of restricted food supply, an animal can invest its limited diet into both somatic growth and maintenance or to shunt energy into egg production at the expense of body weight (Coward, 1997; Ireland and Wootton, 1977).

To summarise, the present study provides valuable and novel information regarding the effect of dietary lipid sources on egg lipid and fatty acid composition, fecundity, egg size, spawning periodicity, larval weight and length, ovarian dynamics and reproductive endocrinology in *O. niloticus*; a species that represents 95% of farmed tilapia and is currently the second most popular cultured species worldwidely. Dietary lipid and doubling of ration from half to full had no significant effect on reproductive performance. In conclusion, the results of this study suggest that under controlled conditions, lipids of non-marine origin, such as palm oil, can be used successfully for broodstock diets at 1.5% of body weight. In addition, comparable performance with commercial control diets and halving of feed requirement should increase profitability of seed production.

Chapter 7 - References

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APPENDICES

Appendix 1 Published Paper

1.1 Hajizadeh, A., Jauncey, K., & Rana, K. (2008). Effect of dietary lipid sources on egg and larval quality of Nile tilapia *Oreochromis niloticus*, In The Eighth International Symposium on Tilapia in Aquaculture, Abasab,Ag, press,ARC, 965 - 977. Egypt.

Appendix 2 Fixatives preparation

2.1 Preparation of Bouin's fluid

40% formaldehyde 25ml

Saturated aqueous picric acid 75ml

Glacial acetic acid 5ml

2.2 Preparation of 10% BNF

Formalin, full strength (37-40% formaldehyde) 100.0 ml

Sodium phosphate dibasic (anhydrous) 6.5gr

Sodium phosphate monobasic 4.0gr

Distilled water 900 ml

Appendix 3 Staining procedures for histological slides

Appendix Table 3.1 Staining protocol for haematoxylin and eosin

Chemical/Solution	**Immersion Time (Minutes)**
Alcohol 1	2.0
Methylated Sprit	1.5
Wash-running tap water	1.0
Haematoxylin	5.0
Wash-running tap water	1.0
Acid alcohol	4 swift dips
Wash well-running tap water	1.0
Scott's tap water	1.0
Examine slide under microscope	
Wash-running tap water	1.0
Eosin	5.0
Wash-running tap water	20 seconds
Alcohol 2	2.0
Alcohol 1	2.0

Appendix Table 3.2 Staining schedule for polychrome stain modified by from Coello (1989) by Striakultiew (1993)

Chemical/Solution	**Immersion Time (Minutes)**
1% Periodic acid	10
Wash-running tap water	5
Schiff's Reagent	30
Wash-running tap water	5
0.1% Bromophenol Blue (in 95% ethanol)	25
0.5% Acetic acid	20
Wash-running tap water	5
Haematoxylin	40
Phosphate buffer pH 7.4-8*	2 washes(renew buffer after each wash)

Preparation of phosphate buffer used in polychrome stain

Contents:

0.2M Disodium hydrogen orthophosphate 12 Hydrate GPR (BDH/Merck Ltd.)

0.1M Sodium dihydrogen orthophosphate GPR (BDH/Merck Ltd.) Add 217.5ml of (1) to 32.5ml of (2) and make up to 500ml in a volumetric flask and ensure that pH is within the range 7.4-8.0 (adjusted if necessary).

Appendix 4 Effect of dietary lipid sources in the egg lipid contents

Appendix Table 4.1 Percentage total lipid composition in egg from *O. niloticus* fed different diets, ration, and spawning numbers. The means are significantly different are shown as "s" and non ignorantly different are shown as "ns" (P≤.05)

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
D2R1Spaw1	2	1	2	0.9486	ns
	2	1	3	0.1986	ns
	2	2	1	0.1307	ns
	2	2	2	0.9933	ns
	2	2	3	1.00	ns
	3	1	1	0.0622	ns
	3	1	2	0.6485	ns
	3	1	3	0.9989	ns
	3	2	1	1.00	ns
	3	2	2	0.9986	ns
	3	2	3	1.00	ns
	4	1	1	0.9943	ns
	4	1	2	1.00	ns
	4	1	3	0.9147	ns
	4	2	1	0.8765	ns
	4	2	2	0.042	ns
	4	2	3	1.00	ns
D2R1Spaw2	2	1	3	0.998	ns

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
	2	2	1	0.8912	ns
	2	2	2	1.00	ns
	2	2	3	0.9874	ns
	3	1	1	0.6765	ns
	3	1	2	0.9999	ns
	3	1	3	1.00	ns
	3	2	1	0.9985	ns
	3	2	2	0.1597	ns
	3	2	3	0.9904	ns
	4	1	1	1.00	ns
	4	1	2	0.5849	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	0.5958	ns
	4	2	3	0.9989	ns
D2R1Spaw3	2	2	1	1.00	ns
	2	2	2	0.9982	ns
	2	2	3	0.616	ns
	3	1	1	1.00	ns
	3	1	2	1.00	ns
	3	1	3	0.9962	ns
	3	2	1	0.7571	ns
	3	2	2	0.0234	s

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
	3	2	3	0.6241	ns
	4	1	1	0.9978	ns
	4	1	2	0.1389	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	1.00	ns
	4	2	3	0.7277	ns
	2	2	2	0.6754	ns
	2	2	3	0.0645	ns
	3	1	1	1.00	ns
	3	1	2	1.00	ns
	3	1	3	0.659	ns
	3	2	1	0.0897	ns
	3	2	2	0.0001	s
D2R2Spaw1	3	2	3	0.0455	s
	4	1	1	0.647	ns
	4	1	2	0.002	s
	4	1	3	0.9234	ns
	4	2	1	0.9751	ns
	4	2	2	1.00	ns
	4	2	3	0.0312	s
D2R2Spaw2	2	2	3	0.986	ns
	3	1	1	0.404	ns

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
D2R2Spaw3	3	1	2	0.9988	ns
	3	1	3	1.00	ns
	3	2	1	0.999	ns
	3	2	2	0.1143	ns
	3	2	3	0.9918	ns
	4	1	1	1.00	ns
	4	1	2	0.5432	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	0.297	ns
	4	2	3	0.9992	ns
	3	1	1	0.0246	ns
	3	1	2	0.5268	ns
	3	1	3	0.9985	ns
	3	2	1	1.00	ns
	3	2	2	0.9867	ns
	3	2	3	1.00	ns
	4	1	1	0.9908	ns
	4	1	2	1.00	ns
	4	1	3	0.8575	ns
	4	2	1	0.8042	ns
	4	2	2	0.013	s
	4	2	3	1.00	ns

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
D3R1Spaw1	3	1	2	0.9959	ns
	3	1	3	0.404	ns
	3	2	1	0.0395	s
	3	2	2	0.0001	s
	3	2	3	0.0195	s
	4	1	1	0.3852	ns
	4	1	2	0.0009	s
	4	1	3	0.7115	ns
	4	2	1	0.8411	ns
	4	2	2	1.00	ns
	4	2	3	0.0135	s
D3R1Spaw2	3	1	3	0.9973	ns
	3	2	1	0.6848	ns
	3	2	2	0.0061	s
	3	2	3	0.5197	ns
	4	1	1	0.9985	ns
	4	1	2	0.0708	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	0.9936	ns
	4	2	3	0.5915	ns
D3R1Spaw3	3	2	1	0.9999	ns
	3	2	2	0.2647	ns

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
	3	2	3	0.9991	ns
	4	1	1	1.00	ns
	4	1	2	0.7538	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	0.3114	ns
	4	2	3	1.00	ns
D3R2Spaw1	3	2	2	0.8901	ns
	3	2	3	1.00	ns
	4	1	1	0.9992	ns
	4	1	2	0.9985	ns
	4	1	3	0.9503	ns
	4	2	1	0.9183	ns
	4	2	2	0.0206	s
	4	2	3	1.00	ns
D3R2Spaw2	3	2	3	0.9602	ns
	4	1	1	0.1207	ns
	4	1	2	1.00	ns
	4	1	3	0.0203	s
	4	2	1	0.017	s
	4	2	2	0.0001	s
	4	2	3	0.5634	ns
D3R2Spaw3	4	1	1	0.9931	ns

Characteristics	Comparison			Significant level between (D*R*Spa)	List significant difference (LSD)
	Diet	Ration	Spawning No		
	4	1	2	0.9999	ns
	4	1	3	0.8606	ns
	4	2	1	0.8063	ns
	4	2	2	0.0093	s
	4	2	3	1.00	ns
D4R1Spaw1	4	1	2	0.5802	ns
	4	1	3	1.00	ns
	4	2	1	1.00	ns
	4	2	2	0.2796	ns
	4	2	3	0.9994	ns
D4R1Spaw2	4	1	3	0.2039	ns
	4	2	1	1.00	ns
	4	2	2	0.0003	s
	4	2	3	0.972	ns
D4R1Spaw3	4	2	1	1.00	ns
	4	2	2	0.6075	ns
	4	2	3	0.9258	ns
D4R2Spaw1	4	2	2	0.8074	ns
	4	2	3	0.8819	ns
D4R2Spaw2	4	2	3	0.0044	s

Appendix 5 Set-up/shut-down procedure for atomic absorption spectrophotometer

(a) preliminary set-up

(1) Check drain vessel, empty if necessary.
(2) Ensure that calcium bulb is fitted to spectrophotometer.
(3) Ensure that GAIN and LAMP1 settings are turned to zero.
(4) Switch on power. Allow bulb to warm-up for 15-30 minutes.
(5) Reset the machine as follows:
    (a) BGKD to AA
    (b) SLIT to 0.7 (normal)
    (c) WAVELENGHT to 423nm
    (d) SIGNAL to lamp1
    (e) MODE to CONT
(6) Adjust lamp/energy to 20 with LAMP1.
(7) Set SIGNAL to ABS.
(8) Adjust lamp/energy to 50 with GAIN.
(9) Maximise lamp/energy by moving calcium bulb within its mounting and by adjusting the two position knobs.
(10) Set lamp/energy to 75.
(11) Return burner head to lowest setting with horizontal alignment knob.
(12) Clear absorption reading with AZ.
(13) Rise burner head until a reading is obtained on absorption display, then lower burner until display read zero.

(b) Ignition of flame and warm-up procedure

(1) Open acetylene bottle, switch on extractor-fan and air compressor.
(2) Place sample tube in de-ionised water.
(3) Turn on air, fuel and ignite flame. Ensure that fuel flow=20 and air flow=50.
(4) Allow to warm-up for 15-30 minutes.
(5) Maximise flame position with flame orientation knob and vertical burner alignment knob.
(6) Maximise appropriation rate of sample tube.

(c) Calibration and sample measurement

(1) Place sample tube in de-ionised water and ensure that lamp energy is still set at 75.
(2) Set SIGNAL to CONC and MODE to HOLD.
(3) Key in: 2.0 S1, 4.00 S2, 2.0 t
(4) Calibrate machine by aspirating prepared standards (lowest first) and keying S1/S2 then set MODE to CONT.
(5) Re-check each standard measure samples (ensure lamp/energy remains at 75 and recheck standard often.

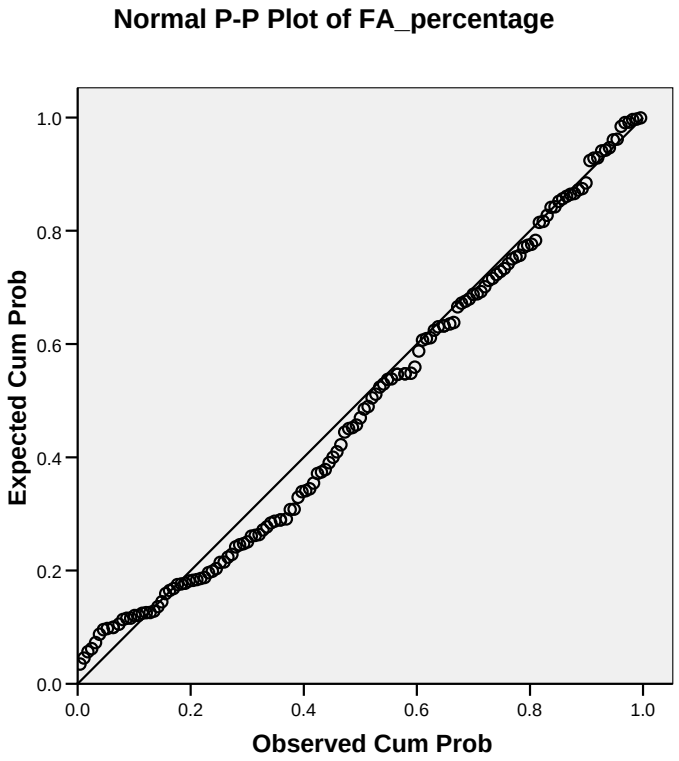
(d) Shut down procedure

(1) Place sample tube in deionised water for several seconds.
(2) Turn off fuel, air, and compressor and extractor fan.
(3) Turn GAIN and LAMP1 to zero.
(4) Close acetylene bottle.

Calculation of total serum calcium and expression of result

Serum calcium levels recorded from the atomic absorption spectrophotometer were multiplied by the dilution used (1:150) and divided by 10 to obtain the total plasma calcium concentration (expressed as mg %).

Appendix 6 Normality plot of fatty acid analysis using SPSS



Appendix 7 Fatty acid analyses in eggs

Appendix Table 7.1 Total mean MUFA in eggs from *O. niloticus* fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in MUFA (data are mean ± SD, n=3 and P<0.05).

Diet	Ration	Spawning No.	Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
			Spawning No.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				Ns																	
2	1	3		1	1	x															
				Ns	NS																
2	2	1		1	0.9999	1	x														
				Ns	NS	NS															
2	2	2		1	0.9898	1	1	x													
				Ns	NS	NS	NS														
2	2	3		1	0.9967	1	1	1	x												
				Ns	NS	NS	NS	NS													
3	1	1		1	1	1	1	1	1	x											
				Ns	Ns	NS	NS	NS	NS												
3	1	2		1	0.9999	1	1	1	1	1	x										
				Ns	NS	NS	NS	NS	NS	NS											
3	1	3		1	1	1	1	1	1	1	1	x									
				Ns	NS	NS	NS	NS	NS	NS	NS										
3	2	1		1	1	1	0.9989	0.9636	0.9987	0.9998	0.9994	1	x								
				Ns	NS	NS	NS	NS	NS	NS	NS	Ns									
3	2	2		1	1	1	1	1	1	1	1	1	1	x							
				Ns	Ns	NS	NS	NS	NS	NS	Ns	NS	Ns								
3	2	3		1	1	1	1	1	1	1	1	1	1	1	x						
				Ns	NS	NS	NS	NS	NS	NS	NS	Ns	NS	NS							
4	1	1		0.011	0.0717	0.0088	0.0006	0.0001	0.00009	0.0032	0.0021	0.0057	0.1236	0.0097	0.0088	x					
				S	NS	S	S	S	S	S	S	S	NS	S	S						
4	1	2		0.0369	0.1562	0.0307	0.0045	0.0006	0.0043	0.0138	0.0098	0.0219	0.2349	0.0333	0.0309	1	x				
				S	NS	S	S	S	S	S	S	S	NS	S	S	NS					
4	1	3		0.4341	0.8442	0.3871	0.098	0.0162	0.0829	0.2224	0.1711	0.3096	0.9293	0.407	0.38888	0.981	0.9902	x			
				NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS				
4	2	1		0.0002	0.002	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0042	0.0002	0.0002	0.9975	0.9999	0.3062	x		
				S	S	S	S	S	S	S	S	S	S	S	S	NS	NS	NS			
4	2	2		0.7127	0.9748	0.6631	0.243	0.0501	0.1961	0.4486	0.3665	0.5706	0.994	0.6846	0.6649	0.7508	0.8679	1	0.0536	x	
				Ns	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
4	2	3		0.3457	0.3911	0.7389	0.6342	0.046	0.4992	1	0.9842	0.9781	0.7037	0.9343	1	0.999	1	0.8697	0.5134	1	x
				NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Appendix Table 7.2 The mean arachidonic acid (ARA) in eggs from O. niloticus fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in ARA (data are mean ± SD, n=3 and P<0.05).

			Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
Diet	Ration	Sp no.	Sp no.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		X																	
2	1	2		1	X																
				NS																	
2	1	3		0.8172	0.9648	x															
				NS	NS																
2	2	1		0.4043	0.7097	1	x														
				NS	NS	NS															
2	2	2		0.9998	1	0.9988	0.9256	x													
				NS	NS	NS	NS														
2	2	3		0.7734	0.9443	1	1	0.9967	x												
				NS	NS	NS	NS	NS													
3	1	1		0.0001	0.0001	0.046	0.0853	0.0002	0.107	x											
				S	S	S	NS	S	NS												
3	1	2		0.0001	0.1786	0.0003	0.0004	0.0001	0.001	0.9897	x										
				S	NS	NS	S	S	S	NS											
3	1	3		0.0641	0.7273	0.9945	0.9999	0.3491	0.9992	0.7475	0.0357	x									
				NS	NS	NS	NS	NS	NS	NS	S										
3	2	1		0.4405	0.0002	1	1	0.9305	1	0.1916	0.0019	1	x								
				NS	S	NS	NS	NS	NS	NS	S	NS									
3	2	2		0.0001	0.9207	0.06	0.1105	0.0003	0.1333	1	0.9811	0.8041	0.2345	x							
				S	NS	NS	NS	S	NS	NS	S	NS	NS								
3	2	3		0.7118	0.0001	1	1	0.994	1	0.0744	0.0005	0.9988	1	0.0954	x						
				NS	S	NS	NS	NS	NS	NS	S	NS	NS	NS							
4	1	1		0.0001	0.0001	0.0002	0.0003	0.0001	0.0009	0.9989	1	0.042	0.0017	0.9972	0.0004	x					
				S	S	S	S	S	S	NS	NS	S	S	NS	S						
4	1	2		0.0001	0.0001	0.0031	0.0057	0.0001	0.0096	1	1	0.1893	0.019	0.9999	0.0056	1	x				
				S	S	S	S	S	S	NS	NS	NS	S	NS	S	NS					
4	1	3		0.0001	0.0001	0.0083	0.0149	0.0001	0.0283	1	0.9926	0.4631	0.0555	1	0.0156	0.9994	1	x			
				S	S	S	S	S	S	NS	NS	NS	NS	NS	S	NS	NS				
4	2	1		0.0001	0.0001	0.0055	0.0099	0.0001	0.0189	1	0.9994	0.3544	0.0375	1	0.00103	1	1	1	x		
				S	S	S	S	S	S	NS	NS	NS	S	NS	S	NS	NS	NS			
4	2	2		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.5496	1	0.0004	0.0001	0.4733	0.0001	0.9959	0.9997	0.4771	0.7081	x	
				S	S	S	S	S	S	NS	NS	S	S	NS	S	NS	NS	NS	NS		
4	2	3		0.0001	0.0001	0.0001	0.001	0.0001	0.0001	0.7703	1	0.0011	0.0001	0.7003	0.0001	0.9999	0.9999	0.7279	0.9159	1	x
				S	S	S	S	S	S	NS	NS	S	S	NS	S	NS	NS	NS	NS	NS	

Appendix Table 7.3 Total mean n-6 in eggs from O. niloticus fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in total n-6 (data are mean ± SD, n=3 and $P<0.05$).

Diet	Ration	Spawning No	Spawning No	2 1 1	2 1 2	2 1 3	2 2 1	2 2 2	2 2 3	3 1 1	3 1 2	3 1 3	3 2 1	3 2 2	3 2 3	4 1 1	4 1 2	4 1 3	4 2 1	4 2 2	4 2 3
2	1	1		x																	
2	1	2		1 NS	x																
2	1	3		0.808 NS	0.6816 NS	x															
2	2	1		0.9928 NS	0.9715 NS	1 NS	x														
2	2	2		1 NS	1 NS	0.3367 NS	0.7966 NS	x													
2	2	3		0.999 NS	0.9946 NS	1 NS	1 NS	0.944 NS	x												
3	1	1		0.0001 S	0.0001 S	0.0012 S	0.0001 S	0.0001 S	0.0001 S	x											
3	1	2		0.0001 S	0.0001 S	0.0228 S	0.0005 S	0.0001 S	0.0024 S	1 NS	x										
3	1	3		0.0028 S	0.0014 S	0.6523 NS	0.1073 NS	0.0001 S	0.1959 NS	0.6574 NS	0.9894 NS	x									
3	2	1		0.3764 NS	0.259 NS	1 NS	0.9912 NS	0.068 NS	0.9946 NS	0.0111 S	0.1342 NS	0.9584 NS	x								
3	2	2		0.004 S	0.0002 S	0.2954 NS	0.0218 S	0.0001 S	0.0549 NS	0.9328 NS	0.9999 NS	1 NS	0.7276 NS	x							
3	2	3		0.0189 S	0.0098 S	0.941 NS	0.3854 NS	0.0009 S	0.5192 NS	0.2792 NS	0.8406 NS	1 NS	0.9994 NS	0.9998 NS	x						
4	1	1		0.004 S	0.0018 S	0.8401 NS	0.1744 NS	0.0001 S	0.3165 NS	0.1846 NS	0.7769 NS	1 NS	0.9961 NS	0.9998 NS	1 NS	x					
4	1	2		0.0001 S	0.0001 S	0.0266 S	0.0008 S	0.0001 S	0.0031 S	1 NS	1 NS	0.9857 NS	0.142 NS	0.9999 NS	0.8286 NS	0.7697 NS	x				
4	1	3		0.0001 S	0.0001 S	0.131 NS	0.0036 S	0.0001 S	0.0162 S	0.9051 NS	0.9999 NS	1 NS	0.5056 NS	1 NS	0.9984 NS	0.9974 NS	0.9999 NS	x			
4	2	1		0.2641 NS	0.1669 NS	1 NS	0.9804 NS	0.0292 S	0.9893 NS	0.0046 S	0.0799 NS	0.9319 NS	1 NS	0.6312 NS	0.9988 NS	0.9916 NS	0.0894 NS	0.3727 NS	x		
4	2	2		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	1 NS	0.9911 NS	0.1513 NS	0.0002 S	0.4783 NS	0.0264 S	0.0073 S	0.9981 NS	0.3279 NS	0.0001 S	x	
4	2	3		0.0001 S	0.0001 S	0.0117 S	0.0001 S	0.0001 S	0.0009 S	0.9962 NS	1 NS	0.9969 NS	0.102 NS	1 NS	0.8759 NS	0.7891 NS	1 NS	1 NS	0.0459 S	0.7168 NS	x

Appendix Table 7.4 The mean EPA in eggs from O. niloticus fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in EPA (data are mean ± SD, n=3 and P<0.05).

			Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
Diet	Ration	Spawning	Spawning	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				NS																	
2	1	3		1	1	x															
				NS	NS																
2	2	1		1	1	1	x														
				NS	NS	NS															
2	2	2		1	1	1	0.999	x													
				NS	NS	NS	NS														
2	2	3		1	1	1	1	1	x												
				NS	NS	NS	NS	NS													
3	1	1		0.9786	0.9853	0.9956	0.9995	0.7863	0.991	x											
				NS	NS	NS	NS	NS	NS												
3	1	2		0.9998	0.9999	1	1	0.985	1	1	x										
				NS	NS	NS	NS	NS	NS	NS											
3	1	3		0.9999	1	1	1	0.9893	1	1	1	x									
				NS	NS	NS	NS	NS	NS	NS	NS										
3	2	1		1	1	1	1	1	1	0.9994	1	1	x								
				NS	NS	NS	NS	NS	NS	NS	NS	NS									
3	2	2		1	1	1	1	0.9987	1	1	1	1	1	x							
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS								
3	2	3		0.9668	0.9765	0.9992	0.9989	0.7338	0.9853	1	1	1	0.9987	1	x						
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS							
4	1	1		0.063	0.0757	0.2354	0.1523	0.0041	0.1202	0.9531	0.6537	0.6177	0.2144	0.4724	0.9697	x					
				NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS	NS						
4	1	2		0.5723	0.6149	0.869	0.8297	0.2047	0.6858	1	0.9931	0.9905	0.8487	0.9711	1	1	x				
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
4	1	3		0.0049	0.0062	0.0287	0.0125	0.0002	0.0128	0.5083	0.1623	0.1441	0.0245	0.0867	0.5691	1	0.9934	x			
				S	S	S	S	S	S	NS	NS	NS	S	NS	NS	NS	NS				
4	2	1		0.0011	0.0014	0.0071	0.0027	0.0001	0.0031	0.2222	0.0506	0.0439	0.006	0.0243	0.2632	0.9926	0.9068	1	x		
				S	S	S	S	S	S	NS	NS	S	S	S	NS	NS	NS	NS			
4	2	2		0.1069	0.1273	0.3586	0.2472	0.007	0.1936	0.99	0.8089	0.7785	0.3263	0.6393	0.9948	1	1	0.9997	0.9082	x	
				NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		

Appendix Table 7.5 The mean DHA in eggs from *O. niloticus* fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in DHA (data are mean ± SD, n=3 and P<0.05).

Diet	Ration	Spawning No	Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
			Spawning	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				NS																	
2	1	3		1	0.9996	x															
				NS	NS																
2	2	1		1	1	1	x														
				NS	NS	NS															
2	2	2		1	1	0.9664	0.9963	x													
				NS	NS	NS	NS														
2	2	3		0.9995	0.9925	1	1	0.8655	x												
				NS	NS	NS	NS	NS													
3	1	1		0.0035	0.0011	0.0521	0.0064	0.0001	0.1942	x											
				S	S	NS	S	S	NS												
3	1	2		0.1214	0.0531	0.5879	0.2177	0.0041	0.8776	0.9996	x										
				NS	NS	NS	NS	S	NS	NS											
3	1	3		0.1578	0.0719	0.6665	0.2764	0.0041	0.9181	0.9987	1	x									
				NS	NS	NS	NS	S	NS	NS	NS										
3	2	1		0.0001	0.0001	0.008	0.0001	0.0062	0.0062	0.999	0.6493	0.5657	x								
				S	S	S	S	S	S	NS	NS	NS									
3	2	2		0.0012	0.0004	0.0211	0.0021	0.0001	0.097	1	0.9933	0.9857	1	x							
				S	S	S	S	S	NS	NS	NS	NS	NS								
3	2	3		0.7297	0.0001	0.0001	0.9001	0.0001	0.9999	0.776	0.9999	1	0.08881	0.5708	x						
				NS	S	S	NS	S	NS	NS	NS	NS	NS	NS							
4	1	1		0.0001	0.0001	0.0001	0.001	0.1317	0.0001	0.0001	0.0001	0.0001	0.0006	0.0001	0.0001	x					
				S	S	S	S	NS	S	S	S	S	S	S	S						
4	1	2		0.0001	0.0001	0.0001	0.001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0012	0.0001	0.0001	1	x				
				S	S	S	S	S	S	S	S	S	S	S	S	NS					
4	1	3		0.0001	0.0001	0.0001	0.001	0.001	0.0001	0.0111	0.0001	0.0001	0.3811	0.0317	0.0001	0.6003	0.5429	x			
				S	S	S	S	S	S	S	S	S	NS	S	S	NS	NS				
4	2	1		0.0001	0.0001	0.0001	0.001	0.001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.021	0.2706	0.0001	x		
				S	S	S	S	S	S	S	S	S	S	S	S	S	NS	S			
4	2	2		0.0001	0.0001	0.0001	0.001	0.001	0.0001	0.0673	0.0009	0.0006	0.8148	0.1602	0.0001	0.0964	0.1187	1	0.0001	x	
				S	S	S	S	S	S	NS	S	S	NS	NS	S	NS	NS	NS	S		
4	2	3		0.0001	0.0001	0.0001	0.001	0.001	0.0001	0.0001	0.0001	0.0001	0.0023	0.001	0.0001	1	0.9998	0.923	0.0007	0.3117	x
				S	S	S	S	S	S	S	S	S	S	S	S	NS	NS	NS	S	NS	

Appendix Table 7.6 Total mean n-3 in eggs from *O. niloticus* fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in total n-3 (data are mean ± SD, n=3 and P<0.05).

Diet	Ration	Spawning No	Spawning No	2 1 1	2 1 2	2 1 3	2 2 1	2 2 2	2 2 3	3 1 1	3 1 2	3 1 3	3 2 1	3 2 2	3 2 3	4 1 1	4 1 2	4 1 3	4 2 1	4 2 2	4 2 3
2	1	1		x																	
2	1	2		1 NS	x																
2	1	3		0.9603 NS	0.9994 NS	x															
2	2	1		1 NS	1 NS	0.9998 NS	x														
2	2	2		1 NS	0.9985 NS	0.5053 NS	0.9845 NS	x													
2	2	3		0.9965 NS	1 NS	1 NS	1 NS	0.8096 NS	x												
3	1	1		0.0007 S	0.0051 S	0.1719 NS	0.0034 NS	0.0001 S	0.1142 S	x											
3	1	2		0.1304 NS	0.4037 NS	0.9878 NS	0.3986 NS	0.0068 S	0.9526 NS	0.9792 NS	x										
3	1	3		0.1635 NS	0.469 NS	0.9938 NS	0.468 NS	0.0096 S	0.9705 NS	0.9646 NS	1 NS	x									
3	2	1		0.0003 S	0.0021 S	0.0944 NS	0.0013 S	0.0001 S	0.0613 NS	1 NS	0.9275 NS	0.8937 NS	x								
3	2	2		0.0009 S	0.0065 S	0.2017 NS	0.0044 S	0.0001 S	0.1351 NS	1 NS	0.9867 NS	0.9761 NS	1 NS	x							
3	2	3		0.7104 NS	0.9584 NS	1 NS	0.9693 NS	0.1592 NS	1 NS	0.491 NS	1 NS	1 NS	0.3281 NS	0.5422 NS	x						
4	1	1		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	x					
4	1	2		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	1 NS	x				
4	1	3		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.02 S	0.0001 S	0.0001 S	0.0451 S	0.0156 S	0.0001 S	0.2847 NS	0.6356 NS	x			
4	2	1		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0155 S	0.0644 NS	0.0001 S	x		
4	2	2		0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0001 S	0.0173 S	0.0001 S	0.0001 S	0.0406 S	0.0133 S	0.0001 S	0.1698 NS	0.5127 NS	1 NS	0.0001 S	x	
4	2	3		0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.0001 s	0.999 Ns	1 Ns	0.9195 Ns	0.0001 s	0.8244 s	x

Appendix Table 7.7 Total mean PUFA in eggs from *O. niloticus* fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in total PUFA (data are mean ± SD, n=3 and $P<0.05$).

Diet	Ration	Spawning No	Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
			Spawn No	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				NS																	
2	1	3		1	1	x															
				NS	NS																
2	2	1		1	1	1	x														
				NS	NS	NS															
2	2	2		1	1	1	1	x													
				NS	NS	NS	NS														
2	2	3		1	1	1	1	1	x												
				NS	NS	NS	NS	NS													
3	1	1		1	1	1	1	1	1	x											
				NS	NS	NS	NS	NS	NS												
3	1	2		1	1	1	1	1	1	1	x										
				NS	NS	NS	NS	NS	NS	NS											
3	1	3		1	1	1	1	1	1	1	1	x									
				NS	NS	NS	NS	NS	NS	NS	NS										
3	2	1		0.5213	0.8527	0.7284	0.3222	0.2039	0.8722	0.8022	0.2619	0.698	x								
				NS	NS	NS	NS	NS	NS	NS	NS	NS									
3	2	2		0.9989	1	1	0.9935	0.9773	1	1	0.9763	0.9999	0.9977	x							
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS								
3	2	3		1	1	1	1	1	1	1	1	1	0.413	0.9958	x						
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS							
4	1	1		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0125	0.0001	0.0001	x					
				S	NS	S	S	S	S	S	S	S	S	S	S						
4	1	2		0.0005	0.0027	0.0013	0.0001	0.0001	0.0043	0.002	0.0001	0.0011	0.5263	0.0288	0.0003	0.9997	x				
				S	NS	S	S	S	S	S	S	S	NS	S	S	NS					
4	1	3		0.0051	0.0318	0.0152	0.0009	0.0003	0.0476	0.0231	0.001	0.0129	0.9876	0.2499	0.0028	0.3308	0.998	x			
				S	NS	S	S	S	S	S	S	S	NS	NS	S	NS	NS				
4	2	1		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0369	0.0059	0.0001	x		
				S	NS	S	S	S	S	S	S	S	S	S	S	S	S	S			
4	2	2		0.1467	0.4573	0.3004	0.0469	0.0183	0.5209	0.3842	0.0434	0.2716	1	0.9412	0.0948	0.0041	0.5204	0.994	0.0001	x	
				NS	NS	NS	S	S	S	NS	S	NS	NS	NS	NS	S	NS	NS	S		
4	2	3		0.0001	0.0006	0.0003	0.0001	0.0001	0.0013	0.0004	0.0001	0.0002	0.5136	0.012	0.0001	0.8919	1	0.9998	0.0001	0.4369	x
				S	S	S	S	S	S	S	S	S	NS	S	S	NS	NS	NS	S	NS	

Appendix Table 7.8 The n-3:n-6 ratio in eggs from *O. niloticus* fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in n-3:n-6 ratio (data are mean ± SD, n=3 and $P<0.05$).

Diet	Ration	Spawning No.	Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
			Spawn No	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				NS																	
2	1	3		0.7473	0.953	x															
				NS	NS																
2	2	1		0.9999	1	0.9961	x														
				NS	NS	NS															
2	2	2		1	0.999	0.1656	0.9182	x													
				NS	NS	NS	NS														
2	2	3		0.9842	0.9997	1	1	0.6349	x												
				NS	NS	NS	NS	NS													
3	1	1		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	x											
				S	S	S	S	S	S												
3	1	2		0.0001	0.0002	0.0607	0.0003	0.0001	0.0168	0.586	x										
				S	S	NS	S	S	S	NS											
3	1	3		0.0014	0.0073	0.586	0.0154	0.0001	0.2741	0.0607	0.9998	x									
				S	S	NS	S	S	NS	NS	NS										
3	2	1		0.0001	0.0001	0.033	0.0001	0.0001	0.0087	0.7323	1	0.998	x								
				S	S	S	S	S	S	NS	NS	NS									
3	2	2		0.0001	0.0001	0.0035	0.0001	0.0001	0.0009	0.9834	1	0.885	1	x							
				S	S	S	S	S	S	NS	NS	NS	NS								
3	2	3		0.0532	0.1766	0.9964	0.3248	0.0015	0.9198	0.0017	0.7764	0.9998	0.6363	0.2064	x						
				NS	NS	NS	NS	S	NS	S	NS	NS	NS	NS							
4	1	1		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0016	0.0001	0.0001	0.0001	0.0001	0.0001	x					
				S	S	S	S	S	S	S	S	S	S	S	S						
4	1	2		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.9018	x				
				S	S	S	S	S	S	S	S	S	S	S	S	NS					
4	1	3		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.1326	0.0001	0.0001	0.0001	0.0005	0.0001	0.9858	0.1319	x			
				S	S	S	S	S	S	NS	S	S	S	S	S	NS	NS				
4	2	1		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.3846	1	0.0068	x		
				S	S	S	S	S	S	S	S	S	S	S	S	NS	NS	S			
4	2	2		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	1	0.9951	0.6832	0.7436	x	
				S	S	S	S	S	S	S	S	S	S	S	S	NS	NS	NS	NS		
4	2	3		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	1	0.9977	0.5519	0.8192	1	x
				S	S	S	S	S	S	S	S	S	S	S	S	NS	NS	NS	NS	NS	

Appendix Table 7.9 ARA: EPA ratio in eggs from O. niloticus fed different dietary lipid sources. The results are interaction between diets, rations and spawnings. The diets are considered: diet 2 containing palm oil; diet 3 containing mixed palm and cod liver oil (9:1); and diet 4 a control diet. The diet, ration and spawning number (Diet, ration Spawning no.) given both along the horizontal and vertical axis of the table are compared at each single point to asses whether there were significant (s) or non-significant (ns) in ARA:EPA ratio (data are mean ± SD, n=3 and P<0.05).

Diet	Ration	Spawning No.	Diet	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4
			Ration	1	1	1	2	2	2	1	1	1	2	2	2	1	1	1	2	2	2
			Spawn No	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	1		x																	
2	1	2		1	x																
				NS																	
2	1	3		1	0.9844	x															
				NS	NS																
2	2	1		1	0.9987	1	x														
				NS	NS	NS															
2	2	2		1	1	0.9669	0.9965	x													
				NS	NS	NS	NS														
2	2	3		1	1	0.9997	1	1	x												
				NS	NS	NS	NS	NS													
3	1	1		0.9792	0.7301	1	0.9996	0.5947	0.9536	x											
				NS	NS	NS	NS	NS	NS												
3	1	2		0.983	0.7511	1	0.9997	0.6204	0.9603	1	x										
				NS	NS	NS	NS	NS	NS	NS											
3	1	3		1	1	0.999	1	1	1	0.9095	0.9208	x									
				NS	NS	NS	NS	NS	NS	NS	NS										
3	2	1		1	1	0.9965	0.9999	1	1	0.8479	0.8636	1	x								
				NS	NS	NS	NS	NS	NS	NS	NS	NS									
3	2	2		0.9993	0.9358	1	1	0.8805	0.9968	1	1	0.991	0.9774	x							
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS								
3	2	3		1	0.9993	1	1	0.9983	1	0.9998	0.9999	1	0.9999	1	x						
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS							
4	1	1		0.9787	0.6862	1	0.9997	0.5067	0.9513	1	1	0.8973	0.8241	1	0.9999	x					
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS						
4	1	2		1	0.9991	1	1	0.9981	1	1	1	1	0.9999	1	1	1	x				
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
4	1	3		0.9094	0.4761	1	0.9939	0.2919	0.8496	1	1	0.7428	0.6358	1	0.9978	1	0.9994	x			
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS				
4	2	1		0.7337	0.2726	0.9986	0.9416	0.1403	0.65	1	1	0.5102	0.4039	1	0.9723	1	0.989	1	x		
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS			
4	2	2		0.7543	0.2657	0.9995	0.9553	0.1195	0.6711	1	1	0.5183	0.4048	1	0.9817	1	0.9938	1	1	x	
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		
4	2	3		1	0.9814	1	1	0.9482	0.9999	0.9999	1	0.9993	0.9966	1	1	1	1	0.9978	0.9618	0.9714	x
				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

EFFECTS OF DIETARY LIPID SOURCE ON EGG AND LARVAL QUALITY OF NILE TILAPIA, *OREOCHROMIS NILOTICUS* (L.)

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Abstract

This study investigated the effects of dietary lipid sources on reproductive performance of Nile tilapia for three consecutive spawnings with the goal of replacing dietary fish oil with palm oil. In this study tilapia were fed solely with the selected experimental diet during their entire life, from onset of exogenous feeding until termination of spawning. Three isonitrogenous (40% crude protein), isoenergitic (20 KJg^{-1}) experimental diets were made containing either 10% cod liver oil (CO), palm oil (PO) or mixed palm and cod liver oil (9:1 ratio; PO&CO) using soybean protein concentrate as the protein source. In addition a commercial trout diet was used as a control. The influence of dietary lipid on spawning intervals, fecundity, relative fecundity (egg number per unit weight), egg size, fertilisation and hatching rate and larval quality was investigated. Dietary lipid sources had no significant effect on egg diameter, egg volume and egg dry weight. However, relative fecundity was significantly ($P<0.05$) different in fish fed control diet while those fish fed PO and mixed PO&CO were not different ($P>0.05$). Similar results were observed for egg weight to body weight ration (EW: BW) and inter spawning interval (ISI) for fish fed diet 4. Moreover, total fecundity (number of eggs produced per fish) obtained from fish fed the mixed oil diet (PO & CO) was significantly ($P<0.05$) higher than for those fed the palm oil and control diets. This study suggests that palm oil can replace fish oil with no negative effect on egg and larval quality in *O. niloticus*.

Keywords: Nile tilapia, *Oreochromis niloticus*, Diet, Lipid, Reproduction, Egg and larval quality

INTRODUCTION

The Nile tilapia, *Oreochromis niloticus* is a widely cultured species because it grows and reproduces under a wide range of environmental conditions and tolerates handling stress. Tilapias perform well in extensive, semi-intensive and intensive culture systems. Farmed Nile tilapia production reached 1,703,125 mt, about 84% of total farmed tilapia production, in 2006 (FAO, 2006). Tilapias are now the second most popularly farmed fish after carps, and currently tilapia are cultured in about 100 countries in tropical and subtropical regions. One of the most important aspects in fish seed production is production of fertilized eggs that result in larvae with high survival and growth. Broodstock nutrition affects reproduction and egg and larval quality in fish (Izquierdo *et al.,* 2001). Some feed components are known to greatly

influence spawning quality in several species (Verakunpiriya *et al.,* 1996; Watanabe *et al.,* 1985).

Broodstock productivity remains one of the most significant constraints to commercial production costs and thus knowledge of factors affecting broodstock productivity is of immense importance to further development of tilapia culture. In particular, lipids and essential fatty acids (EFA) are nutritional factors that greatly affect egg and larval quality (Fernandez-Palacios *et al.,* 1995; Furuita *et al.,* 2000; Harel *et al.,* 1994; Navas *et al.,* 1997; Watanabe *et al.,* 1984; Watanabe *et al.,* 1985). Nevertheless, marine fish oils are traditionally used as the main dietary lipid source in many commercial fish feeds. Aquafeeds currently use about 70% of the global supply of fish oil and by the year 2010, fish oil use in aquaculture is estimated to reach about 97% of the world supply (Tacon, 2003).In order to sustain rapid aquaculture development, the industry cannot continue to rely on finite stocks of marine pelagic fish for oil supply. However, one potential replacement for fish oil in aquafeeds is palm oil. In this respect, palm oil is similar to other vegetable oils that have been reported in numerous scientific papers to be able to replace a significant part of fish oil in fish diets without negatively affecting fish growth, feed utilization and survival (Al-Owafeir and Belal, 1996; Bell *et al.,* 2002; Legendre *et al.,* 1995; Ng *et al.,* 2000; Ng *et al.,* 2006; Ng *et al.,* 2003; Ng and Low, 2005; Tortensen *et al.,* 2000). Nevertheless, in addition to its low cost and high availability, palm oil also has many additional advantages over other vegetable oils when used in aqua-feed formulation (Ng *et al.,* 2004).

The effect of dietary lipid source on spawning performance of tilapias has not been sufficiently studied. Only Santiago and Reyes (1993) studied the effects of dietary lipid source on reproductive performance and tissue lipids of Nile tilapia. They found that cod liver oil (rich in *n*–3 HUFA) resulted in poor reproductive performance, while highest fry production was obtained from fish fed a diet supplement with soybean oil (rich in n-6 fatty acids) and El-Sayed *et al*. (2005) studied the effect of dietary lipid source on spawning performance at different salinities and found that tilapia need fish oil for better reproductive performance in brackish water while plant oil (soybean oil) is required for freshwater rearing. However, dietary lipid sources have not been examined under one culture system, including serial spawning and over the entire life cycle of fish. This study investigated the effect of different dietary lipid sources on egg and larval quality over three consecutive spawnings in Nile tilapia *O. niloticus* which had bean reared for their entire life cycle on their respective diet regime in a recirculating system.

MATERIALS AND METHODS

Diet preparation

Three experimental diets in this study were made at the Institute of Aquaculture, University of Stirling. The dry ingredients and the proximate composition for these are presented in (Table 1) and (Table 2), respectively. The dry ingredients were first mixed for approximately 30 minutes using a Hobart mixer to ensure that the mixture was well homogenised and then blended by adding 10% oil from cod liver oil (CO), palm oil (PO) or a mixture of PO and CO (9:1 ratio), respectively for further 15 minutes. Water was added at 20-30% V/W to give a pelletable mixture. Diets were made as pellets of appropriate size using a California pellet mill (model CL2, San Francisco, California).

Table 1. Feed ingredients and formulation of experimental diets (g/100g total diet)

Ingredients	Diet 1	Diet 2	Diet 3
Soybean concentrate	55	55	55
Casein	0.5	0.5	0.5
Corn starch	17.5	17.5	17.5
Cod liver oil	10	---	---
Palm oil	---	10	---
PO& CO (9:1)*	---	---	10
DCP**	2	2	2
Fish hydrolysate	5	5	5
DL-methionine	0.5	0.5	0.5
Vitamin premix	2	2	2
Minerals premix	2	2	2
Carboxy methyl cellulose	3.5	3.5	3.5
α-cellulose	2	2	2
TOTAL	100	100	100

PO &CO = combination of cod liver and palm oil**DCP= dicalcium phosphate.

Table 2. Proximate composition of experimental diets (composition of diet expressed as %)

Proximate analysis	Diet 1	Diet 2	Diet 3
Dry mater	15.1±0.18	14.3±1.05	14.2±1.1
Crude protein	40.5±.0.28	41.01±0.07	40.8±0.19
Crude lipid	10±0.15	9.8±1.05	9.7±1.25
Carbohydrate	24.11	22.2	23.1
Ash	5.3 ±0.006	5.3 ±0.045	5.1±0.25
Crude fibre	7.7±01	7.3±.79	7.3±.84
Gross energy (KJg^{-1})	20.4±0.121	20.4±0.112	20.3±0.23

Culture system and experimental design

Female broodstock were maintained in glass tanks, each tank incorporated two, three or four (depending on fish size) vertical dividers constructed from translucent Perspex, thus respectively creating three or four separately partitioned 'holding spaces' within each tank into which female broodstock could be introduced and maintained individually (Coward and Bromage, 1999). All fish were maintained in gravity-fed recirculation aquaria incorporating various sizes of covered fish holding tanks linked to several settling tanks, faecal traps and filtration units incorporating filter brushes and bio-rings (Dryden aquaculture, UK) for particulate filtration and maximizing bio-filtration. Water was pumped from the system collector tank to a sand filter tank and then sent to a header tank (227-l capacity) via a water pump (Beresford Pumps, UK) (Figure 1).

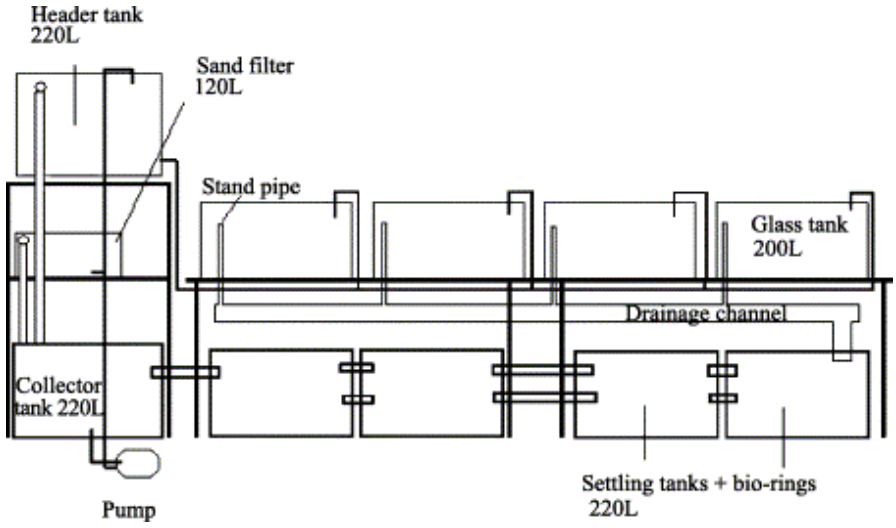


Figure 1. Lateral view of the closed recirculating system used to hold experimental fish

Water temperature was maintained at 27±1 °C (using a 3-kW thermostatically controlled water heater). Water was oxygenated via airstones in the header tank and each aquarium by a low-pressure blower. The water inflow was constant at 252 l h^{-1} $tank^{-1}$. Water quality was monitored twice a month, including dissolved oxygen (O_2) and water temperature. The levels of pH, nitrate, nitrite and ammonia were evaluated with aquarium water quality kits (C-Test kits, New Aquarium Systems, UK). To maintain good water quality, a partial change of water (10% of total volume) was carried out once a week; the system was refilled with fresh, aerated and preheated water.

Before starting the experiment, the female broodstock, *O. niloticus* were previously reared under experimental conditions for their entire life from onset of exogenous feeding until spawning; female broodsocks were then collected randomly from their respective populations and measured (weight and total length) and tagged with Passive Integrated Transponder-PIT tags (Trovan, UK) under anaesthesia by immersion in 1:10 000 ethyl 4-aminobenzoate (Sigma, UK). The fish were allowed to recover completely in clean aerated water prior to being placed to their respective glass tank.

Fish were fed three times daily (9:13:17) at 3% of body weight with the experimental diets and a commercial pelleted trout feed (Skretting, UK) as a control.

Spawning investigation

Fish were checked at two hourly intervals during the day for the evidence of spawning. In females undergoing ovulation and oviposition the genital papilla were considerably swollen and extended. Fish were manually stripped under anaesthesia and eggs were fertilised on a Petri-dish by adding the sperm from males maintained under the same diet regime as well as the same method of females. Fish were measured and weighed prior to returning into experimental tank after recovering in clean aerated water and all data recorded.

Petri-dishes containing fertilised eggs were scanned using a scanner and the scanned picture analysed using MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) to determine total fecundity (Rana ,1988) where total fecundity is the number of eggs in a freshly spawned batch of eggs. Fertilised eggs were then placed in round-bottomed plastic containers (Rana, 1986) supplied with clean, U.V. sterilised water and left until hatching. A sub-sample of 50 eggs per spawning was taken, prior to incubation and each egg individually measured to the nearest 0.1mm with a dissecting microscope (Olympus Optical Ltd., U.K.) connected to a video camera by specific calibration utilising Image Pro software (Macromedia V. 4). Since tilapia eggs are ellipsoid it was important to measure both axes (long and short axis) in order to

calculate egg diameter and volume according to method of (Coward and Bromage, 1999). The fertilisation (%) and hatching rate (%) and inter-spawning-interval (ISI time elapsed between one spawn and the next) were also determined.

After measuring egg size, eggs were then weighed and subsequently oven dried at 70ºC for 24h. Mean egg dried weight was determined to the nearest 0.1mg. The EW: BW ratio was determined (Coward and Bromage, 1999).

(EW: BW= (EDW*TF/W*100)

Where: EW: BW= egg weight to body weight ratio (%), EDW=egg dry weight (mg), TF= total fecundity and W= fish weight (g).

Larval quality

Larvae from each individual fish at 10 days post-fertilisation were sacrificed by overdose of anaesthetic and weighed to the nearest 0.1mg. The length was also measured to the nearest 0.1mm utilising MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001).

Feeding procedure

In the present study four diets, including the control, were examined as shown in (Table 3). Diet 1(D1) containing cod liver oil , diet 2 (D2) containing palm oil , diet 3 (D3) containing palm an cod liver oil (9:1 ratio) and diet 4 (D4) a commercial trout feed containing fish meal and fish oil as control.

Table 3. Experimental design

Lipid source of diets	Diet	Protein source	Feeding rate (%BW $day^{-1)}$	No. replicate	Spawning no. per fish
Cod liver oil	D1	Soybean Concentrate	3	2	3
Palm oil	D2	Soybean Concentrate	3	2	3
Mixed PO*&CO** (9:1)	D3	Soybean Concentrate	3	2	3
Control	D4	Fish meal	3	2	3

*PO=palm oil and **CO=cod liver oil

Statistical analysis

Statistical analyses were performed using SPSS for windows (version 15) and Minitab (version 15). Statistical significance between treatments was evaluated at the 5% probability level. General linear model (GLM) ANOVA was used further analysis of data. Values are expressed as means ± S.E.M.

RESULTS

Fecundity and egg size

A total of 125 spawns were recorded over three consecutive spawnings for all diet treatments. In diet CO (diet 1) only one fish spawned three times, however, due to high mortality of fish from the previous phase of the experiment and poor egg quality data obtained from the group of fish fed diet 1 this was discarded from the analyses. Egg size and fecundity were analysed among the dietary treatment and spawning numbers using two-way ANOVA. As a result of no significant ($P>0.05$) interaction being observed between diet and spawning numbers, spawning data were pooled and analysed using GLM one-way ANOVA comparing differences between diet treatments. There were no significant ($P>0.05$) differences between egg diameter, egg volume, egg wet and dry weight and total egg volume from fish fed diet 2, 3 and 4, respectively (Table 4).

Relative fecundity ranged from 5.5± 1.84, 5.5 ± 2.17 and 3.6± 1.68 for fish fed diet 2, 3 and 4, respectively. However, a significant ($P<0.05$) difference occurred in relative fecundity for fish fed diet 4 (control) but for fish fed diets 2 and 3 were not significant ($P>0.05$) (Table 4). Similar results were observed when comparing the EW: BW which ranged from 1.4±0.06, 1.3±0.08 and 0.9±0.08 (Table 4). Mean total fecundity in the present study ranged from 629 to 823, the effect of dietary lipid source on total fecundity for fish fed diet 3 was significantly ($P<0.05$) higher than fish fed diet 2 and 4, respectively, but for fish fed diet 2 and 4 was not significant (Table 4).

Table 4. Spawning performance of *O. niloticus* fed different dietary lipid sources

Parameters	Treatments		
	Palm oil diet (Diet 2)	P&CL oil diet (9:1) (Diet 3)	Control (diet 4)
Total Fecundity	752.6±32.01^{b}	823.3±46.59^{a}	662.9±36.10^{b}
Relative fecundity (no. /g)	5.5±0.23^{a}	5.5±0.38^{a}	3.6±0.31^{b}
Egg Diameter (mm)	2.2±0.03^{a}	2.2±0.03^{a}	2.2±0.03^{a}
Egg volume (mm^3)	5.2±0.22^{a}	5.4±0.22^{a}	5.6±0.24^{a}
Total egg volume (mm^3)	3902.7±236.45^{a}	4385.7±267.11^{a}	3654.6±237.07^{a}
Egg dry weigh (mg)	2.6±0.05 ^{a}	2.5±0.09^{a}	2.7±0.09^{a}
Egg wet weight (mg)	6.1±0.1^{a}	6.1±0.16 ^{a}	6.6±0.21 ^{a}
EW: BW (%)	1.4±0.06^{a}	1.3±0.08^{a}	0.9±0.08^{b}
Fertilisation rate (%)	76.3±1.40 ^{a}	78.5±1.82 ^{a}	75.9±2.2 ^{a}
Hatchability (%)	59.5±1.04 ^{a}	60.1±1.75 ^{a}	61.4±1.35 ^{a}
ISI (day)	14±0.71 ^{a}	19±1.52 ^{b}	24±2.74 ^{c}

In each raw means with different superscripts are significantly different (ANOVA, Tukey's test, $P < 0.05$).

Data are means ± SEM of two replicates.

Larval quality

Larval batches of each fish were recorded individually for three consecutive spawnings and grouped as fish fed diet 2, 3 or 4 respectively. Mean values of larval length and weight were analysed using GLM two-way ANOVA. The effects of dietary lipid sources on larvae length and weight over three serial spawnings were significant. However, these significance levels were not constant and due to no significant difference in egg dry weights between treatments these slight differences could not be due to diets; therefore the larvae length and weight data were pooled together to determine mean differences between the diets. Table 5 shows that both larval length and weight from fish fed diet 2 were significantly ($P<0.05$) lower than for larvae obtained from fish fed diet 3 and 4 but between diet 3 and 4 the difference was not significant ($P>0.05$).

Table 5. Larval performance of Nile tilapia (*O. niloticus*) fed different dietary lipid sources over three consecutive spawning.

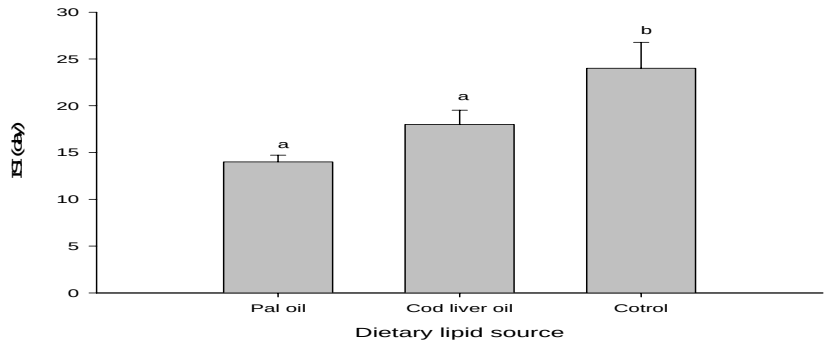
Parameters	Treatments		
	Diet 2	Diet 3	Diet 3
Larvae length (mm)	9.3 ± 0.64^{a}	9.6 ± 0.67^{bc}	9.5 ± 0.66^{c}
Larvae weight (mg)	9.8 ± 1.43^{a}	10.2 ± 1.57^{bc}	10.3 ± 1.61^{c}

Values are means ± S.D In each row means with different superscripts are statistically different (ANOVA, Tukey's test, $P<0.05$).

Inter spawning intervals (ISI)

The average spawning intervals in the present study ranged from 14-24 days. Significant ($P<0.05$) differences were detected when comparing ISI between the diet groups. The longest ISI was found in fish fed diet 4 (control) and the shortest was found for fish fed diet 2 (PO), however, ISI in fish fed diets 2 and 3 was not significantly ($P>0.05$) different.

Figure 2. Inter -Spawning- -Intervals (ISI day-1) of *O. niloticus* fed on different dietary lipid source



Values are mean ± S.E.M. In each column means with different superscripts are statistically different (ANOVA, Tukey's test, $P<0.05$).

DISCUSSION

One of the principal objectives of the present study was to investigate fish oil based diets, commonly used by industry, with alternative oil sources. Fish oil is produced from small marine pelagic fish and represents a finite fishery resource (Ng *et al.,* 2003). Because of several factors, including over fishing, resulting in dwindling catch and environmental changes which necessitate tight regulations, future demand for wild-caught fish will exceed supply (Sargent *et al.,* 1999). Hence the need to evaluate potential substitutes for fish oil, an important ingredient in the formulation of aquafeeds. Palm oil, currently the second most abundant vegetable oil in the world, presents a viable alternative to fish oil in aquafeeds (Ng, 2002).

A fishmeal based diet contains approximately 6-7% fish oil. Therefore to avoid any effect of fish oil in the experimental diet, the protein sources of diets were changed to soybean concentrate containing 65% protein and a trace amount of lipid. Previous studies revealed that palm oil could be used as a dietary lipid source with no negative effect on fish growth (Al-Owafeir and Belal, 1996; Bell *et al.,* 2002; Legendre *et al.,* 1995; Ng *et al.,* 2000; Ng *et al.,* 2006; Ng *et al.,* 2003; Ng *et al.,* 2004; Ng and Low, 2005; Tortensen *et al.,* 2000). However, limited information is available on the effect of lipid sources on tilapia reproductive performance. The present study is the first attempt to investigate the effect of dietary lipid source of the reproductive performance of tilapia fed solely their respective experimental diets for their entire life cycle. The present study shows that tilapia broodstock can be maintained and spawned successfully on different dietary lipid sources. The spawning performance of the Nile tilapia fed the two formulated dietary lipid sources (Palm and mixed PO&CO) was comparable to those fed a control diet. No significant differences were found in egg wet and dry weights, egg diameter and volume, fertilisation and hatching rate obtained the fish fed diet 2, 3 and 4 respectively. The fish group fed diet 1 (cod liver oil) had high mortality in the on-growing stage and only one fish spawned during the experiment which had poor egg quality; the growth gain was lower than other diets, this might be due to the high concentration of (n-3) HUFA in cod liver oil. The results of the present study are in agreement with the previous studies (Kanazawa *et al.,* 1980; Ng, 2004; Ng *et al.,* 2004; Takeuchi *et al.,* 1983) that reported depressed growth of tilapia with oils having high levels of n-3 PUFA and (Santiago and Reyes, 1993; Watanabe, 1982) who found that fish fed a cod liver oil diet had poor egg quality but this result contradicted the results of growth gain of tilapia that reported by Santiago and Reyes (1993). On the other hand the reason for lower growth gain could be due to the palatability of the diet which consisted of soybean meal and cod liver oil. However, further investigations are required to support this assumption.

Usually fertilised eggs of *O. niloticus* take about 4 days to hatch at 28°C and development time takes about 6 days (Macintosh and Little ,1995).In the present study, eggs from all treatments were kept at 28±1°C and 3-4 days were required for hatching and a further 6 days to absorb the yolk-sac . Usually, yolk-sac is absorbed gradually over 6 days after hatching at 28°C when eggs are orally incubated (Coward and Bromage, 1999; Macintosh and Little ,1995). The results showed that total fecundity of the group of fish fed the mixed oil diet was significantly higher than those fed palm oil or the control diet, this could be due to the ratio of n-6 and n-3. The results indicated that tilapia need tiny amounts of n-3 for growth and enhanced reproductive performance; similar results were found by Watanabe (1982) that Nile tilapia fed a basal diet supplemented with soybean oil (high in n-6 fatty acids) had higher fecundity, spawning frequency and fry production and that these were relatively lower in fish fed a 5% cod liver oil supplemented diet (high n-3 fatty acids). In support, Hung *et al* (1998) suggested that *n*–3 HUFA, such as linolenic, EPA and DHA are important for these fish. Similarly, Kanazawa *et al*. (1980) and El-sayed and Garling (1988) found that *T. zillii* reared in freshwater required *n*–6 fatty acids for optimum growth.

Larval quality

Larval length and weight were not significantly affected by parents' dietary lipid sources. Nevertheless, both weight and length of larvae from fish fed palm oil were slightly lower than in larvae from fish fed mixed oil or control diets. The lower weights and lengths from fish fed the palm oil diet could not be affected by diet because no significant difference occurred in egg dry weight. However, this significance could be due to genetic differences within the broodstock or other parameters.

Inter spawning interval (ISI)

Shortest ISI was observed in the group of fish fed the palm oil diet and the longest in fish fed control diet. In the present study there was no relationship between egg size and ISI, but it was apparent that large females had longest ISI and conversely small females the shortest ISI. This might simply imply that ISI was longer, and fish need more energy for maintenance and growth than producing eggs. This result agrees with Rana (1988) who reported that within a group of females of the same age class, there is no significant relationship between body size and egg size.

CONCLUSION

Dietary lipid source (palm oil) had no significant effect on egg and larval quality. In conclusion, the results of this study suggest that under controlled conditions, lipids

of non-marine origin, such as palm oil, can be used successfully for brood stock diets. In addition, comparable performance with commercial control diets and halving of feed requirement should increase profitability of seed production.

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