

Thesis
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**Regulatory Mechanisms Associated with Egg Production
and Egg Activation in *Oreochromis niloticus*.**

A thesis submitted for the Degree of
Doctor of Philosophy

By

Antonio Campos Mendoza

BSc Honours (Biology)
MSc (Conservation of Natural Resources)

Institute of Aquaculture
University of Stirling
Stirling, Scotland
U.K.

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DECLARATION

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ABSTRACT

This study investigated several important aspects of reproductive physiology in the Nile tilapia (*Oreochromis niloticus*). Although tilapias have been cultured for several decades, there has been a rapid increase in production by aquaculture over recent years. Tilapia have thus become one of the main teleosts contributing significantly to world aquaculture. However, significant problems exist that constrain the efficient management of reproduction in these fish. These include low fecundity, and the asynchronous, and hence unpredictable, nature of spawning cycles. Manipulation of photoperiod has proved to be a powerful tool in the control of reproductive cycles in various other fish species, and has become an established aspect of the culture of certain species. We know very little about the ways in which photoperiod might be involved in the control of reproduction in tilapiine species, especially the hormonal rhythms associated with this regulatory mechanism. In addition, manipulation of environmental parameters such as photoperiod has often been reported to influence fertilization rates in some teleosts. Spawning induction using the application of exogenous hormones, a technique often used in aquaculture to produce predictable spawning patterns, is also associated with problems concerning fertilization. It is clear from the literature that there is very little information available concerning the precise mechanisms involved with fertilization and egg activation in fish. Thus, the research described in this Thesis falls into two main sections. The first section investigates how photoperiod may impart a regulatory role over reproduction in *O. niloticus* broodstock, how circulating levels of the hormone melatonin vary in this species, and describes the isolation and partial characterization of a melatonin receptor from this species. In the second part of this Thesis, I have made a preliminary investigation of the precise mechanisms that might be involved at egg activation in fish using tilapia as a

research model; these studies were then extended to three other commercially-important fish.

Tilapia are now a major aquaculture species with production levels of over a million tonnes annually. The hatchery production of fry however, remains very inefficient due to relatively low fecundity and lack of spawning synchrony. Any methodology that enables farmers to synchronise the reproductive cycles of their broodstock would have immense practical advantages. Light is already known to play an important role in the initiation of gonad maturation in other fish species. In this investigation the reproductive performance of 32 sibling Nile tilapia was evaluated under four different photoperiods: short daylength (6L:18D), normal daylength (12L:12D), long daylength (18L:6D), and continuous illumination (24L:0D). Significantly larger eggs ($P < 0.05$) were produced under normal daylength (12L:12D) compared to other treatment groups. Fish reared under long daylength (18L:6D) exhibited significantly higher ($P < 0.05$) total fecundity (2408 ± 70 eggs spawn⁻¹) and relative fecundity (7.2 ± 0.2 eggs g⁻¹ body weight) concomitant with a significant reduction in inter-spawn-interval (ISI, 15 ± 1 days) in comparison with the rest of the trials. This investigation shows that long daylength (18L:6D) helps improve some important reproductive traits in Nile tilapia, and suggests that such methodology may be used to alleviate the production problems caused by low fecundity and poor spawning synchrony, and thus play a valuable future role in tilapia culture.

Plasma melatonin levels were determined by radioimmunoassay (RIA) in fish kept under controlled photo-thermal conditions to investigate how the hormone melatonin varies with environmental change. Six melatonin profiles were described, the first over a 24 hour period (diel cycle), the second describing only changes in melatonin during the night-time, and four further profiles describing the melatonin levels of fish under four different experimental light regimes: short daylength (6L:18D), normal daylength

(12L:12D), long daylength (18L:6D), and continuous illumination (24L:0D). Results showed that in tilapia, melatonin is produced in a rhythmic way; melatonin profiles showed that maximal levels of melatonin were reached as soon as the light went off, then these levels remain high throughout the dark phase and just after the onset of the light phase, melatonin levels were suppressed to base levels. Studies also demonstrated that melatonin levels were very low in *O. niloticus* (50 – 100 pg/ml) compared with salmonids and other species, in which much higher production of melatonin (600 – 1000 pg/ml) has been reported.

Experiments showed that a negative correlation exists between melatonin levels and reproduction in tilapia. Those fish exposed to long daylength (18L:6D) exhibited the lowest melatonin levels but highest fecundity, and lowest ISI; fish reared under short daylength (6L:18D) exhibited the highest melatonin levels but much reduced fecundity and longer ISI. Although, it appears that photoperiod thus seems to play an important role in the reproduction of *O. niloticus*, and is certainly known to impart a strong regulatory effect upon reproduction in other fish, the present investigation also shows that those fish reared under continuous illumination produced the second highest fecundity and exhibited reduced Inter-Spawning-Interval (ISI). However, melatonin levels in these fish were kept constant at very low levels (20 – 30 pg/ml). Interestingly, these observations might suggest that melatonin may not be exerting a strong effect upon reproduction in *O. niloticus*.

Results also showed that the role of melatonin in the reproduction of *O. niloticus* is not as well-defined as in other species of fish. There was clearly a negative relationship between melatonin level and reproductive activity in our experiments; although the results of the continuous illumination treatment produced data that suggested that melatonin might not play a major role in the regulation of reproduction in this species. It was clearly important therefore, to further elucidate the role and function of melatonin in this species.

In a further series of experiments, the melatonin receptor from *O. niloticus* was successfully cloned (Mella), and a partial sequence of this receptor was obtained. This partial sequence was generated using primers based upon known sequence information for the melatonin receptor in rainbow trout. The tilapia melatonin receptor was highly expressed in the brain. However, no expression was found in either gonadal or somatic tissues other than brain after 25 cycles of PCR amplification. In the present study, photoperiod was shown to enhance various reproductive parameters in tilapia, melatonin profiles were defined throughout known periods of light:dark, and a melatonin receptor isolated and partially characterised. However, further research is required to fully characterise the precise function of melatonin in the regulation of tilapia reproduction, especially in terms of its interaction with other endogenous factors, and its relationship with exogenous factors other than photoperiod.

Studies in a variety of organisms including amphibians, fish, ascidians, nemerteans, echinoderms, mammals, and even a species of flowering plant, clearly demonstrate that an increase in intracellular egg calcium is crucial to the process of egg activation at fertilization. Mammalian studies suggest that calcium is released from internal egg stores at fertilization by a sperm-derived cytosolic protein factor. Recent studies in the mouse have identified this sperm-derived factor as being a novel sperm-specific phospholipase C (PLC) isoform (PLC ζ). Homologues of PLC ζ have since been isolated from human and monkey sperm. In addition, sperm factor activity has been detected in non-mammalian species including chicken, *Xenopus*, and a flowering plant. In this thesis, I report novel evidence for the existence of a similar sperm-derived factor in a commercially important species of teleost fish, the Nile tilapia. Using an established bioassay for calcium release, the sea urchin egg homogenate, it was clearly demonstrated that protein extracts obtained from *O. niloticus* spermatozoa exhibited PLC activity similar to that seen in mammalian

sperm extracts, and also induce calcium release when added directly to the homogenate. Further, sperm extracts prepared from *O. niloticus* induced calcium oscillations when injected into mouse oocytes, suggesting that *O. niloticus* sperm contained a similar calcium-mobilizing molecule to that found in mammalian sperm. The same bioassay was used to assay the calcium-releasing properties of sperm extracts prepared from three further commercially important aquaculture species: Atlantic halibut (*Hippoglossus hippoglossus*), African catfish (*Clarias gariepinus*), and rainbow trout (*Onchorhynchus mykiss*). All three of these species exhibited the ability to release calcium in the bioassay, suggesting that the four species of teleost tested in this Thesis appear to use a similar mechanism of egg activation as that reported for mammalian species. However, it was not possible to identify the specific sperm-specific molecule involved, nor the precise cell signalling system used, although present data would support the involvement of a PLC molecule. Several attempts were undertaken to isolate a possible PLC ζ homologue from *O. niloticus*, using molecular techniques such as the Polymerase Chain Reaction (PCR) and screening of a tilapia bacteria artificial chromosome (BAC) library. However, within the time frame imposed by this Thesis, I was unable to successfully isolate a PLC ζ homologue from *O. niloticus*, although research effort in this area is gathering pace and now involves cDNA library screening, and genomic technology. Nevertheless, the results presented herein have provided a valuable insight into the process of egg activation in fish and should provide a stable foundation for future research. Further elucidation of this crucial biological process in fish may help in the reduction of commercial loss post-fertilization, and during early embryonic development.

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LIST OF SPECIES

Scientific names	Common names
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<i>Anguilla anguilla</i>	European eel
<i>Anguilla japonica</i>	Japanese eel
<i>Bidyanus bidyanus</i>	Silver perch
<i>Carassius auratus</i>	Goldfish
<i>Catastomus commersoni</i>	White sucker
<i>Chanos chanos</i>	Snakehead
<i>Cichla ocellaris</i>	Peacock cichlid
<i>Clarias gariepinus</i>	African catfish
<i>Clarias macrocephalus</i>	Asian catfish
<i>Clupea harengus membras</i>	Baltic herring
<i>Clupea pallasii</i>	Pacific herring
<i>Cyprinus carpio</i>	Common carp
<i>Danio rerio</i>	Zebrafish
<i>Dicentrarchus labrax</i>	Sea bass
<i>Epinephelus marginatus</i>	Dusky grouper
<i>Esox lucius</i>	Pike
<i>Fugu rubripes</i>	Fugu
<i>Gadus morhua</i>	Atlantic cod
<i>Gasterosteus aculeatus</i>	Three-spined stickleback
<i>Hippoglossus hippoglossus</i>	Atlantic halibut
<i>Ictalurus furcatus</i>	Blue catfish
<i>Ictalurus punctatus</i>	Channel catfish
<i>Lampetra fluviatilis</i>	River lamprey,
<i>Lates niloticus</i>	Nile perch
<i>Micropterus salmoides</i>	Largemouth bass
<i>Morone saxatilis</i>	Stripped bass
<i>Mystus nemurus</i>	Bagrid catfish
<i>Oncorhynchus gorbuscha</i>	Pink salmon
<i>Oncorhynchus keta</i>	Chum salmon
<i>Oncorhynchus masou ishikawae</i>	Masu salmon
<i>Oncorhynchus mykiss</i>	Rainbow trout
<i>Oreochromis aureus</i>	Blue tilapia
<i>Oreochromis niloticus</i>	Nile tilapia
<i>Oreochromis urolepis hornorum</i>	Zanzibar tilapia
<i>Oreochromis andersonii</i>	Three spotted tilapia
<i>Oreochromis macrochir</i>	Longfin tilapia
<i>Oreochromis mossambicus</i>	Mozambique tilapia
<i>Oreochromis spilurus</i>	Sabaki tilapia
<i>Oryzias latipes</i>	Medaka
<i>Pagrus major</i>	Red sea bream

Scientific name	Common name
<i>Pagrus pagrus</i>	Red porgy
<i>Petromyzon marinus</i>	Lamprey
<i>Plecoglossus altivelis</i>	Ayu
<i>Poecilia veliferata</i>	Sailfin molly
<i>Rhodeus ocellatus ocellatus</i>	Bitterling
<i>Salmo salar</i>	Atlantic salmon
<i>Salvelinus alpinus</i>	Arctic charr
<i>Salvelinus fontinalis</i>	Brook trout
<i>Sarotherodon galilaeus</i>	St. Peter's fish
<i>Sciaenops ocellatus</i>	Red drum
<i>Scophthalmus maximus</i>	Turbot
<i>Solea solea</i>	Sole
<i>Sparus auratus</i>	Gilthead sea bream
<i>Tilapia rendalli</i>	Redbreast tilapia
<i>Tilapia sparrmanii</i>	Banded tilapia
<i>Tilapia zillii</i>	Redbelly tilapia
Non fish species	Common name
<i>Ambystoma mexicanum</i>	Mexican axolotl
<i>Chaetopterus pergamentacetos</i>	Marine worm
<i>Gallus domesticus</i>	Chicken
<i>Homo sapiens</i>	Human
<i>Lytechinus pictus</i>	Sea urchin
<i>Rana perezi</i>	Common frog
<i>Xenopus laevis</i>	Claw frog

an estimate of a 10% growth (1970), there has been an expansion of aquaculture. In the past limited species have been reared in the last few years considerable production was in the order of 10 million tonnes. This is a significant increase in the production of fish and shellfish. The production of fish and shellfish has increased from 10 million tonnes in 1970 to 15 million tonnes in 1975. This is a significant increase in the production of fish and shellfish. The production of fish and shellfish has increased from 10 million tonnes in 1970 to 15 million tonnes in 1975. This is a significant increase in the production of fish and shellfish.

1 Chapter One General Introduction

The first part of the book is a general introduction to the subject of aquaculture. It discusses the history of aquaculture, the different types of aquaculture, and the importance of aquaculture in the world. It also discusses the different species of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The second part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The third part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The fourth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The fifth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The sixth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The seventh part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The eighth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The ninth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them. The tenth part of the book is a detailed description of the different types of aquaculture, and the different methods of rearing them. It discusses the different types of fish and shellfish that are reared in aquaculture, and the different methods of rearing them.

1.1 Introduction

Aquaculture production has shown significant growth in recent years, with a continuous increment of 9.2% since 1970; there has been an expansion of aquaculture in which more than 210 different farmed species have been reported in the last few years. In 2000, total aquaculture production was in the region of 11 millions tonnes (Without China's inputs) (FAO, 2002). On average, this represents consumption of 2.3 kg of fish per person per year, with a nutritional contribution of 15% of the total animal protein intake (FAO, 2002). In 2002, tilapia production by aquaculture alone increased to 1.5 million tonnes. This increase was solely due to the high popularity and acceptance of tilapias around the world. The highest production of tilapias was reported in developing countries such as Taiwan, Bangladesh, Thailand, and Ecuador, (FAO, 2002).

Nile tilapia, *Oreochromis niloticus* is considered to be the most important species of tilapia used in aquaculture; this species was responsible for 70% of total tilapia production in 2000. Other species of tilapia with high potential for aquaculture are the blue tilapia, (*Oreochromis aureus*) and the Mozambique tilapia (*Oreochromis mossambicus*). Tilapia culture has been broadly distributed and introduced into more than 100 countries and now occurs in most continents (Balarin and Hatton, 1979; McAndrew, 2000)

Tilapia consumption has increased in the last years, largely because tilapia is a rich source of animal protein, and that the price of this fish protein is far lower than that other livestock such as cattle, poultry, pork and sea food (Mair, 1993). Tilapias are cultured in both developed and developing countries, and under tropical and temperate conditions, including most aquatic environments such as fresh, brackish and sea water and culture systems including lakes, ponds, earth ponds, concrete tanks, raceways, hapas, etc (Balarin and Haller, 1982). They, therefore, represent a highly versatile species for aquaculture.

Tilapia exhibits a series of positive attributes, such as high growth rate, and a high tolerance to parasites, diseases, and low water quality. 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 species in aquaculture (Cross, 1976; Guerrero, 1982). Nevertheless, tilapia culture suffers from some constraints regarding reproductive biology. These predominantly include early sexual maturation, lack of spawning synchrony, and low fecundity (Little *et al.*, 1993; Mair and Little, 1991; Macintosh and Little, 1995; Coward and Bromage, 1998, 2000; Coward *et al.*, 1998). Early or precocious maturation triggers significant unwanted reproduction and leads to overcrowding of on-growing tanks (Mair and Little, 1991; Macintosh and Little, 1995).

To overcome this problem, fish farmers have adopted a mono-sex culture approach, using just male populations. Many investigations have demonstrated that male tilapia grow faster than females (Guerrero III, 1975, 1982; Guerrero III and Guerrero, 1988; Mair and Little, 1991; Mair *et al.*, 1995, 1997; Bhujel *et al.*, 1998). All-male culture is often routinely applied, and various strategies have been described to achieve this. The first real alternative to all-male culture was the manual segregation of male and females in a normal mixed population. This method is, however, time consuming and often unreliable. Furthermore, this method required a large number of well-trained personnel and reasonable sized fish for sexing to be practical (Guerrero III, 1982; Macintosh *et al.*, 1988; Mair *et al.*, 1991; McAndrew, 1993)

Hormonal sex reversal is the most common method for achieving mono-sex populations in tilapias (Guerrero III, 1975; 1982); this method involves the administration of 17 α -methyltestosterone in the diet. The recommended dose required for successful sex reversal ranges between 25 - 60 mg of 17 α -methyltestosterone per kilo of feed. The feeding regime lasts for one month. This practice requires high numbers of first-feeding fry

to generate the desired level of seed production, as well as special facilities (i.e. culture tanks and laboratories) to achieve positive results. As discussed previously, tilapia culture is also limited by spawning asynchrony and low fecundity. This makes it difficult, if not impossible, for the farmer to produce adequate numbers of similar-sized fish for market, thus creating a significant problem in production. To solve this problem, huge numbers of broodstock are needed to guarantee constant production of similar sized first feeding fry suitable for subsequent hormone treatment (Bhujel, 2000; Coward and Bromage, 2000). Hormone sex reversal has been successfully used in large scale hatcheries, which generate and subsequently sell large numbers of seed suitable for on-growing. Hatcheries that adopt the hormonal sex reversal method appear to receive a better acceptance in the consumer market, which allows an increased price per fry (Bhujel *et al.*, 1998). The price of hormone-treated fry is three-fold higher than that of normal fry; they also exhibit superior quality (Bhujel *et al.*, 1998).

In mixed-sex culture, harvest generally results in the capture of low weight fish, usually lower than 200 g. This has been attributed to the diversion of energy which although originally destined to be used in somatic growth, instead becomes channelled into reproduction, thus, generating a constant supply of new fry every two or three weeks (Campos-Mendoza *et al.*, 2003, 2004). This results in overcrowding of on-growing tanks, resulting in marked competition for food and space. To alleviate this, farms sometimes utilize a piscivorous fish to predate the small fry. This is a good way to reduce overcrowding problems, although the precise ratio between prey and predator in this situation has not yet been evaluated. The predator fish species used varies according to geographical regions. The most common species utilised for such a task are snakehead (*Chanos chanos*), Nile perch (*Lates niloticus*), largemouth bass (*Micropterus salmoides*), and peacock cichlid (*Cichla ocellaris*). Nevertheless, this practice generates further

constraints regarding the availability and size of predators, sometimes even with piscivorous control, the harvest yield is not satisfactory (Guerrero III, 1982; Mair and Little, 1991).

Other methods to generate an all-male culture have been proposed, such as the production of hybrids (e.g., *O. mossambicus* (♀) X *O. urolepis hornorum* (♂); *O. niloticus* (♀) X *O. urolepis hrrnorum* (♂); *O. niloticus* (♀) X *O. macrochir* (♂) and *O. niloticus* (♀) X *O. aureus* (♂)). These crosses produce a very high percentage of male populations, or sterile male or females when pure strains are used. However, this approach involves the manipulation of unmixed strains, a task that in many cases is impossible or difficult to achieve (Hulata *et al.*, 1983; McAndrew, 1993).

The production of monosex male production by genetic manipulation has been reported by Penman and McAndrew, (2000), using the hormone sex reversal methods, it was possible to invert the phenotype sex of tilapia, resulting in the production of neomales (genetically females but phenotypically male) or neofemales (genetically males but phenotypically females), using those neomales or neofemales in combination with chromosome-set manipulation, the production of gynogenesis or androgenesis was achieved. This has allowed the production of genetically male tilapias (GMT) by Mair *et al.*, (1997) and super males 'YY' by Scott *et al.* (1989). The production of gynogenesis involved the UV irradiation of the sperm, then a heat of pressure shock is applied to prevent second polar body extrusion, this result in a diploid gynogenetic fish. This fish will present just the maternal chromosomes. However, UV irradiation of unfertilized eggs followed by fertilization with normal sperm results in a haploid, androgenetic embryo, after that temperature or pressure shock is applied to restore the diploidy in the developing embryo, resulting in androgenetic male.

1.2 Reproductive biology of tilapias

Tilapias exhibit a high degree of parental care over their progeny, this has evolved from ancestral substrate spawners with guarding behaviour (Trewevas, 1983; Rana, 1988). The biogeographical isolation of tilapia populations has resulted in marked differences in the kind of parental care and breeding behaviour evolved (Trewevas, 1983; Rana, 1988). The role of each sex in the brood care provided differs among species, this parental care is geared to provide effective protection for eggs and fry against predators. The physiological mechanisms controlling such parental care behaviour remain poorly understood (Jalabert and Zohar, 1982).

Trewevas (1983) classified the tilapiine group on their reproductive behaviour, feeding habitats, and biogeography. According to this classification, all *Oreochromis* species are maternal mouthbrooders, *Tilapia* species are biparental, substrate spawners, and *Sarotherodon* species are all mouthbrooders in which the male or both parents incubate the eggs (Trewevas, 1983; Macintosh and Little, 1995; Turner and Robinson, 2000) (Table 1.1).

Table 1.1 Reproductive characteristic of the tilapiine genera and the main species of importance in aquaculture

Genus	Mode of reproduction	Important species in aquaculture
<i>Tilapia</i>	Substrate-spawners (Guarded nests)	<i>T. zillii</i> <i>T. rendalli</i>
<i>Sarotherodon</i>	Paternal or bi-parental Mouthbrooders	<i>S. galilaeus</i>
<i>Oreochromis</i>	Maternal mouthbrooders	<i>O. niloticus</i> <i>O. mossambicus</i> <i>O. aureus</i> <i>O. urolepis hornorum</i> <i>O. andersonii</i> <i>O. macrochir</i> <i>O. spilurus</i>

(Source Macintosh and Little, 1995)

According to the above classification, the highest potential for aquaculture belongs to the *Oreochromis* genus. 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; pushing sediment to the side using the snout to create a small nest or fanning the area with pectoral fins, cleaning a large area, and then ejecting and removing sediments using the mouth (Turner and Robinson, 2000). Once the lek is prepared, a receptive female enters and starts a nuptial courtship with a male. After a few hours of courtship, the female releases small batches of eggs. After that, the male realising milt and fertilises the eggs. The female takes the eggs in to her mouth as soon as they are fertilised. Once the spawning and fertilization of eggs is finished the female leaves the lek and moves to special nursery areas where incubation takes place (Trewevas, 1983).

The incubation period lasts about 10 days and the maternal care of the fry after hatching lasts for further 1 to 4 days. After that time, the female begins a recuperation period, in which, she shows intensive feeding behaviour that continues for 2 or 4 weeks (Macintosh and Little, 1995). The mouthbrooding tilapia have adopted the anti-predator tactic of rearing their eggs and fry in the relative safety of the parental buccal chamber (Rana, 1988). However, this has considerable effect on the total fecundity in tilapia, reducing total fecundity, which is governed by the size of the buccal cavity, which dictates the number of eggs that the fish can incubate in its mouth.

Most *Oreochromis* species exhibit a significant degree of sexual dimorphism and dichromatism, males are longer than females at the same age and they present a bright colour 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) observed that in general, tilapias under natural conditions matured in 1 - 3 years, but the age of maturation is strongly influenced by the size of the water body within they live. It was reported that *O. niloticus* matured at 17 cm in Lake Edward, however maturation was reached at 39 cm in a larger water body such as Lake Rudolf (Currently Lake Turkana).

The onset of maturation in tilapia under favourable conditions occurs at early stages, usually when fish have reached an average weight of 15 to 100 g, or within the first 2 to 4 months of age (Mires, 1983; Alvenida-Casauay and Cariño, 1988; Mair and Little, 1991; Macintosh and Little, 1995; de Graaf *et al.*, 1999). As soon as sexual maturity is reached, and environmental conditions are favourable, most tilapias are able to produce a series of spawns, producing new cohort at intervals of 4 to 6 weeks (Jalabert and Zohar, 1982; Macintosh and Little, 1995; Campos-Mendoza *et al.*, 2003, 2004).

1.3 Factors involved in tilapia reproduction

Fish reproduction is influenced by different internal or external factors, the internal factors are related with the endocrinology of the fish. The external factors however, involve a variety of environmental variables, which play an important role in fish reproduction. These include water temperature, salinity, photoperiod, nutritional status of the fish, and in some cases the rain season (Coward and Bromage, 2000; Bromage *et al.*, 2001). These factors (external and internal) will be briefly described in the following section with particular emphasis upon tilapia species.

1.3.1 External factors

1.3.1.1 Temperature

The tropical origin of tilapias is clearly expressed in their ecological physiology, especially in terms of temperature preference during the reproductive period (Chervinski, 1982). Tilapias become inactive in water temperatures below 16°C, which is the minimal temperature reported for normal growth. Reproduction occurs successfully above 22°C, this is the reason why the natural distribution of tilapias is restricted to tropical and subtropical regions (Chervinski, 1982). In some specific areas in subtropical regions, low temperature can inhibit tilapia reproduction during certain parts of the year. In these cases, the duration of reproduction season is constrained and the breeding occurs within the hottest months of the year (Philippart and Ruwet, 1982).

In tropical and subtropical climates at higher altitudes (1300 - 2500 meters above sea level), *O. niloticus* shows a different pattern of reproduction. The age of first maturity is higher under these conditions; an example of this was reported by Hanson *et al.* (1988), in which *O. niloticus* first matured after 6 to 9 months or approximately 231 g in weight. Thus, it seems that temperature is playing a crucial role in the regulation of tilapia reproduction (Lam, 1983).

Temperatures higher than 20°C are required to stimulate tilapia reproduction, however, a variety of temperatures have been reported in some tilapias: 20 - 30°C in the case of the *O. niloticus* and *O. aureus*, and 20 - 35°C for *O. mossambicus* (Rothbard and Pruginin, 1975; Philippart and Ruwet, 1982; Rana, 1988). The reproduction of *O. niloticus* in places with temperatures of 33 – 35°C was affected considerably. High temperature led to reduction in spawning activity, egg quality and hatching success in a hapa-based culture (Little and Hulata, 2000).

Cridland (1962) reported that early maturation and high growth rates could be attributed to high temperatures of around 31.5°C. This author also reported that cold water

temperatures of 19.8°C resulted in immature fish. It was also reported that cold water temperatures of between 19.8 and 22.8°C delayed sexual maturation in the substrate spawner *Tilapia zillii* under controlled conditions. Behrends and Smitherman (1983) reported that, although males of *O. mossambicus*, *O. hornorum*, *O. aureus* and *O. niloticus* reared at 16°C still underwent spermatogenesis, gonadal recrudescence in females was inhibited at temperatures below 22°C.

Terkatin-Shimony *et al.* (1980) reported that *O. aureus* reared under controlled conditions at temperatures of 28°C exhibited increased gonadosomatic index (GSI) in females after three weeks of culture compared with those values reported for fish reared at 17°C.

The physiological limitation on tilapia of breeding at temperatures below 20°C limits tilapia aquaculture in the Southern United States to five or eight months only. Under these circumstances, strains of *O. aureus* and *O. niloticus* are currently cultured successfully, but broodstock need to be over-wintered in heated facilities with a minimum water temperature of 16 to 18°C (Behrends *et al.*, 1990).

1.3.1.2 Salinity

As a general rule, fecundity in tilapias decreases as salinity increases. During a six months period in sea water, *O. aureus* failed to spawn, or build a nest, and a sharp reduction in GSI was observed. Most tilapia species are sensitive to high salinities and reproduction is clearly inhibited by high salinities. Possible reasons for this might be due to osmotic stress on the eggs (Balarin and Hatton, 1979). On the other hand, Chervinski (1982) reported that several species of tilapia can breed successfully at high salinities. An example of this is *Tilapia zillii* and *O. mossambicus*, both species are able to reproduce at high salinities of 10 - 26 and 35 parts per thousand (‰) respectively.

Philippart and Ruwet (1982) reported that several species of tilapias are euryhaline and are able to live and reproduce at salinities greater than 30‰. Tilapias have been found in estuaries and coastal lagoons along the coast of West and East of Africa. Some species are endemic of high salinity lakes such as Lake Magadi, Lake Natron, and Lake Manyara. Some tilapia species such as *T. sparrmani*, *O. andersonii*, *O. macrochir* and *T. rendalli* are less tolerant to salinity and when salinity levels increases these species moves to rivers or tributaries of lakes or water bodies in order to avoid salinity stress (Philippart and Ruwet, 1982). *O. mossambicus* is the most tolerant species of tilapia and is considered a euryhaline organism; *O. mossambicus* grows and reproduces normally in fresh, brackish and seawater (Chervinski, 1982; Villegas, 1990).

Watanabe *et al.* (1997) reported that Florida red tilapia (hybrid of *O. urolepis hornorum* ♀ X *O. mossambicus* ♂) is capable of reproducing in sea water at 36‰, but optimum seed production requires waters with lower salinities. Fertilisation and hatching success and survival of pre-juveniles declined at salinities higher than 18‰. In this species, there is a marked reduction in fertilisation and hatching success at salinities between 27 and 36‰.

Female tilapia broodstock can be kept in saline water during the time of low seed demand. This is a helpful alternative when fish are re-stocked into ponds; thus, reproduction is suppressed in fish held at high salinities, but an increase spawning activity was observed just after fish were re-stocking in fresh water (Balarin and Haller, 1982; Pullin, 1982; Bhujel, 2000).

Finally, Al-Ahmad *et al.* (1988) reported that *O. spilurus* is able to reproduce in brackish ground waters (3 - 4 ‰), and seed production is better than in seawater (38 - 41‰). It was found that fecundity was two to five times higher in brackish waters than in

seawater; hatching rates of eggs, and survival rate of fry, were also twice as high in brackish ground water.

In summary, there is variation in the salinity tolerance in tilapias, in which this ability to tolerate salinity seems to be species specific. In general, there is a tendency to reduce reproduction as salinity increases. However, there are some species capable of reproducing at high salinities (e. g. *O. mossambicus*).

1.3.1.3 Light and photoperiod

Photoperiod is an important factor controlling the seasonal reproductive cycle of many fish species (Lam, 1983, Wootton, 1998; Bromage *et al.*, 2001). Little information is available, however, on the influence of photoperiod and light intensity on reproduction in tilapias. Historically water temperature has been considered to be the most important environmental factor controlling reproduction in tilapia (Rothbard and Pruginin, 1975; Jalabert and Zohar, 1982; Philippart and Ruwet, 1982). However, light intensity and photoperiod are known to influence early maturation in tilapias, although the physiological process involved remains poorly understood (Balarin and Hatton, 1979; Balarin and Haller, 1982). Balarin and Hatton (1979) suggested that further investigation was both necessary and important to understand and use photoperiod manipulation in the control of reproduction in tilapias.

There are reports which suggest that light intensities originated with bulbs of 60 - 100 watts caused inhibition of reproduction in *T. zillii* and that a period of strong illumination delayed sexual maturation in this species (Cridland, 1962). On the other hand, there are also reports that recommend photoperiods of 12L:12D or 14L:10D, with light provided by 100 watt fluorescent bulbs, to improve tilapia reproduction (Rothbard and Pruginin (1975).

Gonad development and subsequent spawning activity in tilapias are strongly correlated with the duration of daylight in natural conditions (Guerrero III, 1982). Pruginin *et al.* (1988) reported that keeping adult fish at temperatures optimal to reproduction (26-28°C) enabled the production of fry all year-round, but that highest production was observed from April to August, corresponding to the months with the greatest amount of daylight. The use of additional light (hours) during December to February when daylength is short could improve reproduction in tilapias (Galman *et al.*, 1988).

Spawning frequency increases as temperature and photoperiod increase. In accordance with this, Behrends and Smitherman (1983) reported that mean GSI in *O. mossambicus* and *O. urolepis hornorum* decreased through November – January, concomitant with a reduction in temperature and photoperiod. However, during this same time period, GSI in *O. aureus* and *O. niloticus* increased, perhaps due to increasing photoperiod through January. It took an additional month for *O. mossambicus* and *O. urolepis hornorum* to exhibit increasing GSI; this coinciding with clear increase in both temperature and photoperiod (Behrends and Smitherman, 1983).

Smith *et al.* (1991) reported that extension of normal short daylength during winter months, by using additional illumination (100 Watt lamps) had no significant effect on seed production and these authors suggested that water temperature probably played a more dominant role than photoperiod in tilapia reproduction. On the other hand, Al-Ahmad *et al.* (1988) reported that an observed reduction in fecundity of *O. spilurus* did not seem to be related to temperature. He reported that a slight decrease in photoperiod during August might have contributed to the observed reduction in fecundity, and suggested that manipulation of photoperiod might provide a means of manipulating fecundity.

Photoperiod manipulation has been applied in order to increase seed production in *O. spilurus*. In Kuwait, reproduction of *O. spilurus* was much reduced during the winter time,

this was attributed to low water temperature and reduced daylength. Using three different photoperiods (13 and 14 hours light and ambient photoperiod) using 4 fluorescent lamps of 60 watts per tank, located 1.8 m above the water surface and a water temperature of $29 \pm 2^\circ\text{C}$. Ridha *et al.* (1998) reported, that seed production was higher under 14 h days with production of 24,724 fry, followed by the ambient spawning conditions with 18,356 fry. The lowest seed production was under 13 h days with just 12,021 fry. This study was the first to provide experimental evidence to suggest that photoperiod did indeed affect tilapia reproduction.

It was not until the year 2000, when the first detailed investigation of the effect of photoperiod and light intensity on tilapia reproduction was reported. Ridha and Cruz (2000) reported a series of experiments using 18L:6D, 15L:9D and 12L:12D with two light intensities, 2500 and 500 lux. The results of this investigation showed that maximum seed production and spawning synchrony were achieved at 18L:6D at both light intensities. These results indicate that photoperiod plays a more important role in seed production than light intensity. These authors suggested that seed production could be improved using a combination of light intensity and photoperiod manipulation.

There is a direct effect of light perception and photoperiod on the blood melatonin levels in fish species (Bromage *et al.*, 2001). This changes in melatonin levels has been suggested to be a key element in fish reproduction, thus in this Thesis the plasma melatonin levels in tilapia will be described.

1.3.1.4 Water exchange

Regular changes of water within the breeding systems tend to lead to improved reproductive activity in tilapias, possibly due to the flushing of metabolite build-up and subsequent increase in dissolved oxygen levels, (Rothbard and Pruginin, 1975; Billard and Breton, 1978; Mires, 1982; Bhujel, 2000). Guerrero, (1982) reported that there is a

hormone like substance in tilapia's mucus which inhibits reproduction; this has been particularly observed under high density culture. 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 long periods of time. This author also suggested that partial water exchanges of 50% of the total volume of water at regular bases of 3 – 4 weeks could help to improve the spawning activity, thus increasing seed production.

1.3.1.5 Rainfall

In tropical and subtropical regions, in which the change in daylength 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 (Philippart and Ruwet, 1982; Lam, 1983). Bhujel (2000) further reported that observed increases in seed production during the rainy season, was most probably due to cool temperatures, increasing water levels, and dilution of hormones, or chemical, and waste metabolites. Hyder (1970) reported that long periods of heavy rainfall had a negative effect upon spawning activity. However, artificial rain, produced by sprinklers in spawning hapas has been reported to increase spawning activity, especially during dry and hot months.

1.3.1.6 Nutritional status of fish and food availability

In tilapia culture, the quantity and quality of food affects both the frequency of spawning and the total seed production per spawn (Hughes and Behrends, 1983; Rana and Macintosh, 1988; Macintosh and Little, 1995; Little and Hulata, 2000).

O. niloticus broodstock fed with protein levels of 20 - 25% of dry weight, exhibit early spawning as well as an improvement in spawning frequency. It is also known that low ration of food have induced early maturation and spawning frequency (De Silva and Radampola, 1990). Dietary protein and lipids levels influence oocyte development and egg

quality (Brooks *et al.*, 1997). The quality of broodfish diet plays an important role in larval production, quality and performance (Gunasekera *et al.*, 1996a,b). *O. niloticus* fed with 35 and 20% of crude protein spawned significantly higher numbers of eggs than those fish fed 10% crude protein levels in the diet. No differences were found in the size of the eggs between treatments, but larval quality was significantly higher in the females fed on 35% of crude protein than those females fed on 20%. The dietary protein levels also affected Inter Spawning Interval (ISI), fish fed with protein levels 35% of dry weight spawned every 16 – 20 days (Gunasekera *et al.*, 1996a).

Food availability has been reported to have considerable effect upon tilapia reproduction. Tilapia hybrids (*O. niloticus* x *O. aureus*) were fed with four different rations (0.5, 1, 2, and 3% of body weight per day). Results showed that total fecundity and relative fecundity (expressed as egg number per cm of female length) decreased with decreasing food ration size. Food availability also plays an important role in first maturation; it was reported that females fed on low ration size (0.5% of body weight per day) spawned more frequently than those fish fed with higher rations (Siddiqui *et al.*, 1997a).

In *T. zillii*, two food ration size were provided from first feeding until first maturity. Total fecundity was higher in those fish fed with high rations than those fed low rations. No differences were found regarding GSI, egg size, or spawning periodicity. However, after histological evaluation of the gonads, it was reported that those females fed at low ration exhibited high proportion of oocytes in stage 2 and 3, corresponding to early and late perinuclear stage of oocyte development, and fewer oocytes in stage 6 or 7, corresponding to late vitellogenic and maturing oocyte, than those ovaries of fish fed at higher ration. No differences were found regarding proportions of atretic eggs (Coward *et al* 1999a).

Bhujel *et al.* (2001) compared three different types of feed in a hapa based system; two feeds were catfish pellets (small pellet, 30% crude protein; large pellet, 25% crude

protein. The third diet was an herbivorous fish feed with just 15.5% of crude protein. The number of spawns produced by *O. niloticus* was higher in those fish fed the catfish diets (i.e. higher protein). Results suggested that increased reproductive activity and seed production was probably attributed to the high levels of crude protein of the catfish pellet, which best fitting the optimum protein level required for tilapia broodfish, in that the higher protein diet (small pellet) was perhaps more palatable, and resulted in increased reproductive activity. Therefore, most of the hapa; based hatcheries utilize diets containing 25 - 30% of crude protein levels for broodstock. This level of protein in the diet is nutritionally adequate, as well as being cost effective (Bhujel *et al.*, 2001).

1.3.1.7 Broodstock size and age

The size and number of eggs produced by tilapias is affected by female age and size (Jalabert and Zohar, 1982; Rana and Macintosh, 1988). According to Rana (1988), large females tend to produce larger eggs; female age rather than weight is more important in determining egg size. Larger tilapia produce larger eggs as well as higher number of eggs than smaller females (Jalabert and Zohar, 1982; Galman *et al.*, 1988; Rana and Macintosh, 1988; Macintosh and Little, 1995).

Rana (1988) reported that there was no correlation between egg size and female weight in one year old females of *O. niloticus* and *O. mossambicus*. Egg size is more probably correlated with the age of fish in these species (Rana, 1988). Bigger eggs were positively correlated with larger fry at hatching time, which exhibited a greater survival rate. There is a strong correlation between egg size and fry length, suggesting that big eggs will produce large fry at hatching time (Rana, 1985). *O. niloticus* and *O. mossambicus* fry produced by large eggs have larger yolk reserves, and are able to grow a greater size by the beginning of exogenous feeding. These fry exhibited high survival rates during starvation

compared with fry produced by small eggs (Rana and Macintosh, 1988; Macintosh and Little, 1995).

Total fecundity in tilapias was defined by Rana (1988) as the number of eggs in a freshly spawned clutch. Relative fecundity is defined as the number of eggs per kg of female body weight. Relative fecundity in tilapia decreases as female size increases (Siraj *et al.*, 1983; Rana and Macintosh, 1988; Smith *et al.*, 1991).

In *O. niloticus*, relative fecundity can increase over a successive spawns (Hughes and Behrends, 1983; Behrends and Smithermann, 1983; Siraj *et al.*, 1983). Although, relative fecundity, expressed as seed per kg of female, is larger in younger and smaller tilapia females, total or absolute fecundity is greater in older and larger females (Hughes and Behrends, 1983; Rana and Macintosh, 1988; Rana, 1988).

Siraj *et al.* (1983) reported an Inter-Spawning-Interval (ISI) of 7 and 21 days for fish of one and two years old females respectively. However, three year old fish spawned at intervals of between 10 and 20 days. Total fecundity was greater in one year old fish, followed by two and three year old fish respectively; egg size was higher in two and three year old fish. A similar pattern was reported by Smith *et al.* (1991), who reported that seed production of the hybrid of *O. urolepis hornorum* x *O. mossambicus* was significantly higher in one year old females than in two year old females. Fecundity is also thought to be strongly influenced by genetic factors, as well as, environmental conditions (Macintosh and Little, 1995; Rana and Macintosh, 1988).

In tilapias, higher seed production is normally generated by smaller broodfish (150 – 200 g), using fish of this size collectively produce more eggs and fry per culture unit. However, bigger females produce more eggs and fry on an individual basis than smaller ones. The higher productivity reported for smaller broodstock is largely explained by their

rapid recovery after spawning, thus resulting in shorter inter-spawning-intervals (Macintosh and Little, 1995).

Hatchery operators normally prefer medium size tilapia broodstock (150 to 200 g), and discard females larger than 250 g due to husbandry difficulties associated with seed collection. Also, larger broodstock require more space and food, thus affecting the seed production cost (Balarin and Haller, 1982; Little and Hulata, 2000; Bhujel, 2000).

1.3.1.8 Stocking density

High stocking densities are known to have an adverse effect upon tilapia reproduction (Balarin and Haller, 1982). Siddiqui *et al.* (1997b) reported that total fecundity of *O. niloticus* x *O. aureus* hybrid females decreased as stocking density increased. The maximum fecundity was observed in females reared at 50 fish per m² and lower fecundity observed in fish reared at 200 fish per m².

High seed production was obtained in *O. niloticus* at low stocking densities (5 fish m²) with low male to female ratios (1:2) (Hughes and Behrends, 1983). Low stocking densities have been reported to improve broodstock productivity; seed production increased compared with those fish stocked at high densities in pond culture (Little and Hulata, 2000). It is worth mentioning that high densities lead to increased pressure on males to find females also restrict hierarchies such that spawning becomes inhibited, this is due to males are unable to make and guard any spawning territory or arena (Little and Hulata, 2000). Under crowded conditions in *T. zillii*, levels of 17 β -oestradiol (E₂) and testosterone (T) become suppressed. This reduction of steroid concentration resulted in considerable reduction in spawning activity (Coward *et al.*, 1998b). Moving fish from crowded conditions to individual aquaria caused steroid levels to rise dramatically, followed by a resumption of spawning activity (Coward *et al.*, 1998b). Coward and Bromage (1999c) reported that 42 days of deprivation of adjacent contact between males

and females of *T. zillii* caused a significant increase in circulating levels of T, but not changes of E₂.

1.3.2 Internal factors

In fish, as well as many other vertebrates, environmental information, also known as external factors or cues, are perceived by the brain, then this information is translated into neural impulses which stimulate the endocrine pathway of the brain-pituitary-gonadal axis (BPGa), the BPGa responds in appropriate way. The response to these stimuli, involves the production and secretion of different forms of hypothalamic gonadotrophin-releasing hormone (GnRH), pituitary gonadotrophin (GTH I and GTH II), sex steroids and prostaglandins (Coward and Bomage, 2000). There are three different groups of gonadotrophin-releasing hormone (GnRH) neuronal group innervating the pituitary gland; these are the terminal nerve, preoptic region and midbrain. The preoptic group appears to be the main innervator of tilapia pituitary (Yamamoto *et al.*, 1998). The GnRH, has several functions in fish, some of them include the regulation and secretion of gonadotrophins and growth hormone (GH).

There are two gonadotrophin hormones in fish: GTH I and GTH II hormones. These hormones are homologues to the mammalian follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Prat *et al.*, 1996). GTH I stimulates ovarian growth and GTH II is responsible for the subsequent ovarian maturation (Prat *et al.*, 1996). GTH I is also involved in the secretion of the sex steroid oestradiol (E₂), then E₂ induces the hepatic production of vitellogenin (VTG). This VTG is then released into the blood stream and sequestered by developing oocytes (Yaron *et al.*, 1983; Wallace *et al.*, 1987).

Profiles of E₂ in tilapias are similar to those already reported for annual spawners such as the rainbow trout (Bromage and Cumaranatunga, 1988). E₂ shows high levels

during vitellogenesis and low levels are reported after the spawning season. It was reported by Rothbard *et al.* (1991) that testosterone (T), but especially (E_2) increased gradually during the onset of nuptial colouring and courtship, however, these high levels decreased during spawning and mouthbrooding. Under crowded conditions in *T. zillii*, steroid levels of 17β -oestradiol (E_2) and testosterone (T) become suppressed. This reduction of steroid concentration resulted in a considerable reduction in spawning activity (Coward *et al.*, 1998b). Moving fish from crowded conditions to individual aquaria caused steroid level to rise dramatically concomitant in resumption of spawning activity (Coward *et al.*, 1998b).

In *O. niloticus*, Srisakultiew, (1993) reported blood levels of total calcium (as an indication of vitellogenin concentration), E_2 and T over a successive spawning cycles (2 -3). This information was then correlated with histological development of the gonads. Results demonstrated that within each spawning cycle, the proportion of maturing oocytes increased from 0 – 15% just a day after spawning to up to 65% by day 10 post-spawning. This increase in oocyte maturation coincided with high levels of total calcium, E_2 and T.

1.4 Spawning induction

Several authors have reported asynchrony in the breeding behaviour of maternal mouthbrooding tilapias, in particular, those species belonging to the *Oreochromis* group. These species exhibit low fecundity and asynchronous spawning behaviour. These are considered to be serious problems for the mass production of eggs and fry (Srisakultiew and Wee, 1988; Mair, 1993; Macintosh and Little, 1995; Little *et al.*, 2000; Coward and Bromage, 1998a, 2000).

Synchronous spawning within a broodstock population would improve broodstock productivity by increasing total egg production over a particular period of time, and make it much easier for farmers to meet market demand as production would be predictable. In

tilapias, various different approaches of hormone manipulation have been attempted to synchronise spawning activity. Srisakultiew and Wee (1988) first reported that no real effect was obtained after hypophysation protocols involving doses of 0.1, 0.25 and 0.50 mg of pituitary gland homogenate (PG) per 100 g of body weight. This first attempt to use hormone induction in tilapias was unsatisfactory and no positive results were found. In a second attempt, this time using human chorionic gonadotropin (HCG), injections of 25 and 50 IU per 100 g of body weight of females successfully induced spawning, although, no spawning was obtained when higher doses were used (100 IU).

Behrends and Smitherman (1983) reported that changes in temperature increased the level of spawning synchrony in tilapias, and this could help in the mass production of eggs and fry. They found that an increase of water temperature from 20°C to 28°C caused improvement in spawning synchrony. In a similar way, manipulation of water temperature could be used to improve spawning activity in *O. niloticus*. For example, synchronised spawning activity was achieved after fish were exposed to 22°C for 6 hours followed by a rise in water temperature to 28°C; this strategy induced a large number of fish to spawn (Srisakultiew and Wee, 1988).

Further study showed that a combination of pregnant mares serum gonadotropin (PMSG) and (HCG) could be used in order to stimulate spawning activity in *T. zillii*. Doses of 500 IU per kg of body weight were injected into the dorsal muscle. Coward *et al.* (2000) recommended that one injection of PMSG should be given a day after spawning of the female and five days later inject HCG. In this study, 8 of 9 females tested were successfully induced to spawn and none of the control females spawned during the treatment (Coward *et al.*, 2000); however, the egg quality was affected and this resulted in a considerable reduction in fertilization rates.

Several other methods to improve spawning synchrony and seed production have been described. These approaches involve (1) total exchange of females after the spawning period by fresh females previously conditioned (females that have been allowed to recover for two to three months) in separated tanks. (2) The exchange of just spawned females by conditioned ones, and 3) return of females to the spawning tank after removal of eggs for female mouth. According to these procedures, the exchange of spawned females for conditioned fish stimulates early spawning, but the exchange of all broodfish was more effective than just the exchange of spawned ones. The removal of eggs and fry from incubating females, as well as exchange of spawned female *O. niloticus* after spawning have increased the number of eggs, as well as improved spawning synchrony (Little *et al.*, 1993). In a similar way, Macintosh and Little (1995) reported that removal of eggs and fry from incubating females increased seed production, compared with undisturbed females. The replacement of spawned females by newly conditioned females, as well as the total exchange or replacement of females every 10 days for previously conditioned females was an efficient technique to improve seed production. However, there are some constraints on this approach. This technique involves the use of three groups of broodstock in which, two groups are maintained in a conditioning tank, while the other is in the spawning tank. Thus, this procedure increases operation costs as well as the requirement for adequate tanks and other facilities.

Holding separate sex stocks of broodstock at high densities in hapas or tanks with adequate feeding regimes and good food quality, improved the synchrony of spawning when both sexes were restocked together at low densities in spawning hapas or tanks (Little *et al.*, 2000).

Coward and Bromage (1998b) reported that the substrate spawner *T. zillii*, under crowded conditions, exhibited reduced levels of sex steroids 17β -oestradiol (E_2) and

testosterone (T), but as soon as these fish were moved to individual aquaria steroid levels increased dramatically. In this work, under crowded conditions fish were conditioned due to inhibition of reproduction, this allowed ovarian cycles to become much more synchronous.

Little *et al.* (2000) recommended the use of exchange methods in hapa-based systems to increase and maintain constant seed production in Nile tilapia. These authors used steroid levels as an indicator of good quality and condition in terms of spawning activity. They found that females exchanged at intervals of 7 days had higher levels of 17β -oestradiol (E_2) and produced larger clutches than females changed at 3.5 days intervals. They observed that the continuous manipulation of broodfish, if changed every 3.5 days incurred spawning interruption and caused reduction in steroid levels.

1.5 Gamete quality and fertilization

One of the major factors governing reproductive success in tilapia, and of course, all other species of fish, is the quality of the gametes involved. Factors affecting egg quality are intrinsic properties of the egg itself and the environment in which the egg is fertilized and subsequently incubated (Brooks *et al.*, 1997). Some of the factors governing egg quality are known, but most, are unfortunately, not known. Some of the more well known factors affecting egg quality are diet, photoperiod, physiochemical properties of water, husbandry practices. A substantial factor controlling egg quality is the genotype of the female. These factors are discussed at length by Brooks *et al.* (1997).

It is of major concern that the manipulation of photoperiod can cause problems with egg quality in a wide variety of teleosts in which photoperiod has been used to advance or delay spawning, to gain a year-round production of eggs. Thus, light manipulation has resulted in high mortalities (60 -80% of the eyed stage egg) in pink salmon (*Oncorhynchus*

gorbuscha), when spawning has been delayed by light manipulation (Brooks *et al.*, 1997). In contrast, Gillet (1994), reported that egg quality was improved in the Arctic charr (*Salvelinus alpinus*) after delay in ovulation using changes in photoperiod. This improvement in egg quality could be explained by the fact that oviposition occurred at low water temperatures (2°C colder than normal).

Factors affecting sperm quality in fish include seasonal variation, the nutritional and endocrinological status of the fish, and also genetic make up (Billard, 1990a; Billard *et al.*, 1995; Pustowka *et al.*, 2000). In terms of sperm quality, which is defined as being the ability of sperm to successfully fertilise an egg (Rurangwa *et al.*, 2004), there are two principal variables that should be considered: those variables involving sperm motility, and those concerning duration of movement (Billard *et al.*, 1995; Kime *et al.*, 2001).

It is clear, therefore, that there are a multitude of factors controlling gamete quality in teleost fish, and our knowledge of these factors in tilapiine species remains scant at best. Above all others, perhaps the mechanism most crucial to reproductive success in fish is that occurring when the two gametes unite at fertilization. However, the fertilization and activation of fish oocytes are vital, but unfortunately overlooked, processes in fish research. This is surprising given that the commercial culture of many important freshwater, but especially marine, teleosts is beset by problems associated with fertilization, hatching and early embryonic development (Coward *et al.*, 2002). These problems have been particularly acute in certain species leading to the application of spawning induction technologies in an effort to optimize production. Although successful in some species, spawning induction experiments have not generally proved satisfactory in the tilapias. For example, the asynchronous nature of spawning in *T. zillii* could be controlled using a combination of pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) administered at specific times during the ovarian cycle, but

unfortunately appeared to result in very poor fertilization rates (Coward *et al.*, 2000). Other commercially important fish species, notably marine species, suffer from low fertilization and hatching rates; examples include Atlantic halibut (*Hippoglossus hippoglossus*) (Norberg *et al.*, 1991, 2001; Holmefjord *et al.*, 1993; Bromage *et al.*, 1994), sole (*Solea solea*) (Houghton *et al.*, 1985), turbot (*Scophthalmus maximus*) (Bromley *et al.*, 1986), gilthead seabream (*Sparus auratus.*), (Carrillo *et al.*, 1989), and some salmonids (Bromage *et al.*, 1992). Furthermore, similar problems often arise when fish are held captive in artificial environmentally controlled conditions. These problems have serious ramifications for successful and profitable culture.

However, our present knowledge concerning the key intracellular and molecular events accompanying fertilization and egg activation in fish is limited solely to small laboratory species that have no real commercial importance but represent useful laboratory models for developmental biology; these species include zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and bitterling (*Rhodeus ocellatus ocellatus*). In contrast, decades of research in other animal species has revealed much about the chemical messengers and mechanisms involved in fertilization and egg activation (Swann and Parrington, 1999). Most recently, the focus has been on uncovering the molecular identity of the signalling proteins involved (Parrington, 2001).

As manipulative techniques such as photoperiod regulation and hormonal induction become more common place in tilapia culture, such strategies might inadvertently influence gamete quality and/or the mechanisms involved with fertilization. A further aim of this Thesis therefore, was to make a preliminary investigation into the process of fertilization in tilapia applying the lessons learnt from the far more advanced mammalian research, and the mechanisms discovered therein.

1.6 Aims and structure of this Thesis

The underlying remit behind this Thesis was the obvious lack of detailed research into the possible role of photoperiod in the control of reproduction in tilapia, and the mechanisms involved in the synchronization and optimization of egg production. It was also evident from the literature that manipulative techniques such as photoperiod manipulation can, in other species, cause decline in gamete quality. Earlier work on tilapia, using the application of hormones to induce spawning, has already shown a significant adverse effect on egg quality and fertilization success. In view of the paucity of information concerning fertilization in tilapia, and other teleosts generally, a further aim of this Thesis was to make a preliminary investigation into the mechanism (s) underlying fertilization in tilapia and a selection of other commercially-important species, using mammalian research as a guideline. Specific objectives are outlined below.

1. To evaluate the effects of photoperiod upon the reproductive performance of Nile tilapia, *O. niloticus*
2. To investigate and describe the variation in plasma melatonin levels in tilapia reared under controlled environmental conditions.
3. To isolate and characterize a receptor for melatonin in *O. niloticus*.
4. To investigate and describe the tissue-specificity of the melatonin receptor Mella in *O. niloticus*.
5. To make a preliminary investigation of the mechanisms involved in egg activation at fertilization in tilapia, and a selection of other commercially-important specie

on in this chapter. Further details of materials and methods specific
to the present study are given in the following chapters.

2 Chapter Two General Materials and Methods

The experimental system was designed to simulate natural conditions
as closely as possible. The system had four settling tanks filled with shavings which act as bio-
films. Water was pumped to a leader tank in which the water was heated and recirculated
through the settling tanks.

2.1.3 Experimental system

Fish were maintained in a gravity fed recirculation system consisting of a
leader tank (100 cm x 45 cm x 45 cm) linked to four settling tanks and a fish
tank appropriate to the system size and capacity. Settling tanks incorporated bio-film
organisms. The bio-film promote filtration and improve biofiltration.

2.1 General materials and methods

Only details of those materials and methods commonly utilized throughout this study are given in this Chapter. Further details of materials and methods specific to discrete sections are given in each chapter.

2.2 Fish aquaria design and fish maintenance

2.2.1 Fish

The Nile tilapia *Oreochromis niloticus*, were obtained from pure broodstock (free from hybridization with other species or genus) held at the Tropical Aquarium, Institute of Aquaculture, University of Stirling, Stirling, Scotland, U.K. Further details of age and size of fish are given in the methods section of relevant experiments.

2.2.2 Stock system

Fish were kept in a gravity-fed recirculation system, which consisted of sixteen square fibreglass tanks (1.20 m²). These tanks were connected to each other with a PVC pipeline. The system had four settling tanks filled with biorings which act as biofilters. The water was pumped to a header tank in which the water was heated and redirected to the main culture tanks.

2.2.3 Experimental system

Fish were maintained in a gravity-fed recirculation system incorporating eight glass aquaria (105 cm x 46 cm x 46 cm) linked to four settling tanks and a filtration unit appropriate to the system size and capacity. Settling tanks incorporated bio-rings (Dryden Aquaculture, U.K.) to aid particulate filtration and improve biofiltration. Water was

pumped from system settling tanks to header tank (200 l capacity) via a water pump appropriate to the system capacity (Beresford Pumps Ltd. U.K).

The system was covered with a special frame built with iron and PVC foam, the light source was provided with a lamp (60 watts bulb) attached to the ceiling of the frame (Figure 2.1). All light were controlled with digital timers (Smiths Industries, U.K.) in order to obtain the desired photoperiod.

Light intensity in each aquarium was evaluated with a lux meter (Photometric sensor SKL310, Skye Instruments, Llandrindod Wells). Measurements were taken centrally at the surface of the water column. Light intensity was constant at 530 lux in each chamber over the full experiment.

2.2.4 Fish maintenance

All systems experienced a constant daily photoperiod regimes as dictated by the experiment being undertaken and a water temperature of $27 \pm 1^{\circ}\text{C}$. The water was oxygenated via airstones attached to a low pressure air blower. The fish were fed *ad libitum* twice a day with a commercial pelleted trout feed (Trouw Aquaculture Ltd., U.K), pellet size number 4 (see Table 2.1). Water quality was monitored twice a month; Levels of pH, nitrate, nitrite and ammonia were evaluated with aquarium water quality test kits (C-Test kits, New Aquarium Systems, U.K). Water quality was maintained satisfactorily by the biofilters and other filter systems. Nevertheless, a water change (10% of total volume) was performed once a week and the system refilled with fresh, aerated and preheated water.

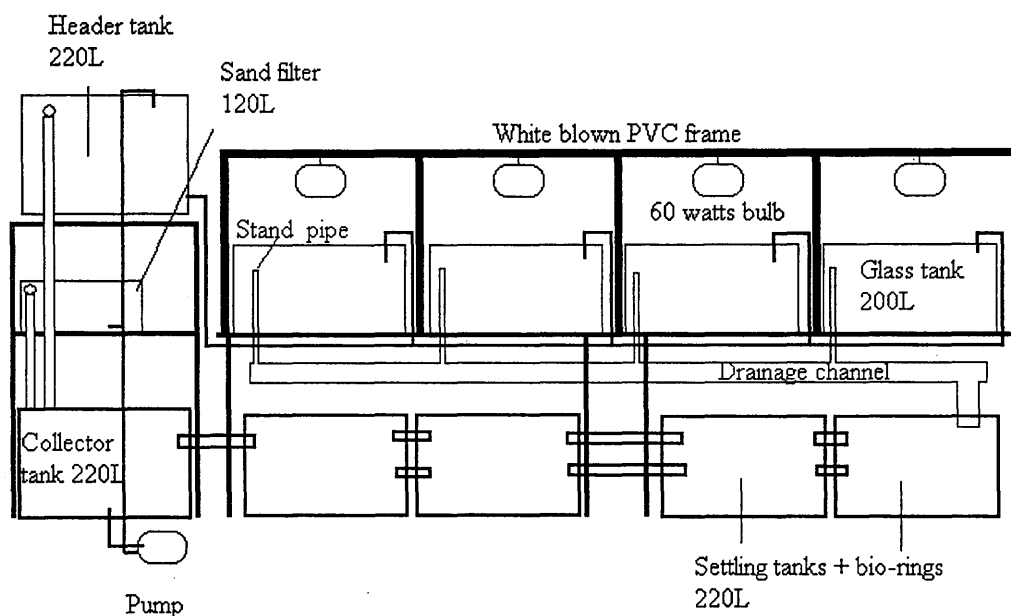


Figure 2.1 Lateral view of the closed recirculation system used to hold experimental fish.

Table 2.1 Proximate analysis of the diets used in this research. (IU = international units)

Feed stuff	Proportion
Oil	8.0%
Protein	40.0%
Ash	8.0%
Fibre	2.0%
Phosphorus	1.2%
Copper	10 mg/kg
Vitamin A	12000 IU/kg
Vitamin D3	2000 IU/kg
Vitamin E	100 IU/kg

Source: Trouw Aquaculture, Ltd.

2.3 General husbandry

2.3.1 Fish handling and anaesthesia

All procedures requiring fish handling such as stripping, tagging, weighing and blood sampling were undertaken under anaesthesia to minimize stress and scale damage. All the procedures were carried out under personal and project licence according to Home Office regulations. 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 1:10,000 in fresh water. After the sampling procedure, fish were allowed to recover completely in clean aerated water. Once the fish were totally recovered they were returned to the tanks.

2.3.2 Fish identification

Each fish were individually tagged with a Passive Integrated Transponder (PIT) tag (Trovan, Ltd., Koln, Germany). Tags were implanted through a special syringe with a modified needle directly into the peritoneal cavity. All the material utilized in the tagging process was previously sterilized with absolute ethanol. All the tagging procedure was carried out under anaesthesia.

2.3.3 Fish production

In order to produce stock fish required for this research, five two year old females (all sisters) were transferred into large a glass aquarium and allowed to breed with one male. This male was taken from a different stock totally unrelated to the females. Three days after the fish were stocked, three females spawned. The eggs were robbed (removed from the buccal cavity of mouth-brooding females) and incubated artificially to provide fry for subsequent experiments.

2.4 Blood samples collection

2.4.1 Blood collection procedure

Blood samples were taken from the caudal dorsal aorta by using 23G sterile hypodermic needles and 1ml sterile syringes (Terumo Europe N.V., Belgium) that had been previously rinsed with heparin ammonium salt from porcine intestinal mucosa (4 mg/ml, 140 units/mg: Sigma Chemicals, Ltd. UK). Blood samples (1 ml) were removed from the fish. Immediately after sampling, the needle was removed from the syringe and the blood sample expelled into a clean Eppendorf microcentrifuge tube (Fisons Scientific Equipment, U.K) and placed on ice until sampling had been completed. Blood samples were spun at 2500 rpm for 15 minutes at 4°C in a chilled centrifuge (Jouan, CT422). The resultant plasma (supernatant) was removed and transferred to clean Eppendorf tubes and stored at -70°C for future analysis.

2.5 Determination of total fecundity, relative fecundity, egg size and estimation of spawning periodicity

2.5.1 Determination of total fecundity

Eggs were removed from the fish's mouth under anaesthesia. The eggs were manually counted with the aid of a tally counter (B.D.H./Merck Ltd., U.K) according to Coward (1997) with this method 1000 eggs could be counted within 10 minutes. To evaluate consistency and accuracy of this technique, the total fecundity of one female was counted a total of 10 times allowing calculation of a coefficient of variation (CV). The CV for this method was 0.34 %.

2.5.2 Determination of relative fecundity

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

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

Where: RF: Relative fecundity (Eggs/g)
 TF: Total fecundity (number of eggs in a freshly spawned clutch)
 W: Female body weight (g)

2.5.3 Determination of mean egg size, diameter and validation 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 variables were measured: egg long axis and egg short axis. Egg long axis refers to the length of the longest axis of the egg and egg short axis is the maximum width of the egg, perpendicular to the longest axis. The diameter of the eggs was easily calculated by this method. All egg measurements were carried out according to the method reported by Coward and Bromage, (1999b).

2.5.3.1 Determination of mean egg size and mean diameter

The maximum length of both short and long axes in a sub-sample of 50 randomly chosen eggs from each spawned egg batch were measured to the nearest 0.01 mm with a dissection microscope (Olympus Optical Co. Ltd., U.K.) incorporating a calibrated eye piece graticule. The mean egg diameter was calculated as follow:

$$d = l + s / 2$$

Where: d = Egg diameter (mm)
 l = Mean length of egg long axis (mm)
 s = Mean length of egg short axis (mm)

2.5.3.2 Validation of egg measurement procedure

Maximum length of both long and short axes of a sub-sample of 50 randomly chosen eggs from a water-hardened egg clutch was measured to the nearest 0.01 mm as described earlier (Section 2.5.3.1). The same sub-sample was re-measured a further four times allowing calculations of the coefficient of variation (CV). The CV of this method was 0.85 % and 0.44 % for the long and short axes respectively.

2.5.4 Estimation of spawning periodicity

Spawning periodicity was estimated using the parameter Inter-Spawning-Interval (ISI) which is based upon completed reproductive cycles of repeat spawning fish, (i.e. the time between one spawn to the next one).

2.6 Determination of mean egg volume and total egg volume

2.6.1 Determination of mean individual egg volume

Mean egg volume was calculated using the following equation:

$$v = \pi \times l \times h^2 / 6$$

Where: v = Volume of egg (mm^3)

l = Mean length of egg long axis (mm)

h = Mean length of egg short axis (mm)

2.6.2 Determination of total egg volume

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

$$\text{TEV} = \text{TF} \times \text{MEV}$$

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

TF = Total fecundity

MEV = Mean egg volume (mm^3)

2.7 Melatonin radioimmunoassay

Plasma samples were analysed for melatonin by a direct radioimmunoassay adapted from the method designed by Randall (1992) for the measurement of melatonin in rainbow trout and salmon.

2.7.1 Assay buffer

The following buffer was made up in 150 ml of nanopure water (Millipore®, Incorporation, USA), in a sterile polystyrene container (Sterilin Ltd., Hounslow, Middx., UK):

Tricine (N-tris(hydroxymethyl)methylglycine)	2.68 g
Sodium chloride	1.35 g
Gelatine	0.15 g

The buffer was submerged in warm water (~ 40°C) for 30 minutes in order to dissolve the gelatine. The solution was hand shaken to enhance gelatine incorporation in the buffer. After that, the buffer was kept in the refrigerator at 4°C until required. All reagents were supplied by BDH Chemicals Ltd.

2.7.2 Antiserum

Sheep anti-melatonin antiserum (Guildhay Antisera Ltd./Stockgrand Ltd., Guildford Surrey, UK) was raised against N-acetyl-5-methoxytryptophan conjugated through the side chain to bovine thyroglobulin. Two batches were available; batch number 704/6483 was used for all measurements described in this Thesis. Comparative percentages cross reactions (melatonin taken as 100%) are 0.91% for N-acetyltryptamine, 0.33% for 6-hydroxymelatonin, 0.22% for N acetyltryptophan, and $\leq 0.06\%$ for all other structurally-

related compounds (Randall, 1992). Supplied freeze-dried, the antiserum was dissolved in 2 ml of nanopure water to provide an intermediate dilution of 1:10. Aliquots of 100 μ l were transferred into polystyrene tubes (LP3, Luckhams Ltd) and stored at -20°C . The working solution was prepared by diluting one 100 μ l aliquot in 20 ml of assay buffer. This provided sufficient reagent for 100 tubes with a final dilution of 1:2000.

2.7.3 Radiolabel

The radiolabel was obtained from Amersham, Pharmacia Biotech, Ltd, UK. The radiolabel, [O-methyl- ^3H]melatonin, with a specific activity of 13.1 GBq/mg, and a radioactivity concentration of 37 MBq/ml, and it was 99.0% pure radio-chemically. An intermediate solution was prepared by diluting 20 μ l of stock label to 2 ml of absolute ethanol (Fisher Scientific International), the intermediate solution in a 20 ml glass vial (Packard, BioScience, B.V. Groningen, The Netherlands). The stock and intermediate solution was stored at -20°C . The working solution was freshly prepared for each assay by further diluting the intermediate solution with assay buffer to give approximately 4000 dpm in 100 μ l.

2.7.4 Standards

A stock standard solution of 1 mg/ml was prepared by dissolving 10 mg of melatonin (N-acetyl-5-methoxytryptamine, Sigma Chemicals, Ltd U.K) in 10 ml absolute ethanol. This solution was stored at -20°C . The standards were freshly prepared for each assay as follows:

Standard A) 100 μ l of 1 mg/ml made up to 10 ml with assay buffer (= 10 μ g/ml)

Standard B) 100 μ l of standard A made up to 10 ml with assay buffer (= 100 ng/ml)

Standard C) 100 μ l of standard B made up to 10 ml with assay buffer (= 1 ng/ml)

Standard D) 100 μ l of standard B made up to 5 ml with assay buffer (= 2 ng/ml)

A serial dilution (1:1) of 250 μ l of standard C with 250 μ l of assay buffer provided several standards in the range of 3.9 to 250 pg/tube for the standard curve. Standard D was used to allow the inclusion of 500 pg/tube.

2.7.5 Melatonin-free plasma

Melatonin-free plasma was prepared by charcoal stripping of plasma collected from fish during the light period according the protocol described by Randall, (1992).

Briefly:

- 1.- Prepare a 10% (W/V) suspension of charcoal (Activated, untreated, Sigma Chemicals, Ltd U.K) in serum plasma in 150 ml polystyrene universal containers (Sterilin Ltd., Hounslow, Middx., UK)
- 2.- Shake for 1 hour on ice in a magnetic stirrer.
- 3.- Centrifuge at 1500 rpm at 4°C for 30 minutes (Centrifuge Jouan, CT422).
- 4.- Decant supernatant and resuspend in charcoal 10% (w/v).
- 5.- Repeat steps 2 and 3.
- 6.- Decant supernatant and centrifuge at 3000 rpm at 4°C for 15 minutes.
- 7.- Decant supernatant and centrifuge at 20,000 rpm at 4°C for 30 minutes. (Centrikon, T-1170 Ultracentrifuge, Kontron Instruments)
- 8.- Filter supernatant through Millex-GV 0.45 and 0.22 μ m filter units (Millipore, S.A., Molshelm, France).
- 9.- Divide pooled plasma into sterile containers with a 6 ml portions (sufficient for one standard curve) and store at -20°C.

Before use, each pool was checked against the previous pool to ensure the plasma was free of melatonin (percentage binding should be indistinguishable from that of the zero standard)

2.7.6 Melatonin radioimmunoassay (RIA)

Standard curve and samples were analyzed in duplicate according to the following protocol.

2.7.6.1 Standard curve

Tube N°	Standard (pg)	+	Standard (μ l)	+	Buffer (μ l)
1 & 2	500		250 (Std D)		none
3 & 4	250		250 (Std C)		none
5 & 6	125		250 (Std C)		250
7 & 8	62.5		250 (5 & 6)		250
9 & 10	31.3		250 (7 & 8)		250
11 & 12	15.6		250 (9 & 10)		250
13 & 14	7.8		250 (11 & 12)		250
15 & 16	3.9		250 (13 & 14)		250 (remove 250)
17 & 18	0		none		250
19 & 20	NSB		none		450

(NSB = Non-specific binding)

2.7.6.2 Assay protocol

- 1.-Add 500 μ l of plasma sample to each sample tube.
- 2.- Add 250 μ l of melatonin free plasma to each of the standards and non-specific binding tubes (start from tube 20 backwards). Vortex tubes.
- 3.- Add 200 μ l of antiserum to all tubes except non-specific binding tubes (19 and 20). Vortex and incubate at 20°C for 30 min.
- 4.- Add 100 μ l of tritiated melatonin to all tubes, vortex tubes and incubate at 4°C for 18 hrs including 2 scintillation vials (totals).

- 5.- Dissolve 0.48 g dextran-coated charcoal (Sigma Chemicals, Ltd U.K) in 50 ml buffer and stir on ice for 30 minutes.
- 6.- Add 500 μ l charcoal to each tube, vortex and incubate at 4°C for 15 minutes.
- 7.- Centrifuge at 2000 rpm at 4°C for 10 min.
- 8.- Transfer 1 ml supernatant to scintillation vials (6 ml Polyethylene vials, Packard, BioScience, B.V. Groningen, The Netherlands) with 4 ml scintillation fluid (Packard, BioScience, B.V. Groningen, The Netherlands) and vortex. Include a blank containing only 4 ml scintillation fluid.
- 9.- Vortex vials and count radioactivity for 10 minutes in a scintillation counter (Canberra, Packard) including 1 blank and 2 total counts.

2.7.6.3 Calculation of melatonin levels

- 1.- Multiply the mean total dpm by 1/1.3 in order to correct the differences between the total reagent volume per tube and the volume of the supernatant counted.
- 2.- Subtract the mean non-specific binding dpm from that of the standards and samples.
- 3.- Calculate the percentage binding of standards and samples relative to the corrected total counts ($\% \text{ binding} = (\text{standard of sample dpm} / \text{mean total dpm}) \times 100$)
- 4.- Plot the percentage binding of the standards against concentration on log linear graph paper (Figure 4.1 in Chapter Four) and read the concentrations of the samples from the standard curve.
- 5.- Correct to pg/ml plasma

The sensitivity of the assay was defined as the smallest quantity of melatonin statistically distinguishable from the zero standards; this was 3.9 – 7.8 pg/tube.

2.7.6.4 Quality control

Aliquots of pooled plasma taken from one year old *O. niloticus* during the dark phase and containing approximately 90-100 pg/ml of melatonin were used as quality controls. The intra-assay coefficient of variation was 5.6% and the inter-assay coefficient of variation was 7.3%.

Chapter Three The Effect of Photoperiod Manipulation on Reproduction in Nile Tilapia, *Oreochromis niloticus*

in, 1979; Sultan and Miller, 1982; Julabert and Zohar, 1982; Nara, 1983; Jahan, 1984; Mariani and Lutz, 1995; Coward and Bromage, 2000).
to help females develop stable individual patterns of mating interaction.

3 Chapter Three The Effect of Photoperiod Manipulation upon Reproduction in Nile Tilapia, *Oreochromis niloticus*

with and thus satisfy market demand in on-growing forms (Baqel and Saad).
through the use of increased numbers of production helps overcome the problem
of low fecundity and poor survival. This method is far from ideal. I
increased numbers of brood fish are significantly higher costs.

Photoperiod manipulation has been applied successfully in several fish
production systems. The first reported use of Bromage et al. (1961). Using three

of the fish production system. The first reported use of hatchery operators
in aquaculture systems. The first reported use of produce sufficient
to meet the demand. This is only one of the many production. In
the world, the first reported use of hatchery operators.

3.1 Introduction

The reproductive physiology of tilapias has been reviewed extensively (Babiker and Ibrahim, 1979; Balarin and Haller, 1982; Jalabert and Zohar, 1982; Rana, 1988; Baroiller and Jalabert, 1989; Macintosh and Little, 1995; Coward and Bromage, 2000; see Chapter One). In brief, females tilapia exhibit individual patterns of ovarian recrudescence, thus in breeding populations they tend to spawn asynchronously every three to four weeks, depending upon environmental conditions (Coward and Bromage, 2000). Low fecundity and asynchronous spawning behaviour are the most important economic constraints on tilapia seed production (Mires, 1982; Rana, 1988; Macintosh and Little 1995; Coward and Bromage, 1998b; Baroiller and Jalabert, 1989; Bhujel, 2000; Little and Hulata, 2000). To optimise tilapia seed production and obtain homogeneous stocks of first-feeding fry suitable for sex reversal, farmers have tended to resort to simply increasing the number of broodfish (Macintosh and Little, 1995; Little *et al.*, 1997; Coward and Bromage, 2000; Bhujel *et al.*, 1998; 2001; Bhujel 2000). In Thailand, for example, a commercial hatchery operator required over 60,000 broodfish in order to guarantee production of 10 million fry per month and thus satisfy market demand to on-growing farms (Bhujel and Suresh, 2000). Although the use of increased numbers of broodfish helps overcome the problems created by low fecundity and poor spawning synchrony, this method is far from ideal. Maintaining increased numbers of broodstock incurs significantly higher costs.

Photoperiodic manipulation has been applied successfully in several fish species in order to control their reproductive cycle (Bromage *et al.*, 2001). Using these techniques, for example in rainbow trout (*Oncorhynchus mykiss*) culture, hatchery operators are able to control the reproductive cycle with great precision and thus produce sufficient numbers of eggs and fry at desired times, thereby ensuring all-year round production. In the case of tilapias, the effect of photoperiod on reproduction is very poorly understood although

several authors have reported that photoperiod and light intensity might play important roles (Cridland, 1962; Hyder, 1970; Rothbard and Pruginin, 1975; Balarin and Haller, 1982; Jalabert and Zohar, 1982; Bhujel, 2000). Currently, only two published papers report the use of photoperiod on tilapias. Ridha *et al.* (1998) first showed that photoperiodic manipulation improved seed production in *O. spilurus* using 14L:10D light regimes. However, Ridha and Cruz (2000) reported that longer and brighter days (18L:6D with light intensities of 2500 lux) produced more fry and improved spawning synchrony in Nile tilapia compared with short days (12L:12D; 15L:9D) and lower light intensity (500 lux). Considering the constraints currently imposed upon tilapia culture by poor spawning synchrony, any method that helps to improve hatchery efficiency should be investigated.

3.1.1 Photoperiod

In most living organisms, reproduction occurs in relation to seasonal changes in the environment as well as food availability. In fish, these seasonal changes occur according to geographical location to generate a specific pattern in reproductive activity such that progeny are produced at the most suitable times. In teleosts, the synchronisation of reproduction is affected by several environmental factors such as photoperiod, temperature, rainfall, and food availability (Bromage *et al.*, 2001). Both photoperiod and temperature are considered to be the most important environmental cues in the control of reproduction in finfish (Billard, 1983; Bye, 1984; Bromage and Cumaranatunga, 1988).

Daylength is the principal factor regulating reproduction in salmonids, bass, flatfish, breams, mullet, sciaenids, cyprinids and serriolids (Bromage and Cumaranatunga, 1988; Poncin, 1989; Bromage *et al.*, 1993a, b, 2001). In those species that are important for aquaculture, the seasonality of spawning is one of the major constraints, due to the consequent restriction on the supply of eggs and fry. On-growing farms require a

continuous supply of fry in order to produce a marketable size-fish throughout the year (Bromage, 1995). According to this, hatchery operators should artificially control the spawning times of their broodstock to produce eggs and fry all year round (Bromage and Cumaranatunga, 1988; Bromage *et al.*, 1992, 1993a, b).

The control of reproduction using photoperiod is a common practice in order to get a continuous supply of eggs and fry (Bromage and Cumaranatunga, 1988; Bromage *et al.*, 1992, 1993b, 2001). Photoperiod is reported to be a relatively cheap and simple method to install on commercial farms, and shows several advantages compared with other methods used to induce spawning (i.e. hormone treatment), due to the reduced levels of broodstock handling involved. In photoperiod manipulation, the pattern of daylength is changed, allowing with this, the synchrony of gonad recrudescence, oocyte ovulation and spawning times (Bromage *et al.*, 1992). Using photoperiod manipulation, it was possible to advance or delay the maturation of fish on a commercial scale in several species (Bromage and Cumaranatunga, 1988; Bromage *et al.*, 1992, 1993b, 2001).

3.1.2 Photoperiod in salmonids

3.1.2.1 The rainbow trout

Most of the information currently available on photoperiod has been generated from salmonid species; in these species, photoperiod has become a powerful tool to produce a continuous supply of egg and fry (Bromage *et al.*, 1992, 1993b, 2001), and is used extensively in the commercial culture of salmonid species.

In rainbow trout, the use of long daylength at the beginning of the reproductive cycle followed by short daylength just before the summer solstice triggered spawning time within 3 or 4 months. However, the opposite was found when fish were exposed to short daylength in the first few months of the reproductive cycle, or long daylength after the summer solstice resulting in delays in gonadal development. Spawning season, however,

could be predictably advanced with the use of 10L:14D (Light:Dark) photoperiods, followed by a change to short photoperiods of 6L:18D (Duston and Bromage, 1986; Bromage *et al.*, 1992; Alvarino *et al.*, 1993). In similar way, fish exposed to long daylength at the beginning of the reproductive cycle followed by short daylength spawned two months in advance, but in contrast, fish exposed to short daylength followed by constant long daylength delayed ovulation by 4 months (Bromage *et al.*, 1984).

Duston and Bromage (1986) and Bromage *et al.* (1992) reported that photoperiod history, and the direction of photoperiod changes are the most important factors compared with the duration of daylength, in the onset of reproduction in rainbow trout. This species exhibits a peculiar spawning behaviour, in which some fish respond to photoperiod changes such as 6L:18D changing to 22L:2D. When fish were exposed to total darkness (DD) or continuous illumination (LL), fish continued to spawn. These results suggested that rainbow trout have an internal “clock” or endogenous rhythm (Duston and Bromage, 1986; Davies *et al.*, 1991). Under controlled conditions and over several reproductive cycles, the rainbow trout clearly shows the presence and stimulation of the endogenous rhythm or clock. This clock is believed to control the onset of reproduction, so under these conditions, the internal clock exerts strong periodicity over the year, demonstrating that this internal clock is running in a circannual mode (Bromage *et al.*, 1992). This endogenous rhythm or “clock” is thought to be entrained by photoperiod under natural conditions. However, this entrainment could be adapted under controlled conditions (Bromage and Cumaranatunga, 1988; Duston and Bromage, 1986; Randall *et al.*, 1991a, b; Randall *et al.*, 2000).

In rainbow trout, the spawning season can be advanced through photoperiod manipulation, the use of long daylength in February and then short daylength in May advanced the spawning season by three to four months (Davies and Bromage, 1991). It was

also reported that water temperature did not play an important role in the control of rainbow trout reproduction. The effect of photoperiod upon total fecundity and egg size has been reported in rainbow trout; fish exposed to photoperiodic manipulation produced significantly smaller eggs compared with those fish reared under natural conditions, but total fecundity was not affected (Davies and Bromage, 1991).

It has been reported that short photoperiods and continuous light could also advance, and delay spawning in rainbow trout as required. Randall (1991ab) reported the use of two months of continuous illumination during mid January to mid March; this resulted in an advancement of spawning activity under farming conditions. On the other hand, two months of continuous illumination throughout July to September caused a delay in spawning activity. In further investigations, it was demonstrated that one month of continuous illumination during January was enough to trigger an advance in spawning activity in 80% of the rainbow trout females subjected to these conditions (Randall, 1991a, b). Thus, the use of additional light in the middle of winter, just after the spawning season, has the power to trigger a subsequent spawning season in the following summer, creating two spawning seasons within the space of just one year (Randall, 1991a). Previous investigations reported that three reproductive cycles could be obtained in a 16 month period when fish were exposed to constant long days (Bromage *et al.*, 1984), without compromising total fecundity or egg quality.

Photoperiodic manipulation thus has the power to alter gonad recrudescence, but several adverse effects have been reported in this respect. Bon *et al.* (1999) reported a reduction in spawning activity in rainbow trout when fish were exposed to a long photoperiod. This was caused by a reduction in the early and middle stages of gonadal development. Thus, photoperiod is clearly the main modulating factor affecting the biochemical processes involved in reproduction in rainbow trout, but in contrast, the late

stage of the reproductive cycle was governed by an endogenous biological clock in rainbow trout.

3.1.2.2 Other salmonids

Light manipulation has also been used in other salmonid species. Photoperiod caused either an advancement or delay in spawning activity in the in brook trout (*Salvelinus fontinalis*) (Carlson and Hale, 1973), pink salmon (*Oncorhynchus gorbuscha*) (MacQuarrie *et al.*, 1979; Beacham and Murray, 1993), Masu salmon (*O. masou ishikawae*) (Takashima and Yamada, 1984; Amano *et al.*, 2000). Photoperiod manipulation has also been used to improve growth rates and reproduction in Atlantic salmon (*Salmo salar*) (Endal *et al.*, 1991, 2000; Hansen *et al.*, 1992, 2001; Taranger *et al.*, 1991, 1995; Duncan *et al.*, 2000). In Atlantic salmon, photoperiod manipulation has been used in order to reduce grilsing (precocious maturation) in in sea cages; the additional light reduced the grilse from 63% in untreated fish groups to just 6% in fish exposed to additional nighttime illumination (Porter *et al.*, 1999).

3.1.3 Use of photoperiod in non salmonid species

The use of light manipulation to improve fish reproduction has been growing in popularity over the last ten years; this is the result of significant research taking place in the salmon and trout industry. Light manipulation has now been expanded to several non-salmonid species with high potential to aquaculture.

In European sea bass (*Dicentrarchus labrax*), several studies involving photoperiodic control have been reported by Carrillo and co-workers, (Zanuy *et al.*, 1986, 1991; Carrillo *et al.*, 1989). These authors reported that photoperiodic manipulation delayed maturation by three months in this species, although, hormone manipulation was needed in order for this fish to complete spawning (i.e. Luteinizing Hormone Releasing Hormone analogue

(LHRHa) and Human Chorionic Gonadotropin (HCG)). Sea bass react positively to light manipulation. Fish were maintained under constant long daylength for six months after the summer solstice and then transferred to short daylength. This caused a delay in spawning activity by at least 3 months. If fish are maintained under constant short photoperiods (9L:15D), spawning activity is advanced (Carrillo *et al.*, 1989, 1991). The use of light manipulation has not yet been shown to cause any detrimental effect upon egg quality in sea bass, and no hormone treatment was needed for these fish to complete spawning (Carrillo *et al.*, 1989, 1991).

Zanuy *et al.* (1991) reported a similar pattern to that of Carrillo (1989), in which *D. labrax* were exposed to different photoperiods. Results showed that fish held under constant long daylength (15L:9D) for two months and then transferred to constant short daylength (9L:15D), resulted in an advance of spawning by 3 - 4 months. On the other hand, those fish exposed to constant long daylength (15L:9D) for two months in the second half of the reproductive cycle, exhibited a delay in spawning by up to 4 months. In this study, the use of constant long daylength (15L:9D) or short daylength (9L:15D) throughout the entire reproductive cycle resulted in delay or advance in spawning time of about 1.5 - 2 months. These studies demonstrated that photoperiod was the most important environmental cue in the reproduction of sea bass (Carrillo *et al.*, 1989) and that this species responded to an endogenous rhythm or internal "clock" that is entrained by photoperiodic information (Carrillo *et al.*, 1989, 1993).

The use of photoperiod to improve growth has been used in some species such as the turbot (*Scophthalmus maximus*) and Atlantic halibut (*H. hippoglossus*). Photoperiod is also used to advance or delay maturation in the Atlantic halibut (Imstrand *et al.*, 1997; Björnsson *et al.*, 1998; Simensen *et al.*, 2000; Jonassen *et al.*, 2000), Atlantic cod (*Gadus morhua*) (Norberg *et al.* 1995, 2001; Dahle *et al.*, 2000). The use of photoperiod is also reported to

enhance reproduction in some fish species, for example, the three-spined stickleback, in which reproductive activity is stimulated by the use of continuous illumination, this allowed the study of reproductive performance in this species (Wootton, 1973 a, b; Wootton and Evans, 1976).

The aim of this chapter therefore was to further investigate the effects of photoperiodic manipulation on the reproductive performance of *O. niloticus* broodstock to see whether this might identify possible broodstock management strategies that may be adopted by hatcheries to improve egg production rates and improve spawning synchrony.

3.1.3. Fish husbandry

Before starting the experiment, fish were individually identified, as described with a PIT tag (Section 2.1.3). The fish were checked daily, every morning (daylight) for the evidence of spawning. Fish do display a swelling of the abdomen just before spawning, but were also observed for breeding behaviour such as an over-extension of the lower jaw, the lowering of the body height. Spawning fish were carefully anaesthetised with a fine mesh net into a bucket of water to expel the eggs. Eggs were checked, individually counted, and the average clutch measured for egg length, egg diameter, and clutch volume (Table 3.1). The Inter-Spawning Interval (ISI) was determined (Section 2.1.4) regularly, and water quality was monitored as described previously (Section 2.2.1).

3.2 Materials and methods

3.2.1 Fish supply

Thirty two sibling of *O. niloticus* were taken from the tilapia collection (McAndrew and Majumdar, 1983) held at the Tropical Aquarium, Institute of Aquaculture, University of Stirling. Fish were 18 months old at the beginning of the experiment.

3.2.2 Experimental system

The fish were maintained in eight (114x 45x 42 cm) glass aquaria. Each glass aquarium was sub-divided into four individual areas with Perspex sheets. The system was sub-divided into four individual chambers with two aquaria in each chamber (see Chapter Two for further explanation, Section 2.2.4).

3.2.3 Fish husbandry

Before starting the experiment, fish were anaesthetised, measured, weighed and implanted with a PIT tag (Section 2.3.2). The fish were checked daily (every two hours during daylight) for the evidence of spawning. Tilapia display a swelling of the genital papillae just before spawning, fish were also observed for brooding behaviour which involves an over-extension of the lower jaw; the brooding fish hardly open its mouth. Spawning fish were carefully transferred with a fine mesh net into a bucket and gently forced to expel the eggs. Eggs were washed, individually counted, and a sub sample of 50 eggs per clutch measured for egg long axis, short axis, diameter, and volume (Section 2.5.3 and 2.6). The Inter-Spawning-Interval (ISI) was determined (Section 2.5.4.). Feeding regimes and water quality were maintained as described previously Section 2.2.4.

3.2.4 Photoperiod

In this experiment, four photoperiods were studied. First one consisted of 6 hours light and 18 hours dark (6L:18D), this trial was identified as the shorter daylength. The second photoperiod consisted of 12 hours of light and 12 hours of dark (12L:12D), this trial was identified as the normal daylength assuming that in the tropics, under natural conditions, this will be the most common light regime, in which just a minor variation occurred throughout the year (Philippart and Ruwet, 1982). The third photoperiod consisted of 18 hours light and 6 hours dark (18L:6D); this trial was identified as the long daylength treatment. Finally, the fourth photoperiod consisted of 24 hours of continuous illumination (24L:0D). All the above photoperiods were kept constant in accordance with Section 2.2.3. A summary of the light regimes used in this investigation are given in Table 3.1. The duration of this experiment was six months (180 days).

Table 3.1 Light regimes used in this investigation.

Trial general description	Photoperiod	Number of tanks (Replicates)	Number of fish per tank replicate
Short daylength	6L:18D	2	4
Normal daylength	12L:12D	2	4
Long daylength	18L:6D	2	4
Continuous illumination	24L:0D	2	4

L= hours light, D= hours dark

3.2.5 Statistical analysis

Statistical analyses were performed using MINITAB (version 13.1). Statistical significances between treatments were evaluated at the 5% probability level. General linear model (ANOVA), (ANCOVA) and regression analyses (linear) were used to further analyse data. Data were \log_{10} or Arcsine transformed as appropriate. Values are expressed as the mean \pm standard error of the mean (S.E.M).

3.3 Results

3.3.1 Fish growth

In relation to fish size (weight and length), no differences were found among treatments ($P > 0.05$; Figure 3.1). The fish weight at the beginning of the experiment varied between 255 ± 20 to 305 ± 40 g, and total length ranged from 23 ± 1 to 24 ± 1 cm. At the end of the experiment, the final weight observed was 560 ± 50 g in short daylength treatment (6L:18D), 537 ± 40 g under normal daylength (12L:12D), 477 ± 40 g under long daylength treatment (18L:6D) and finally, 577 ± 40 g under continuous illumination (24L:0D). According to the mean total length observed at the end of the experiment, these results showed that short daylength treatment (6L:18D), normal daylength (12L:12D), and continuous illumination (24L:0D) all resulted in the same final length of 30 ± 1 cm. The small fish were found in the long daylength treatment (18L:6D) these fish exhibited a final length of 29 ± 1 cm. No differences were found between photoperiods in relation to fish weight and length. Nevertheless, strong differences were found in terms of growth rates ($P > 0.05$).

In the case of the absolute growth rates (AGR) (Final weight – Initial weight), significant differences were found. Fish under the short daylength (6L:18D) treatment increased by a total of 305 ± 25 g, under normal daylength (12L:12D) this increment was 281 ± 25 g, in weight. The long daylength photoperiod (18L:6D) showed the lowest AGR with just 201 ± 22 g. Fish under continuous illumination (24L:0D) exhibited a total weight increase of 272 ± 28 g (Figure 3.2 -A)

Relative growth rate (RGR) is a parameter that expresses the total increase of weight as a percentage of the initial weight (Hopkins, 1992). In the present experiment, significant differences were detected between photoperiods in terms of RGR ($P < 0.05$).

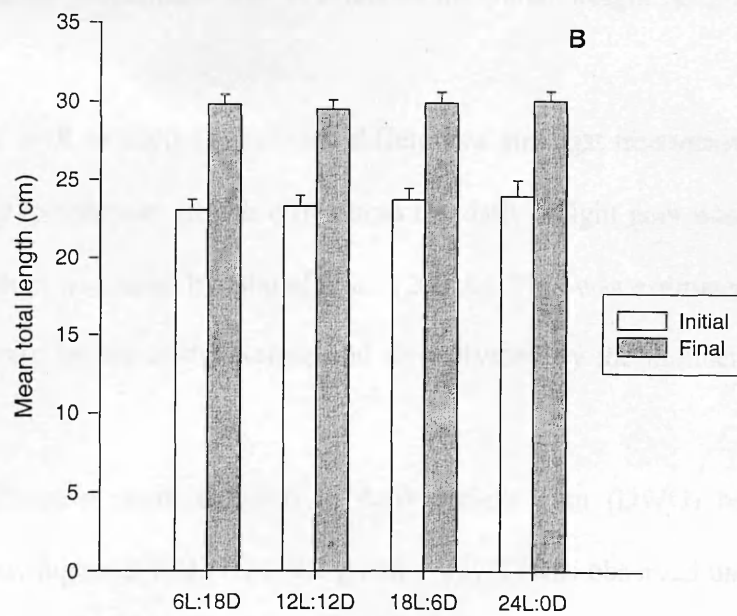
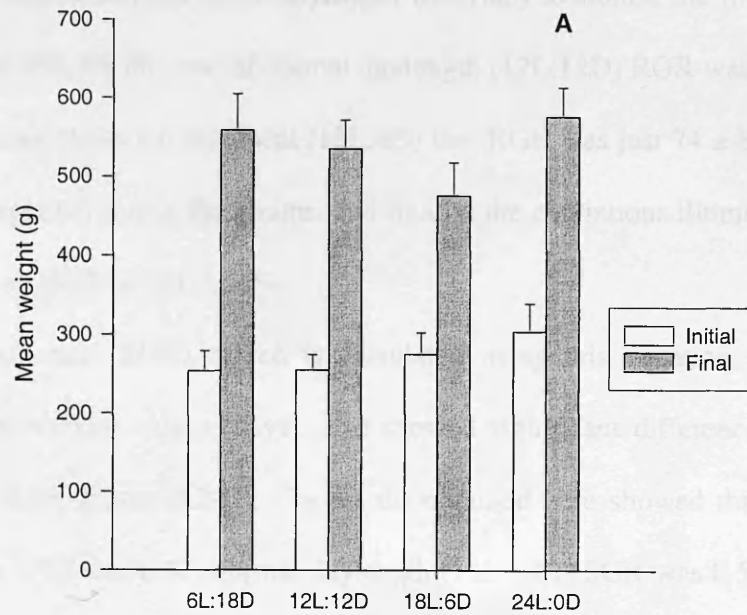


Figure 3.1 A) Mean initial and final weight of experimental fish, B) Mean initial and final total length of experimental fish. Values are Mean \pm S.E.M.

Figure 3.2-B shows that the short daylength (6L:18D) exhibited the highest RGR which reached $122 \pm 8\%$, for the case of normal daylength (12L:12D) RGR was $114 \pm 2\%$, for the case of the long daylength treatment (18L:6D) the RGR was just $74 \pm 8\%$ and was the smallest RGR recorded across the groups, and finally, the continuous illumination trial (24L:0D) exhibited an RGR of $101 \pm 16\%$.

Specific growth rate (SGR), which is calculated using this equation, $(\text{Log final weight} - \text{Log initial weight}) / \text{time (days)}$, also showed significant differences between photoperiods, ($P < 0.05$; Figure 3.2-C). The results obtained here showed that the short daylength (6L:18D) SRG was 0.47, normal daylength (12L:12D) SGR was 0.51, the long daylength (18L:6D) exhibited 0.31, and continuous illumination (24L:0D) exhibited a SGR of 0.38. This result was confirmed with analysis of covariance, using the initial weight as a covariate, in which there was no effect of the initial weight on the SGR ($P > 0.05$).

Both RGR and SGR exhibited significant differences amongst treatments, however. As an additional way to compare growth differences the daily weight gain was evaluated, according to the method described by Bhujel *et al.* (2001b). This was estimated using the final weight subtracted by the initial weight and then divided by the number of culture days.

Significant differences were detected in daily weight gain (DWG) between the groups. A significantly higher DWG (1.9 ± 0.2 g fish⁻¹ day⁻¹) was observed under normal daylength (12L:12D) treatment compared with long daylength (18L:6D) treatment (1.1 ± 0.1 g fish⁻¹ day⁻¹) over the 180 day experimental period (Figure 3.2-D).

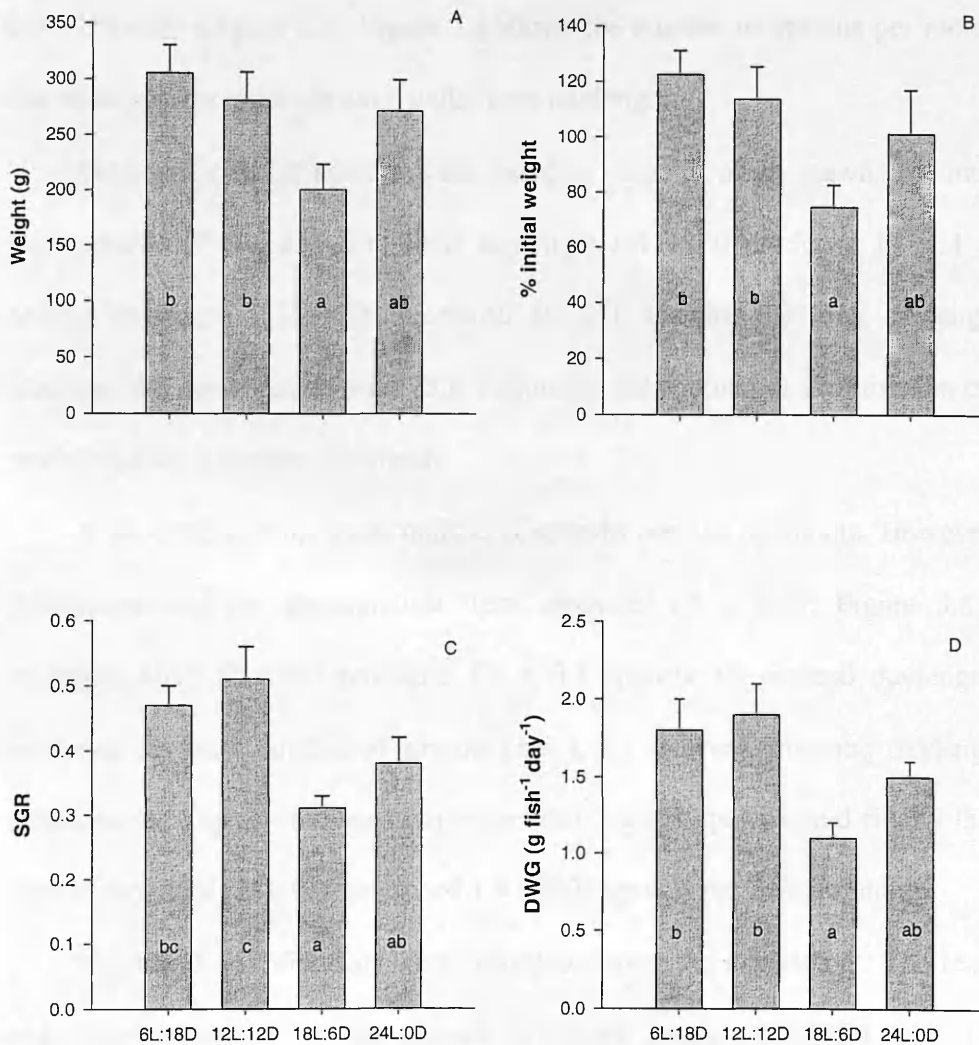


Figure 3.2 Growth rates of *O. niloticus* exposed to different photoperiods. A) Absolute growth rate (AGR), B) Relative growth rate (RGR), C) Specific growth rate (SGR) and D) Daily weight gain (DWG). Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

3.3.2 Spawning, fecundity and egg size

A total of 291 spawns were observed during the six months of experiment (180 days); 65 spawns were observed under short daylength (6L:18D) (22% of total spawns), 61 spawns reported under normal daylength (12L:12D) (21%), 90 spawns (31%) under long daylength (18L:6D), and 75 spawns (26%) were recorded under continuous illumination trial (24L:0D) (Figure 3.3). Figure 3.4 shows the number of spawns per month; it is clear that most spawns were obtained under long daylength.

No significant differences were found in terms of mean spawns per month between photoperiods ($P > 0.05$). The short daylength (6L:18D) produced 11 ± 1 spawns, The normal daylength (12L:12D) produced 10 ± 1 spawns, the long daylength (18L:6D) produced the most spawns with 15 ± 1 spawns, and continuous illumination trial (24L:0D) produced 12 ± 1 spawns per month.

If we compared the mean number of spawns per fish per month. However, significant differences between photoperiods were observed ($P < 0.05$; Figure 3.5). The short daylength trial (6L:18D) produced 1.6 ± 0.1 spawns, the normal daylength (12L:12D) produced the least number of spawns (1.5 ± 0.1 spawns), the long daylength (18L:6D) produced the highest number of spawns with 2 ± 0.1 spawns, and finally the continuous illumination trial (24L:0D) produced 1.9 ± 0.01 spawns per fish per month.

A total of 647,976 eggs were collected during the experiment. The least number of eggs were produced by the normal daylength group (12L:12D) with 124,675 eggs (representing 19% of total egg production), followed by the short daylength treatment (6L:18D) with 129,269 eggs (20%), the continuous illumination treatment (24L:0D) produced a total of 177,331 eggs (27.5%), and finally the long daylength treatment (18L:6D) which exhibited the highest production of eggs with a total of 216,701 eggs (33.5%) (Figure 3.6). The total egg production per month for each treatment for the whole experiment (180 day) is presented in Figure 3.7., and in a cumulative way in Figure 3.8.

Figure 3.7 shows that the long daylength group (18L:6D) consistently produced the highest number of eggs per month over course of the experiment, with the exception of October. Mean egg production was significantly reduced under the normal daylength (12L:12D) with a mean of $20,347 \pm 3,188$ eggs month⁻¹, while the highest was in the long daylength treatment (18L:6D) with $36,988 \pm 1,667$ eggs month⁻¹.

Total fecundity showed significant differences between photoperiods ($P < 0.05$). Short daylength (6L:18D) produced a mean fecundity of 2020 ± 80 eggs, normal daylength (12L:12D) exhibited a mean fecundity of 2043 ± 70 eggs, the long daylength treatment (18L:6D) produced the highest fecundity of 2408 ± 70 eggs, and finally the continuous illumination treatment (24L:0D) exhibited a fecundity of 2396 ± 80 eggs (Figure 3.9). The analysis of covariance (ANCOVA) using fecundity as a dependent variable, photoperiod as a categorical variable and fish weight or total length after spawning as a covariate showed that in this experiment fish weight and length are highly significant on fish fecundity ($P < 0.001$) in both cases.

Significant differences were found between photoperiods in relation to relative fecundity, it was defined as the number of eggs per gram of body weight ($P < 0.05$; Figure 3.10). Fish reared under short daylength (6L:18D) and normal daylength (12L:12D) exhibited similar relative fecundity with 6.0 ± 0.2 eggs/g in both cases. The long daylength treatment (18L:6D) exhibited the highest relative fecundity with 7.2 ± 0.2 eggs/g, and the continuous illumination (24L:0D) produced 6.4 ± 0.2 eggs/g.

As far as egg size is concerned, strong differences were found in egg diameter between photoperiods (Figure 3.11, $P < 0.05$). In the short daylength treatment (6L:18D) mean egg diameter was 2.40 ± 0.03 mm, in the normal daylength treatment (12L:12D) the biggest eggs were found, with a mean diameter of 2.47 ± 0.02 mm. In contrast, the long daylength treatment (18L:6D), and the continuous illumination (24L:0D) treatment

exhibited the lowest egg diameter with 2.37 ± 0.01 mm and 2.36 ± 0.02 mm respectively. The analysis of covariance (ANCOVA) using egg diameter as a dependent variable, photoperiod as a categorical variable and fish weight and total length after spawning as a covariate showed that in this experiment fish weight and length are not significant on egg diameter ($P > 0.05$) in both cases.

Similarly, results for mean egg volume showed significant differences between photoperiods, and showed the same pattern as that found with egg diameter ($P < 0.05$, Figure 3.12). The short daylength treatment (6L:18D) produced an egg volume of 6.6 ± 0.3 mm³. On the other hand, the normal daylength treatment (12L:12D) produced the highest egg volume with 7.0 ± 0.2 mm³. The long daylength treatment (18L:6D) and the continuous illumination treatment (24L:0D) both exhibited similar egg volumes with 6.2 ± 0.1 mm³ and 6.1 ± 0.1 mm³ respectively. The analysis of covariance (ANCOVA) using egg diameter as a dependent variable, photoperiod as a categorical variable and fish weight and total length after spawning as a covariate showed that in this experiment fish weight and length are not significant on egg volume ($P > 0.05$) for both cases.

No differences were found regarding total egg volume ($P > 0.05$). This value was the product of mean egg volume and mean fecundity per fish. All the photoperiods presented similar values of total egg volume. The short daylength treatment (6L:18D) produced a total egg volume of $13,173 \pm 620$ mm³, the normal daylength (12L:12D) produced $14,058 \pm 476$ mm³, the long daylength treatment (18L:6D) produced $14,804 \pm 376$ mm³ and finally, the continuous illumination treatment (24L:0D) produced a total egg volume of $14,348 \pm 496$ mm³.

3.3.3 Spawning periodicity

Significant differences were found in relation to Inter-Spawning-Interval (ISI) between photoperiods ($P < 0.05$; Figure 3.13). In the case of the short daylength treatment (6L:18D) mean ISI was 19 ± 2 days. In the normal daylength treatment (12L:12D) mean ISI was 20 ± 1 days. However, in the long daylength trial (18L:6D), the shortest ISI was observed at only 15 ± 1 days. Finally the continuous illumination trial (24L:0D) produced a mean ISI of 16 ± 1 days.

3.3.4 Correlation and regression analyses

Correlation and regression analyses were performed between fish size (length and weight) and various egg production variables (fecundity, relative fecundity, egg long axis, eggs short axis, egg diameter, egg volume and total egg volume). The results for all correlation and regression analysis are showed in Tables 3.2. to 3.5.

Correlation and regression analysis were performed using both untransformed (raw) and transformed data (Log_{10}). The transformation of data failed to improve the strength of the relationship (with exception of total fecundity and total egg volume) when it was compared with the untransformed data. In all cases in which data was transformed, the coefficient of correlation (r) and coefficient of determination (r^2) were similar values. Data transformation is strongly recommended in certain cases, such as, fecundity, ISI and total egg volume, which after show raw data to be distributed in a manner that is not normal. Under these circumstances, the data transformation significantly improved the strength of the relationship (Fig. 3.14 and 3.15).

3.3.4.1 Total fecundity

Highly significant positive relationships were found between total fecundity and fish weight in normal, long daylength and continuous illumination treatments ($r^2 = 0.24$; $r^2 = 0.28$ and $r^2 = 0.16$, $P < 0.001$ for all cases respectively). Nevertheless, no significant

relationship was found in the short daylength treatment ($P > 0.05$). The strongest relationship was detected in the long daylength treatment (18L:6D) in which 28% of the data were covered by the regression line (Figures 3.13 - 3.14). On the other hand, the poorest relationship was found in the short daylength treatment (6L:18D). A very similar pattern was found in the relationship between total fecundity and fish length. Strong and positive relationships were found in normal daylength (12L:12D), long daylength (18L:6D) and continuous illumination (24L:0D) treatments ($r^2 = 0.16$, $P < 0.01$; $r^2 = 0.35$ and $r^2 = 0.21$, $P < 0.001$ in the latter two values). The best relationship was found in the long daylength treatment, in which, 35% of the data reported were covered by the regression line. In contrast, no relationship was found in the short daylength treatment (Tables 3.2 – 3.5). The analysis of covariance (ANCOVA) using fecundity as a dependent variable, photoperiod as a categorical variable and fish weight or total length after spawning as a covariate showed that in this experiment fish weight and length are highly significant on fish fecundity ($P < 0.001$) in both cases.

3.3.4.2 Relative fecundity

Highly significant relationships were found in all photoperiods in relation to relative fecundity when compared to fish weight and fish length. In all cases, the probability value P was less than 0.001, with the exception of the long daylength treatment (18L:6D), in which, the P value for the relationship between relative fecundity and fish length was less than 0.01 (Tables 3.2 – 3.5).

3.3.4.3 Egg long axis

A significant positive relationship was found between egg long axis and both fish weight ($P < 0.01$) and length ($P < 0.05$) in the normal daylength treatment. No significant relationships were found in the other photoperiods. Nevertheless, in the particular case of

the long daylength treatment (18L:6D) a negative relationship was detected with fish weight and length, although, these relationships were not statistically significant ($P > 0.05$), (Tables 3.2 – 3.5).

3.3.4.4 Egg short axis, diameter and volume

The relationship found between these three parameters and fish weight and length exhibited the same general pattern as that described above ($P < 0.001$) (Tables 3.2 – 3.5).

3.3.4.5 Total egg volume

Significant positive relationships were found between total egg volume and fish weight and length in normal, long daylength and continuous illumination treatments ($P < 0.001$). In the case of the continuous illumination treatment, the relationship between egg volume and fish weight exhibited a probability value of 0.05. No relationship was found in the short daylength treatment (Tables 3.2 – 3.5).

3.3.4.6 Relationship between ISI and total fecundity

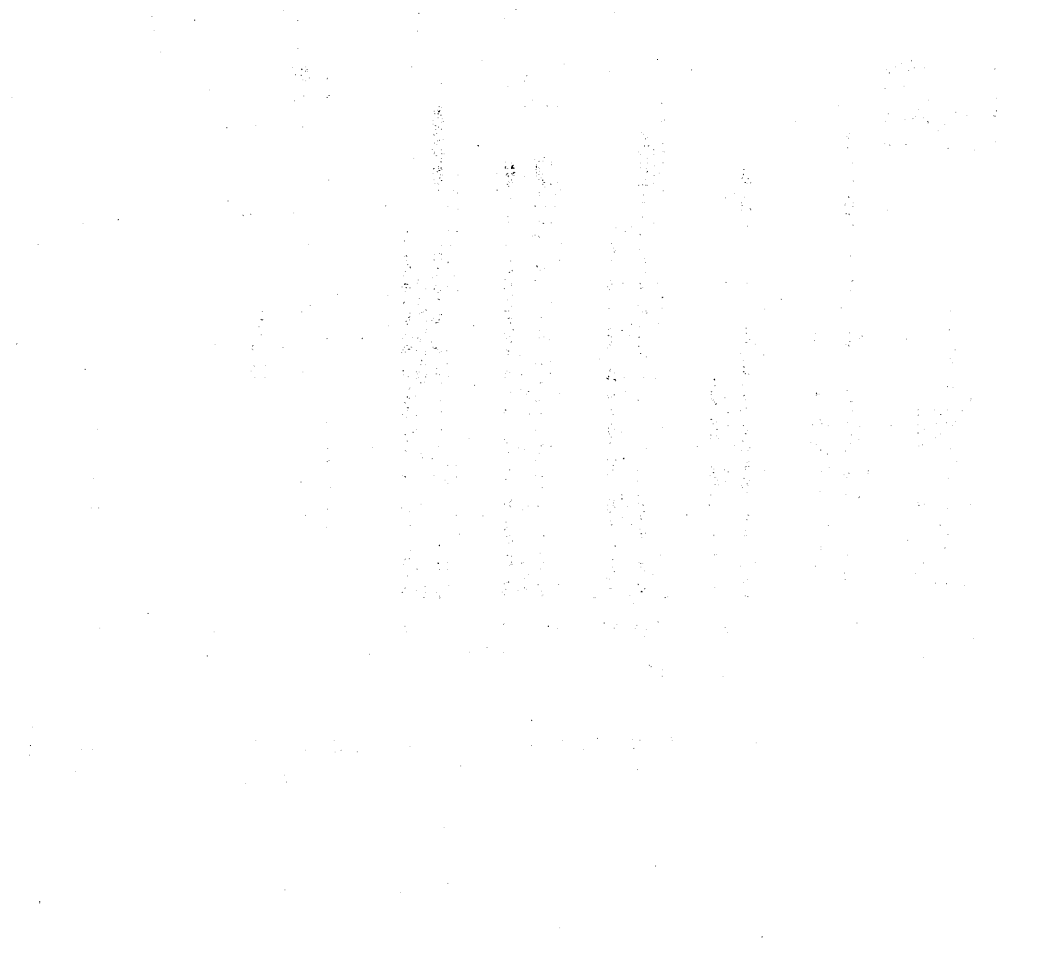
A significant negative relationship was found between ISI and total fecundity in the continuous illumination treatment (24L:0D) ($P < 0.05$). Nevertheless, no significant relationships were found in the other photoperiods (Table 3.6; Figures 3.16 -17). The analysis of covariance (ANCOVA) using ISI as a dependent variable, photoperiod as a categorical variable and total fecundity as a covariate showed that in this experiment the total fecundity is highly significant on the ISI ($P < 0.05$).

3.3.4.7 Relationship between ISI and mean egg volume

A significant positive relationship was found between ISI and mean egg volume in short (6L:18D), normal (12L:12D), and long (18L:6D) daylength treatments ($P < 0.001$ for short and normal daylength treatments, $P < 0.05$ for long daylength treatment). No

significant relationships were found in the continuous illumination treatment (24L:0D) ($P > 0.05$; Table 3.7). The analysis of covariance (ANCOVA) using egg diameter and egg volume as a dependent variable, photoperiod as a categorical variable and ISI as a covariate showed that in this experiment the ISI is highly significant on egg size (diameter and volume ($P < 0.001$)).

Table 3.7. Analysis of covariance (ANCOVA) for egg diameter and egg volume in response to photoperiod and ISI.



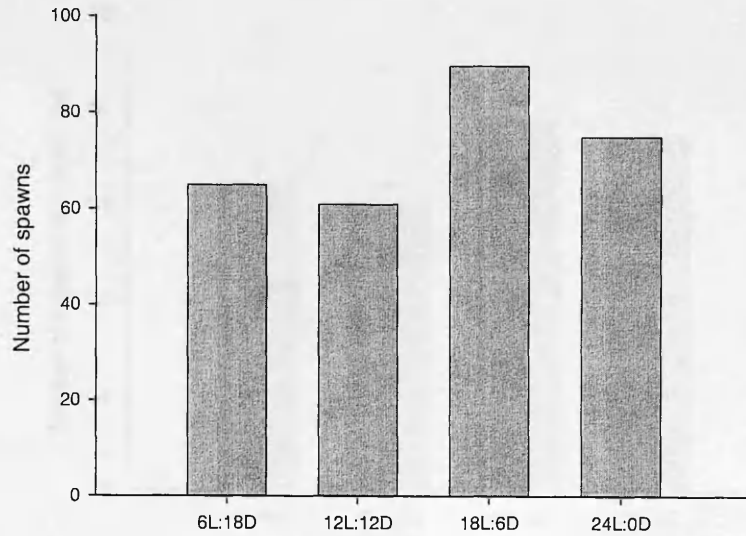


Figure 3.3 Total spawns produced in 180 experimental days by *O. niloticus* reared under four different light regimes.

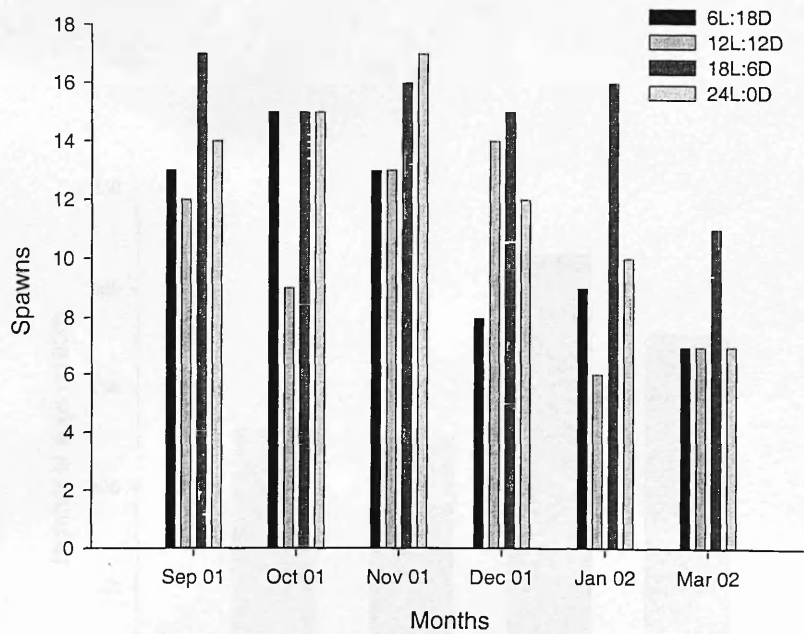


Figure 3.4 Number of spawns produced per month by *O. niloticus* reared under four different light regimes.

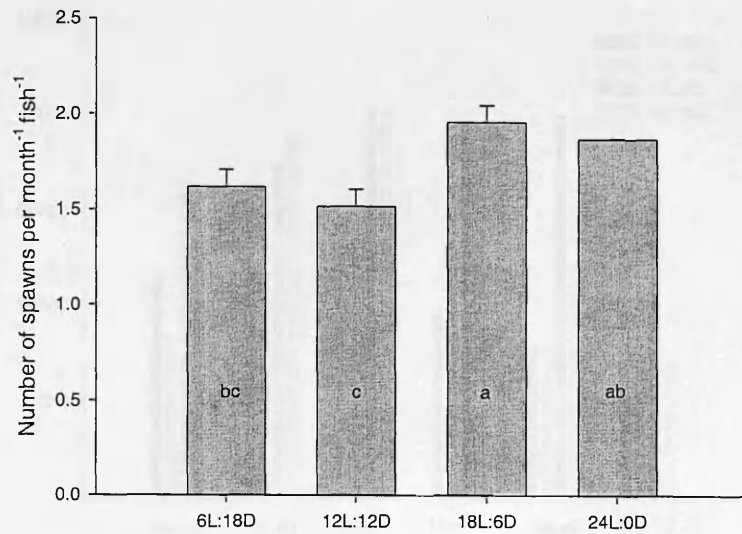


Figure 3.5 Number of spawns per month produced by *O. niloticus* reared under four different light regimes. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

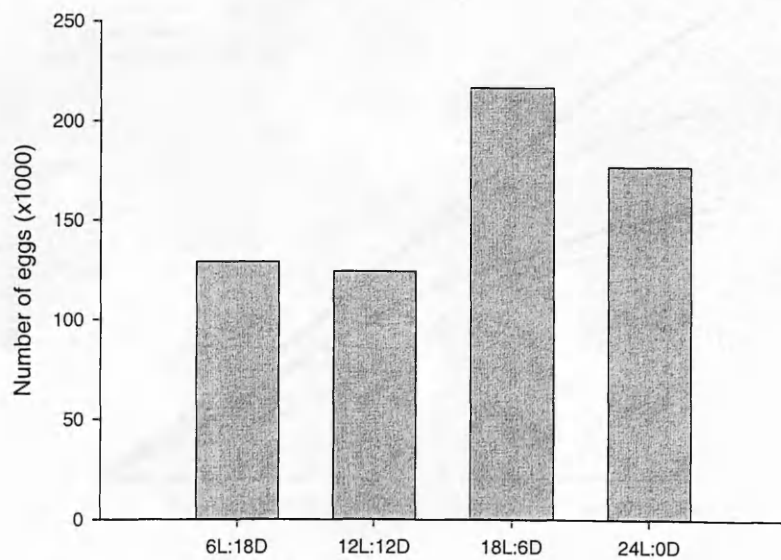


Figure 3.6 Total number of eggs per treatment produced by *O. niloticus* reared under four different light regimes.

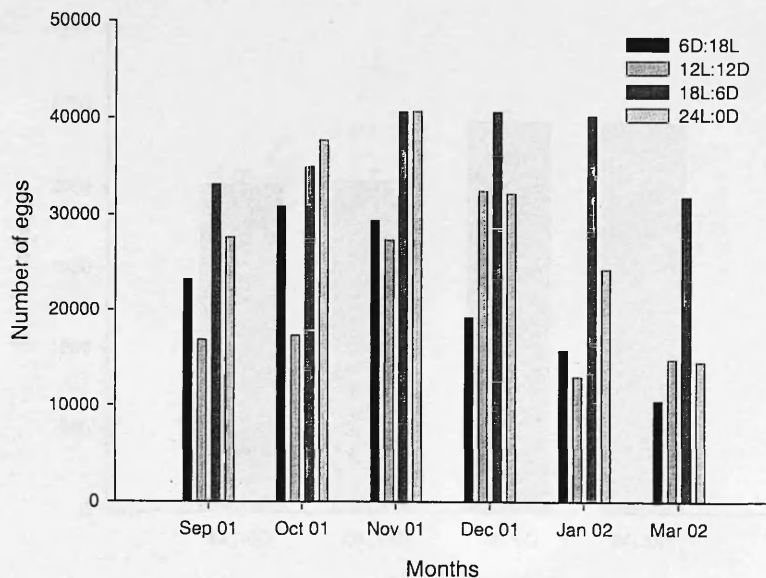


Figure 3.7 Number of egg per month spawned by *O. niloticus* exposed to four different photoperiods.

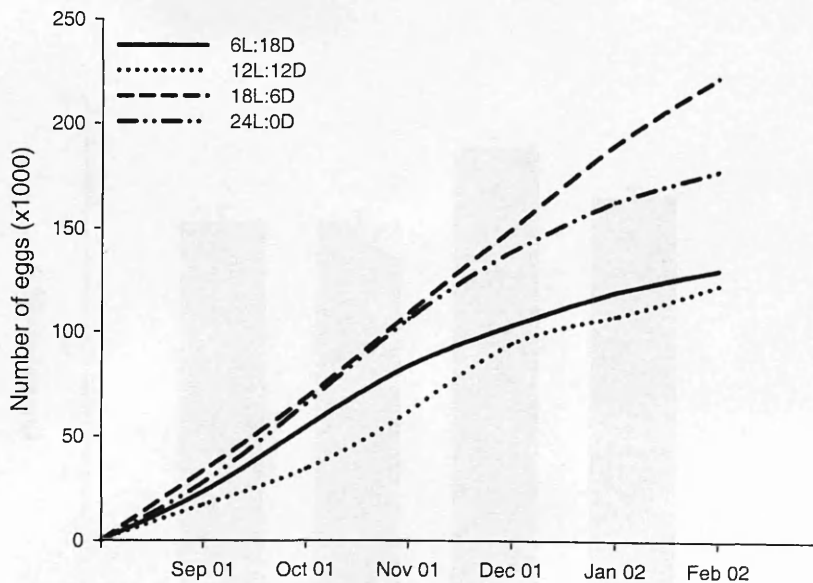


Figure 3.8 Cumulative egg production by *O. niloticus* exposed to four different photoperiods.

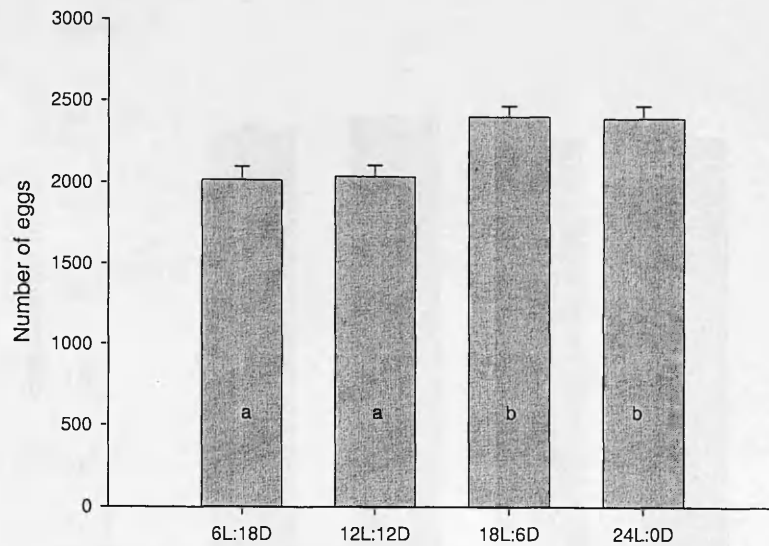


Figure 3.9 Total fecundity of *O. niloticus* exposed to different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

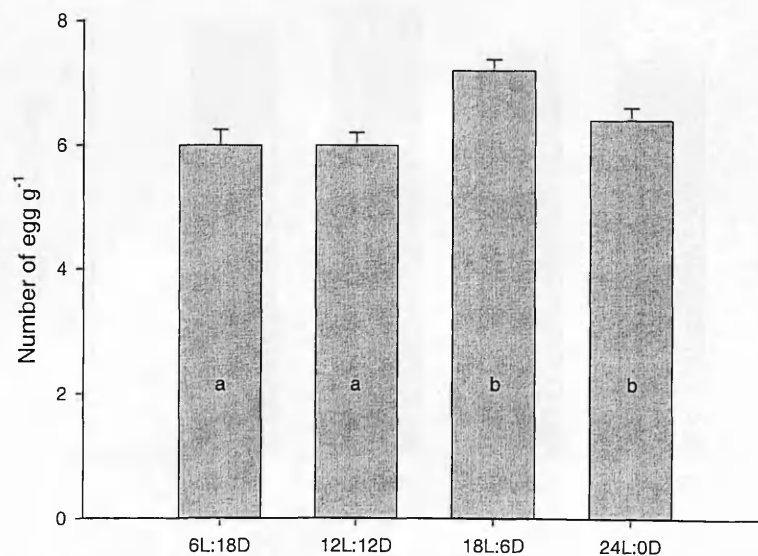


Figure 3.10 Relative fecundity of *O. niloticus* exposed to different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

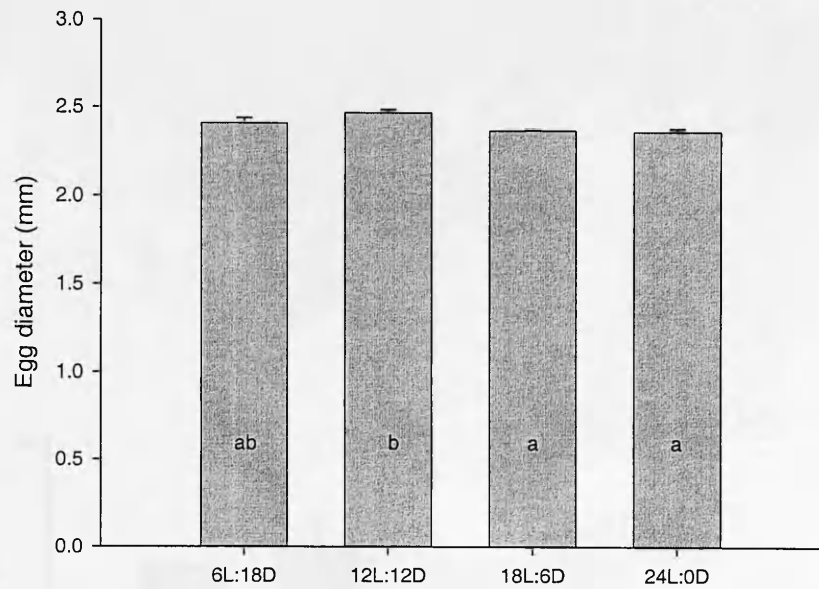


Figure 3.11 Mean egg diameter of *O. niloticus* exposed to different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

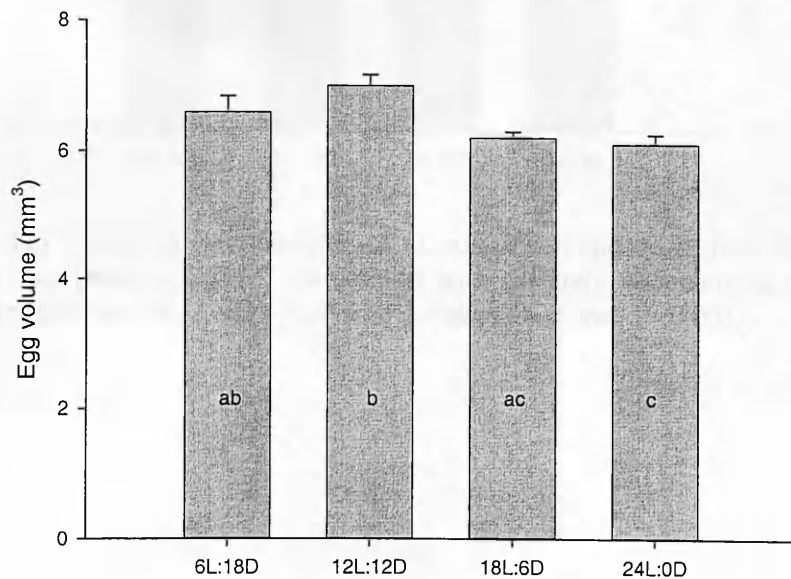


Figure 3.12 Mean egg volume of *O. niloticus* exposed to different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

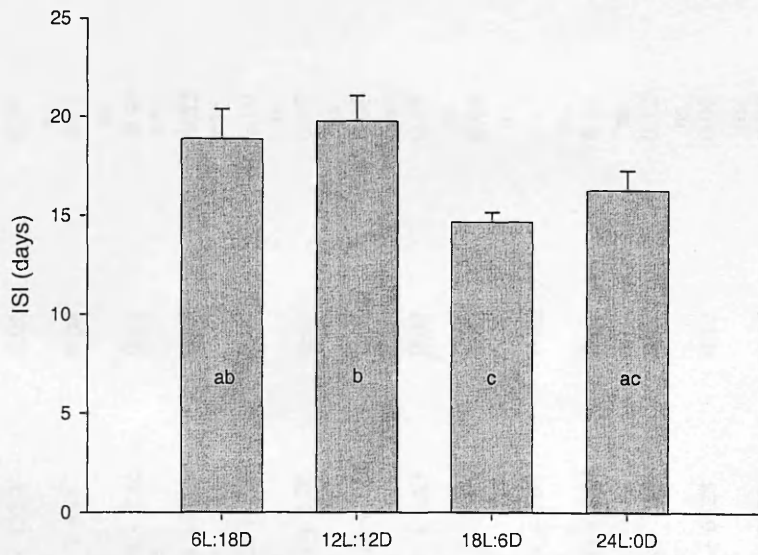


Figure 3.13 Mean inter spawning interval (ISI) of *O. niloticus* exposed to four different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

Table 3.2 Correlation and regression analyses of total fecundity, relative fecundity, egg long and short axis, diameter and volume, and total egg volume, of 65 spawns produced by eight fish under short day treatment (6L:18D)

Dependent variable (Y)	Variable tested	UNTRANSFORMED DATA (6L:18D)				TRANSFORMED DATA			
		Independent variable (X)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	
Total fecundity	Weight		0.22 ns	$y=1.34x + 1539$ ns	0.05	0.06	$y=0.096x + 3.03$ ns	0	
	Length		0.19 ns	$y=56.1x + 564$ ns	0.04	0.02	$y=0.088x + 3.15$ ns	0	
Relative fecundity (egg/g)	Weight		-0.58 ***	$y=-0.0109x + 9.88$ ***	0.34	-0.49 ***	$y=-0.904x + 3.03$ ***	0.25	
	Length		-0.60 ***	$y=-0.558x + 20.5$ ***	0.36	-0.53 ***	$y=-3.36x + 5.48$ ***	0.28	
Egg long axis (mm)	Weight		0.15 ns	$y=0.000392x + 2.58$ ns	0.02	0.10	$y=0.0364x + 0.340$ ns	0.01	
	Length		0.15 ns	$y=0.0199x + 2.20$ ns	0.02	0.15	$y=0.184x + 0.172$ ns	0.02	
Egg short axis (mm)	Weight		0.11 ns	$y=0.000202x + 2.05$ ns	0.01	0.08	$y=0.0239x + 0.264$ ns	0.01	
	Length		0.12 ns	$y=0.0113x + 1.83$ ns	0.02	0.12	$y=0.137x + 0.132$ ns	0.02	
Egg diameter (mm)	Weight		0.13 ns	$y=0.000293x + 2.31$ ns	0.02	0.08	$y=0.0294x + 0.306$ ns	0.01	
	Length		0.13 ns	$y=0.0150x + 2.03$ ns	0.02	0.13	$y=0.153x + 0.166$ ns	0.02	
Egg volume (mm ³)	Weight		0.12 ns	$y=0.00208x + 5.88$ ns	0.01	0.08	$y=0.075x + 0.614$ ns	0.01	
	Length		0.14 ns	$y=0.113x + 3.69$ ns	0.02	0.12	$y=0.422x + 0.207$ ns	0.02	
Total egg volume (mm ³)	Weight		0.42 **	$y=18.8x + 6428$ **	0.17	0.09	$y=0.171x + 3.64$ ns	0.01	
	Length		0.38 **	$y=867x - 9320$ **	0.15	0.08	$y=0.510x + 3.35$ ns	0.01	

Level of significance ns= not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table 3.3 Correlation and regression analyses of total fecundity, relative fecundity, egg long and short axis, diameter and volume, and total egg volume, of 61 spawns produced by eight fish under normal day treatment (12L:12D)

Dependent variable (Y)	Variable tested	UNTRANSFORMED DATA (12L:12D)				TRANSFORMED DATA			
		Independent variable (X)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	
Total fecundity	Weight		0.43**	$y = 2x + 1327$ **	0.18	0.49***	$y = 0.406x + 2.27$ ***	0.24	
	Length		0.39**	$y = 91.4x - 346$ **	0.15	0.40**	$y = 1.16x + 1.65$ **	0.16	
Relative fecundity (egg/g)	Weight		-0.59***	$y = -0.00853x + 9.07$ ***	0.34	-0.64***	$y = -0.594x + 2.27$ ***	0.41	
	Length		-0.59***	$y = -0.434x + 17.4$ ***	0.35	-0.64***	$y = -2.09x + 3.72$ ***	0.41	
Egg long axis (mm)	Weight		0.33**	$y = 0.000568x + 2.57$ **	0.11	0.25	$y = 0.0575x + 0.29$ ns	0.06	
	Length		0.28*	$y = 0.0240x + 2.15$ *	0.08	0.25	$y = 0.201x + 0.159$ ns	0.06	
Egg short axis (mm)	Weight		0.36**	$y = 0.000439x + 2.02$ **	0.13	0.30*	$y = 0.0645x + 0.173$ *	0.09	
	Length		0.33**	$y = 0.0204x + 1.64$ **	0.11	0.31*	$y = 0.231x + 0.009$ *	0.09	
Egg diameter (mm)	Weight		0.39**	$y = 0.000566x + 2.27$ **	0.15	0.31*	$y = 0.0699x + 0.216$ *	0.10	
	Length		0.35**	$y = 0.0257x + 1.80$ **	0.12	0.32*	$y = 0.249x + 0.041$ *	0.10	
Egg volume (mm ³)	Weight		0.41**	$y = 0.00484x + 5.26$ **	0.17	0.31*	$y = 0.194x + 0.345$ *	0.09	
	Length		0.38**	$y = 0.222x + 1.19$ **	0.14	0.32*	$y = 0.711x - 0.169$ *	0.10	
Total egg volume (mm ³)	Weight		0.76***	$y = 24.6x + 5227$ ***	0.58	0.72***	$y = 0.601x + 2.61$ ***	0.52	
	Length		0.68***	$y = 1106x - 14868$ ***	0.46	0.64***	$y = 1.87x + 1.48$ ***	0.41	

Level of significance ns= not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table 3.4 Correlation and regression analyses of total fecundity, relative fecundity, egg long and short axis, diameter and volume, and total egg volume, of 90 spawns produced by eight fish under long day treatment (18L:6D)

Dependent variable (Y)	Variable tested	UNTRANSFORMED DATA (18L:6D)				TRANSFORMED DATA			
		Independent variable (X)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	
Total fecundity	Weight	0.46 ***	$y = 2.95x + 1386$ ***	0.21	0.53 ***	$y = 0.539x + 2.01$ ***	0.28		
	Length	0.58 ***	$y = 168x - 1975$ ***	0.33	0.59 ***	$y = 2.0x + 0.543$ ***	0.35		
Relative fecundity (egg/g)	Weight	-0.51 ***	$y = -0.00894x + 10.3$ ***	0.26	-0.47 ***	$y = -0.461x + 2.01$ ***	0.22		
	Length	-0.35 **	$y = -0.277x + 14.4$ **	0.12	-0.35 **	$y = -1.13x + 2.43$ **	0.12		
Egg long axis (mm)	Weight	-0.12 ns	$y = -0.000162x + 2.71$ ns	0.01	-0.10 ns	$y = -0.0181x + 0.469$ ns	0.01		
	Length	-0.18 ns	$y = -0.0113x + 2.95$ ns	0.03	-0.18 ns	$y = -0.105x + 0.571$ ns	0.03		
Egg short axis (mm)	Weight	-0.09 ns	$y = -0.000104x + 2.14$ ns	0.01	-0.08 ns	$y = -0.0150x + 0.360$ ns	0.01		
	Length	-0.13 ns	$y = 0.00718x + 2.29$ ns	0.02	-0.13 ns	$y = -0.0857x + 0.443$ ns	0.02		
Egg diameter (mm)	Weight	-0.08 ns	$y = -0.000104x + 2.41$ ns	0.01	-0.05 ns	$y = -0.0103x + 0.401$ ns	0		
	Length	-0.14 ns	$y = -0.00782x + 2.58$ ns	0.02	-0.12 ns	$y = -0.0776x + 0.485$ ns	0.02		
Egg volume (mm ³)	Weight	-0.11 ns	$y = -0.00114x + 6.64$ ns	0.01	-0.10 ns	$y = -0.0546x + 0.928$ ns	0.01		
	Length	-0.17 ns	$y = -0.0738x + 8.16$ ns	0.03	-0.17 ns	$y = -0.294x + 1.21$ ns	0.03		
Total egg volume (mm ³)	Weight	0.43 ***	$y = 15.7x + 9368$ ***	0.18	0.50 ***	$y = 0.484x + 2.93$ ***	0.25		
	Length	0.51 ***	$y = 847x - 7248$ ***	0.26	0.53 ***	$y = 1.70x + 1.75$ ***	0.29		

Level of significance ns= not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table 3.5 Correlation and regression analyses of total fecundity, relative fecundity, egg long and short axis, diameter and volume, and total egg volume, of 75 spawns produced for eight fish under continuous illumination treatment (24L:0D)

Variable tested		UNTRANSFORMED DATA (24L:0D)			TRANSFORMED DATA		
Dependent variable (Y)	Independent variable (X)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)	Correlation coefficient	Regression equation	Coefficient of determination (r^2)
Total fecundity	Weight	0.44 ***	$y = 2.54x + 1403$ ***	0.19	0.40 ***	$y = 0.414x + 2.29$ ***	0.16
	Length	0.54 ***	$y = 137x - 1246$ ***	0.29	0.46 ***	$y = 1.51x + 1.22$ ***	0.21
Relative fecundity (egg/g)	Weight	-0.63 ***	$y = -0.00926x + 10.1$ ***	0.40	-0.52 ***	$y = -0.586x + 2.29$ ***	0.27
	Length	-0.54 ***	$y = -0.349x + 15.7$ ***	0.29	-0.45 ***	$y = -1.60x + 3.06$ ***	0.20
Egg long axis (mm)	Weight	0.05 ns	$y = 0.000108x + 2.63$ ns	0	-0.01 ns	$y = -0.0035x + 0.434$ ns	0
	Length	0.00 ns	$y = -0.00012x + 2.67$ ns	0	-0.02 ns	$y = -0.0206x + 0.454$ ns	0
Egg short axis (mm)	Weight	0 ns	$y = -0.000002x + 2.06$ ns	0	-0.05 ns	$y = -0.0119x + 0.343$ ns	0
	Length	0 ns	$y = -0.00244x + 2.12$ ns	0	-0.06 ns	$y = -0.0394x + 0.368$ ns	0
Egg diameter (mm)	Weight	0 ns	$y = 0.000007x + 2.36$ ns	0	-0.06 ns	$y = -0.0142x + 0.410$ ns	0
	Length	-0.05 ns	$y = -0.00332x + 2.46$ ns	0	-0.07 ns	$y = -0.0510x + 0.446$ ns	0
Egg volume (mm ³)	Weight	0 ns	$y = 0.00007x + 6.07$ ns	0	-0.05 ns	$y = -0.0356x + 0.867$ ns	0
	Length	-0.05 ns	$y = -0.0242x + 6.73$ ns	0	-0.07 ns	$y = -0.138x + 0.972$ ns	0
Total egg volume (mm ³)	Weight	0.46 ***	$y = 17.1x + 7650$ ***	0.22	0.35 **	$y = 0.379x + 3.16$ **	0.12
	Length	0.51 ***	$y = 830x - 7676$ ***	0.26	0.40 ***	$y = 1.37x + 2.19$ ***	0.16

Level of significance ns= not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

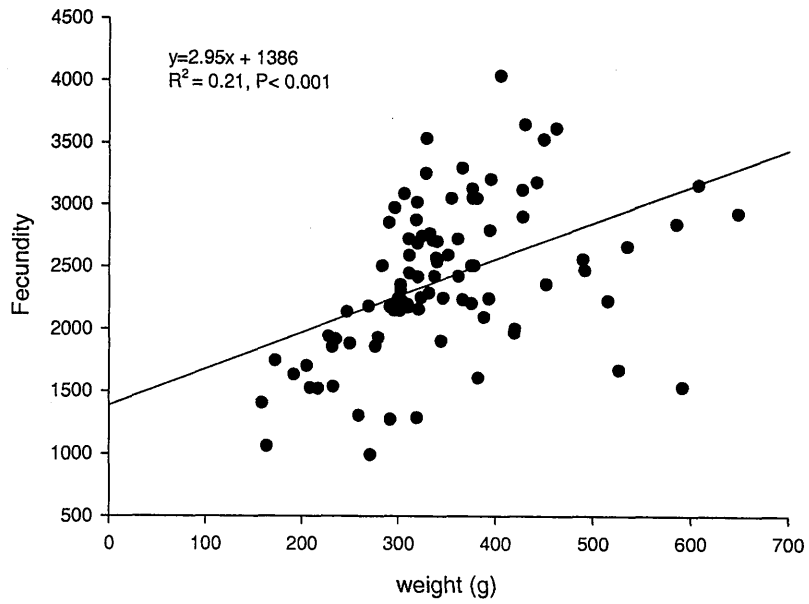


Figure 3.14 Relationship between fecundity and weight (untransformed data) in fish held under long daylength (18L:6D).

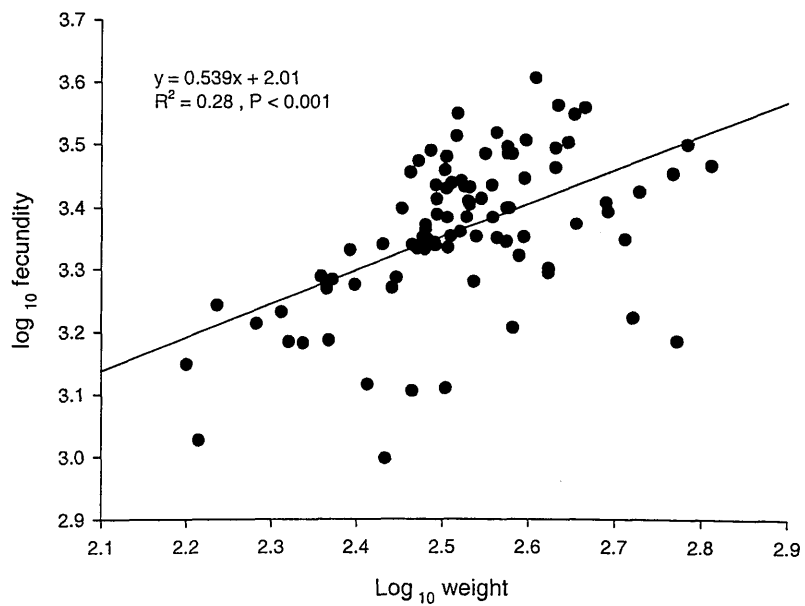


Figure 3.15 Relationship between fecundity and weight (Log_{10} transformed data) in fish held under long daylength (18L:6D).

Table 3.6 Regression analyses of ISI vs. total fecundity

Photoperiod	Independent value (X)	Dependent value (Y)	Test result
Short daylength	ISI	Total fecundity	Ns
(6L:18D)	Log ₁₀ ISI	Log ₁₀ Total fecundity	Ns
Normal daylength	ISI	Total fecundity	Ns
(12L:12D)	Log ₁₀ ISI	Log ₁₀ Total fecundity	Ns
Long daylength	ISI	Total fecundity	Ns
(18L:6D)	Log ₁₀ ISI	Log ₁₀ Total fecundity	Ns
Continuous	ISI	Total fecundity	*
(24L:0D)	Log ₁₀ ISI	Log ₁₀ Total fecundity	*

Level of significance ns= non significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table 3.7 Regression analyses of ISI vs. egg volume

Photoperiod	Independent value (X)	Dependent value (Y)	Test result
Short daylength	ISI	Mean egg volume	***
(6L:18D)	Log ₁₀ ISI	Log ₁₀ mean egg volume	***
Normal daylength	ISI	Mean egg volume	***
(12L:12D)	Log ₁₀ ISI	Log ₁₀ mean egg volume	***
Long daylength	ISI	Mean egg volume	Ns
(18L:6D)	Log ₁₀ ISI	Log ₁₀ mean egg volume	*
Continuous	ISI	Mean egg volume	Ns
(24L:0D)	Log ₁₀ ISI	Log ₁₀ mean egg volume	Ns

Level of significance ns= non significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

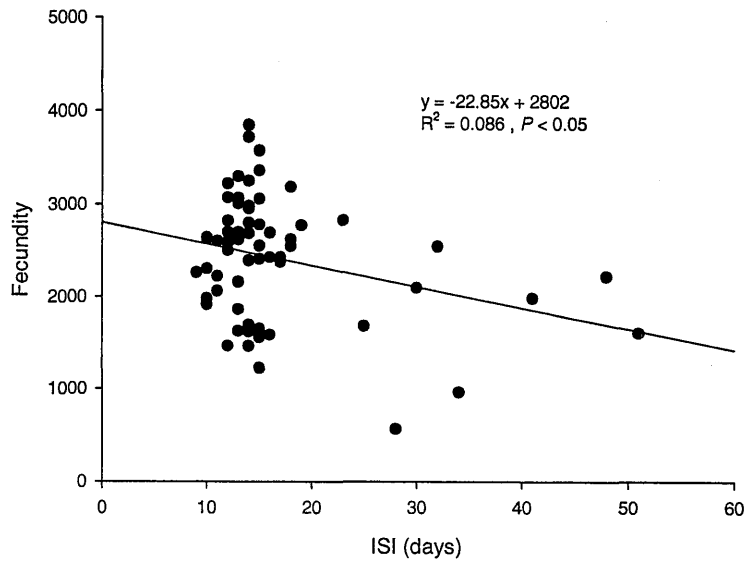


Figure 3.16 Regression analyses of ISI vs. fecundity (untransformed data) in the continuous illumination treatment (24L:0D).

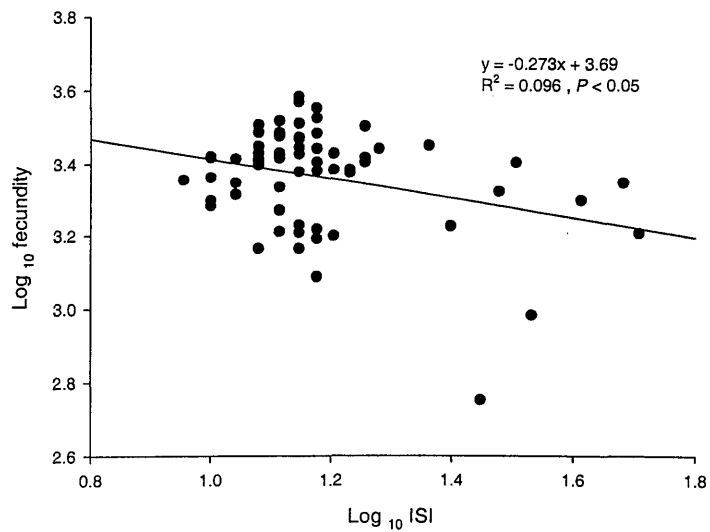


Figure 3.17 Regression analyses of ISI vs. fecundity (Log_{10} transformed data) in the continuous illumination treatment (24L:0D)

3.4 Discussion and conclusion

This study set out to further investigate the possibility of optimising commercial production of tilapia by manipulation of photoperiod. Four photoperiod regimes were chosen to mimic short, normal and long daylengths as well as a further light regime including continuous illumination.

3.4.1 Growth

Although we analysed growth rates in our experiment using conventional growth measures such as absolute growth rate (AGR), relative growth rate (RGR), and specific growth rate (SGR) each showing significant differences between treatments. Additionally, to compare growth differences in this experiment, the daily weight gain (DWG) was evaluated, according to the method described by Bhujel *et al.* (2001b). This information allowed us to successfully compare the differences in weight gained by those fish reared at different light regimes.

There were significant differences in DWG between the four photoperiods which changed the relative ranking of the mean weights and length of the short (6L:18D), normal (12L:12D) and long (18L:6D) daylength treatment groups over the 180 days of the experiment. Fish in this experiment received excess food, and the change in body size appears to be related to the amount of energy given over to reproduction.

At the beginning of this study, fish were 18 months of age and had been sexually mature for about 10 to 12 months. Sexually active tilapia partition more energy into reproduction than somatic growth, (Mair and Little, 1991; Macintosh and Little, 1995). Although there were no significant differences between the mean weights of the fish in the treatment groups at the beginning of the experiment, the means could be ranked. Fish

in the continuous illumination (24L:0D) trial remained the largest mean weight but the relative ranking in size of the 6L:18D, 12L:12D and 18L:6D groups had reversed by the end of the experiment, suggesting that the fish on the short and normal daylength (6L:18D, 12L:12D) were growing more quickly. The 18L:6D treatment was growing at the slowest rate; this group contained smaller broodstock but exhibited the highest reproduction performance. The results suggest that the diet was adequate to maintain both growth and reproductive capacity in these fish for the duration of the experiment. Wootton (1973a, b,) reported that in the three-spined stickleback (*Gasterosteus aculeatus*) the amount of food available could be determinant in the spawning activity of this species, when food supply was adequate, the fish could produce more spawns and the ISI was shorter.

3.4.2 Egg production and spawning periodicity

Figure 3.7 shows that fish under the long day photoperiod (18L:6D) consistently produced more eggs per month than all other treatments, some 58% more eggs than the normal daylength (12L:12D). Fish under the long day (18L:6D) treatment exhibited significantly increased total and relative fecundity concomitant with significantly reduced ISI, thus resulting in more frequent spawning and a greater clutch size than the other treatments. These findings are in accordance with those of Ridha and Cruz (2000), who compared seed production (egg, sac fry and swim-up fry) under three photoperiods (12L:12D, 15L:9D, and 18L:6D) and two light intensities (2500 and 500 lux). The highest number of seed were observed under a 18L:6D photoperiod (Ridha and Cruz, 2000); the 2500 lux treatment gave the highest number of seed but not significantly more

than the 500 lux treatment, which is close to that used in the present experiment. Ridha and Cruz (2000) did not show any significant effect of light intensity on any spawning activity; spawning data appeared to be periodic, probably related to the sampling procedure used. They also found no statistical difference for mean ISI between their six treatment groups; ISI ranged from 14 to 55 days and the lack of statistical significance was most likely due to the lack of data pertaining to individual fish. The shortest ISI observed by Ridha and Cruz (2000) was 14 days in fish under 18L:6D with 500 lux, which is almost identical to the shortest ISI of 15 days in our experiment (also in the long day (18L:6D) group).

The present experiment effectively 'robbed' eggs (removed eggs from the buccal cavities of mouth-brooding females) within a few hours of spawning, or resulted in fish being stripped. Both of these techniques have been shown to reduce ISI in tilapia (Fishelson, 1966; Dadzie, 1970; Siraj *et al.*, 1983; Rana, 1988, Little *et al.*, 1993; Tacon *et al.*, 1996; Coward and Bromage, 2000). The similarity in ISI between the results of Ridha and Cruz (2000) and this present study would suggest that we are getting close to the minimum ISI using these techniques. It is known that ISI is normally shorter in smaller tilapia (Siraj *et al.*, 1983). The fish used in this experiment were two to three times bigger than those females used by Ridha and Cruz (2000) and it would also suggest that the rapid removal of eggs from actively brooding females might have helped to reduce the ISI. Egg robbing is, however, time consuming and labour intensive and may therefore only be a useful contribution to broodstock management on a very intensive commercial scale.

The fish under continuous illumination treatment (24L:0D) were not significantly different to the long daylength (18L:6D) for most of the reproductive traits measured. They produced fewer eggs than the (18L:6D) trial, mainly because of the drop in monthly egg production after the third month that characterised the other treatments (Figure 3.7). This suggests that the use of a long daylength photoperiod (18L:6D) helps to extend spawning activity.

Under natural conditions, and some geographical distributions, tilapias in equatorial regions do not have any seasonality in their reproductive activity. However, under subtropical conditions, seasonality is present with a well defined pattern. These changes in reproductive seasonality are controlled by environmental cues such as water temperature and daylight. The environmental conditions exhibit little variations throughout the year in equatorial regions, but do show considerable variations in subtropical regions, particularly in terms of light and temperature (Lowe-McConnell, 1958, Philippart and Ruwet, 1982).

3.4.3 Egg size and fecundity

Photoperiod manipulation had an effect on egg size. Significant differences in egg size were observed between photoperiods. The largest eggs (diameter and volume) in this experiment were produced by fish under the normal daylength (12L:12D) (Figures 3.11 - 12). This group exhibited the lowest number of spawns and total fecundity, and the longest ISI. Significant relationships were detected in this group, between ISI and egg size (Table 3.7). This might simply imply that because ISI was longer, eggs had more time to sequester vitellogenin from the bloodstream, hence resulting in a larger final egg

size. In contrast, fish from the long daylength treatment (18L:6D) exhibited the shortest ISI but produced the smallest eggs (Figures 3.11 - 12). This is the first clear evidence that ISI directly effects egg size in this species. In previous studies, egg size in tilapia has been related to genotype, nutritional condition, age and size of the broodstock (Rana, 1985).

In this study, it appears that those fish reared under long daylength showed advancement in gonadal recrudescence, producing discrete batches of smaller eggs quickly compared to those fish reared under shorter daylengths. This is in accordance with Wootton (1979), who suggested that in fish, the ovaries have the capability to produce many small eggs or few large eggs suggesting that in fish variations in fecundity could also be reflected in egg size variations. This effect was clearly observed in salmonids and is discussed at length by Bromage and Cumaranatunga (1988), but the impact on ISI is unlikely to be as profound as in a species that can spawn as frequently as tilapia, due to salmonid species use to spawn once a year. The advantage of producing high numbers of smaller eggs has to be balanced against the fact that bigger eggs produce larger and stronger larvae at hatching, with consequent improved chances of survival under some farming regimes (Rana and Macintosh, 1988; Rana, 1985, 1988). It is unclear whether this advantage would outweigh the loss of production associated with having to use larger females or extending the ISI to get larger eggs.

Highest mean total fecundity was recorded in fish experiencing long daylength and continuous illumination in the present study. It is well documented that total fecundity is more related to tilapia size rather than age (Rana, 1988; Coward and Bromage, 1999a). In the case of the substrate spawning *Tilapia zillii*, Coward and Bromage (1999a) reported a

strong relationship between fish size (weight and length) and fecundity but did not find any significant relationship between egg size and maternal weight and length. In the present experiment, all fish were of the same age and were of similar size. The long daylength treatment (18L:6D) produced the highest relative fecundity (7 ± 0.2 eggs g⁻¹), which is very similar to that reported by Bhujel *et al.* (2001a), who reported relative fecundity of $7,331 \pm 618$ eggs kg⁻¹ in a shaded hapa system suspended in outdoor tanks under tropical conditions of light and temperature. It is still unclear why long daylength and continual illumination resulted in increased total fecundity in our experiment.

A further explanation as to why fecundity varied between photoperiods might be simply basic reproductive strategy. The population of *O. niloticus* (Manzala, Egypt) is the most northerly occurring natural population of this species and as such would experience seasonal changes in day length. It would be interesting to see whether species with a much more restricted equatorial distribution respond in the same way to longer daylength. This study, however, also demonstrates that fecundity and egg size may be determined, at least in part, by the ISI, confirming the earlier findings of Coward and Bromage (1999a). The basic mechanisms underlying the dynamics of ovarian development in a substrate-spawning species of tilapia were described in a highly quantitative manner by Coward and Bromage (1998a, 1999b). Further research, however, is needed to assess how these basic dynamics might be influenced by exogenous (e.g. temperature and photoperiod) and endogenous (e. g. melatonin, gonadotropins, sex steroid hormones) factors.

In conclusion, this study has shown that photoperiodic manipulation appears to be a reliable and powerful tool for tilapia broodstock management. Further research is needed

Chapter Three

to fully understand how photoperiod imparts such an effect upon tilapia reproduction. Particular areas of concern include the effects of 'biological' and 'reproductive' age, the precise effects of light intensity, the possible interactions of photoperiod and temperature, and how photoperiod/light intensity might influence the reproductive endocrinology of tilapia, and thus affect the dynamics of ovarian development.

4 Chapter Four Evaluation of Plasma Melatonin Levels in the Nile Tilapia *Oreochromis niloticus*, Reared under Different Light Regimes

4.1 Introduction

4.1.1 The pineal gland

The pineal, is a photosensitive organ which, over the course of 500 million years vertebrate evolution, has become an endocrine gland. It is formed as an evagination of the dorsal roof of the diencephalon, resulting in a small structure which is attached to the telencephalon by a small slender stalk. The lumen of the pineal gland is an extension of the third ventricle of the brain; however, it is still unknown if there is an active circulation of cerebrospinal fluid into the pineal lumen (Figure 4.1 A and B) (Davies *et al.*, 1991; Randall *et al.*, 1991a; Zachmann *et al.*, 1992c; Ekström and Meissl, 1997; Vanecek, 1998; Falcón, 1999; Bromage *et al.*, 2001). The size of the pineal organ varies broadly amongst fish species. In some species the pineal organs are large and cover most of the telencephalon (e.g. pike or salmon) (Ekström and Meissl, 1997). In other species such as the cod, sea bass and tilapia, the pineal gland is less conspicuous and lacks its characteristic vesicle form (Migaud, personal communication).

The wall of the pineal organ is formed by unistratified epithelium, which is strongly folded and almost obliterates the central lumen of the pineal. The pineal epithelium is formed from different cell types, including photoreceptor cells, neurons, ependymal interstitial cells (best known as supportive cells), interstitial cells or glial cells, some oligodendrocytes have been found surrounding neural axons and macrophages are found in the central lumen of the pineal (Zachmann, *et al.*, 1992c; Ekström and Meissl, 1997; Vanecek, 1998; Falcón, 1999), (Figure 4.1 C). It has been reported that melatonin is produced by the photoreceptor cell within the pineal epithelium (Zachmann, *et al.*, 1992c; Ekström and Meissl, 1997). In non mammalian vertebrates like fish, the pineal gland translates or transduces photoperiodic information into a hormonal signal (melatonin)

which then serves as a messenger to every organ in the body (Reiter, 1991, Zachmann, *et al.*, 1992c; Reiter and Tan, 2003).

4.1.2 Melatonin hormone

Melatonin is mainly secreted by the pineal gland in all vertebrates and is produced in large quantities during the dark phase (at night); production is reduced by the onset of the light phase (day). The melatonin hormone is synthesised by the pineal gland, and it is formed by synthesis from amino acid tryptophan, which then after a process of hydroxylation and decarboxylation is transformed into serotonin and then transformed into melatonin by the enzyme N-acetyl-transferase, (Axelrod, 1974; Klein, 1985; Zachmann, *et al.*, 1992c; Ekström and Meissl, 1997; Falcon, 1999; Mazurais *et al.*, 1999) (Figure 4.2).

The rhythmic biosynthesis of melatonin is thought to be controlled by the suprachiasmatic nucleus of the brain (SCN), this SCN or “internal clock” is located in the hypothalamus of most mammals studied so far. The SCN contains an endogenous circadian clock which controls the rhythmic synthesis of melatonin (Axelrod, 1974; Klein, 1985; Zachmann, *et al.*, 1992c; Gillette and McArthur, 1996). This rhythmic secretion of melatonin has an important role in the control and synchronisation of many physiological, biochemical and behavioural rhythms such as locomotor activity, sleep-awake cycles, body temperature, and feeding behaviour (Reiter, 1991; Zachmann, *et al.*, 1992c; Vanecek, 1998; Falcon, 1999). However, melatonin has the ability to reset the activity of the SCN neurons, thus melatonin may act as a clock by itself (Ekström and Meissl, 1997; Falcon 1999). This may suggest that the SCN is regulated and entrained by photoperiodic signals perceived from the environment and also by melatonin (Davies *et al.*, 1991; Randall *et al.*, 1991a; Ekström and Meissl, 1997; Falcón, 1999; Bromage *et al.*, 2001).

Melatonin exhibit a high lipophilic property, which allows it to be transferred in short period of time to fluids other than plasma, including: cerebrospinal fluid, ovarian follicular fluid, saliva, seminal fluid, eye fluid, milk and amniotic fluid (Reiter, 1991).

4.1.3 Melatonin production in fish

In fish, as well as all vertebrates, melatonin shows an increase in blood levels during the night and then a sharp reduction during the day (Matty, 1978; Gern *et al.*, 1978a,b; Delgado and Vivien-Roels, 1989; Kezuka *et al.*, 1989; Zachmann *et al.*, 1992a,b,c; Alvarino *et al.*, 1993; Davies *et al.*, 1994; Randall *et al.*, 1995a; Falcón *et al.*, 1996; Yañez and Meissl, 1996; Porter *et al.*, 1995, 1996, 2000a,b, 2001; Gasser and Gern, 1997; Ekström and Meissl, 1997; Mayer *et al.*, 1997a, 1998; Vernadakis *et al.*, 1998; Mazurais *et al.*, 1999; Pavlidis *et al.*, 1999; Bromage *et al.*, 1995, 2001; Amano *et al.*, 2000).

In the particular case of the rainbow trout (*O. mykiss*), melatonin secretion is controlled by the light cycle of the day rather than the internal clock or pace marker (Duston and Bromage, 1986; Gern and Greenhouse, 1988; Bromage *et al.*, 2001).

Melatonin level varies between species of fish, high night time levels of melatonin have been reported in most salmonids studied thus far. However, lower levels of melatonin have been reported during the night in some other species (Table 4.1).

As a consequence of such alteration in melatonin secretion during light and dark phases, several studies report the use of additional light in order to reduce melatonin levels, which are strongly related to maturation in salmonid species (Bromage *et al.*, 1995, 2001). It was reported that the application of continuous illumination for 5 months (January to May) resulted in an increment in fish weight, but decrease in sexual activity in Atlantic salmon reared in seawater (Taranger *et al.*, 1995).

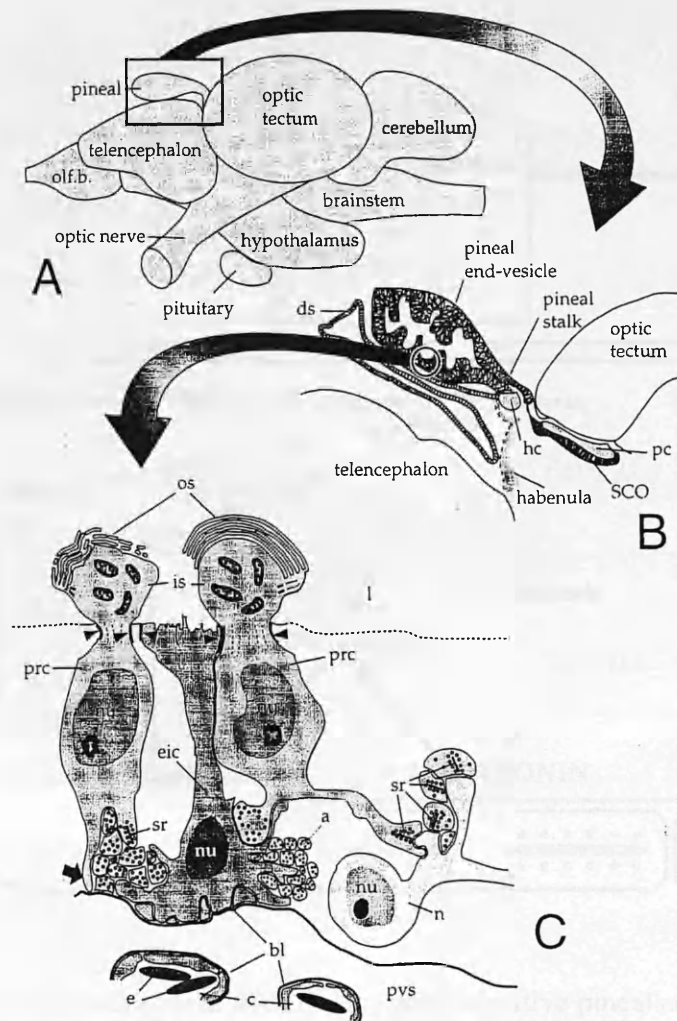


Figure 4.1 (A) Overview of the major subdivision of the teleost brain (Salmonid): olf. B., olfactory bulb. (B) mid-sagittal section of the pineal (Shown inside the rectangle in A), showing the location of the pineal gland with respect to the dorsal diencephalon (habenula and habenular commissure, hc), midbrain optic tectum, and telencephalon: ds, dorsal sac; pc, posterior commissure; SCO, subcommisural organ. (C) schematic representation of the cell types in the epithelium of the pineal end-vesicle (circular frame in B), a, axon of pineal neurons, gathered in a bundle; bl, basal lamina; c, capillaries; e, erythrocytes; eic, interstitial cell; is, photoreceptor inner segment with mitochondria; l, pineal lumen; n, centrally projecting neuron, 'ganglion cell'; nu, nucleus; os, photoreceptor outer segment; prc, photoreceptor cells; pvs, perivascular space; sr, synaptic ribbons; arrows indicate photoreceptor basal process that terminates on the basal lamina; arrowheads indicate tight junctions that form a barrier against the cerebrospinal fluid of the pineal lumen. (Taken from Ekström and Meissl, (1997))

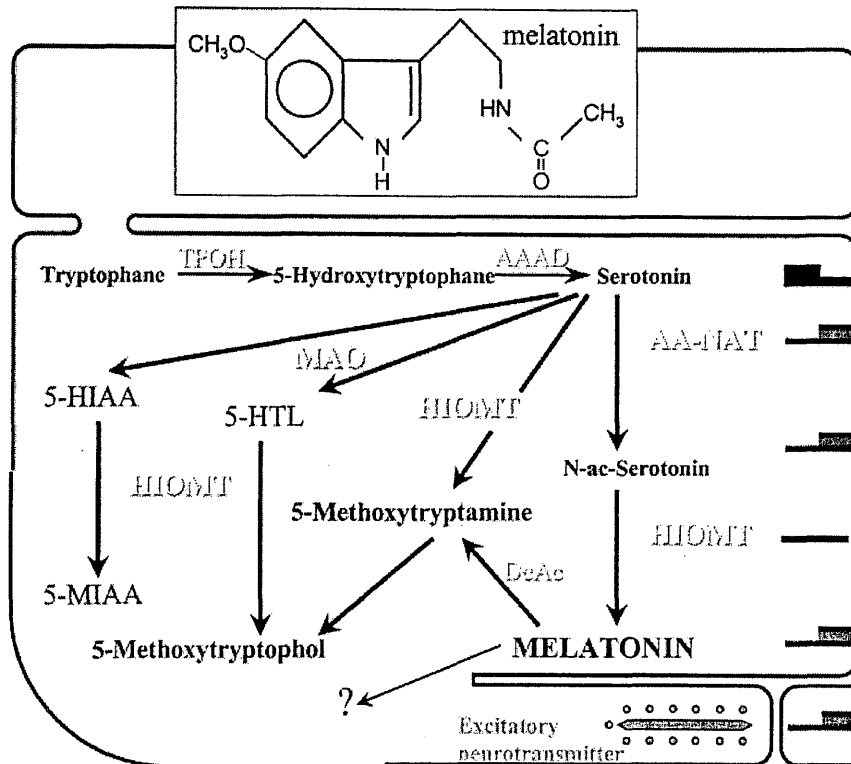


Figure 4.2 Pathways of the indole metabolism in the photosensitive pineal cells. Enzymes: AAAD, aromaticamino acid decarboxylase; AA-NAT, arylalkylamine N-acetyltransferase; DeAc, deacetylase; HIOMT, hydroxyindole-O-methyltransferase; MAO, monoamine oxidase; TPOH, tryptophane hydroxylase. Indoles: N-ac-serotonin, N-acetylserotonin; 5-HIAA, 5-hydroxyindole acetic acid; 5-HTL, 5-hydroxy-tryptophol; 5-MIAA, 5-methoxyindole acetic acid. The chemical structure of melatonin is given in the upper part of the drawing. On the right hand of the scheme, the drawings indicate whether the corresponding compound, or enzyme, peak during day (square on the left), or during night (square on the right), or display no rhythm (horizontal line), (Taken from Falcon, 1999).

The use of additional illumination has also been applied to reduce melatonin levels in Atlantic salmon. Additional light for a 9 month period (November - July) resulted in the secretion of lower levels of melatonin during the night (303.1 ± 6.3 pg/ml) compared with those fish held under natural illumination (600 ± 53 pg/ml). Grilising rates were reduced to 6.1% in those fish exposed to additional light. However, 61.5% of those fish kept in normal photoperiod underwent a precocious maturation (grilising). Thus, the additional light triggered a reduction in melatonin levels of 50% concomitant with a remarkable reduction in grilising (Porter *et al.*, 1999). In a similar attempt to reduce grilising using submersible lights (400 watts), night melatonin levels were decreased and precocious maturation was observed in just 2% of the fish exposed to additional illumination. The use of additional illumination also enhanced fish growth (Porter *et al.*, 2000b). In Masu salmon, melatonin is responsible for transduction of photoperiodic information and that this information is then sent to the brain-pituitary gonadal-axis, resulting in the onset of fish reproduction (Amano *et al.*, 2000).

To identify the amount of melatonin produced by the pineal gland, a surgical technique known as pinealectomy has been applied. In this procedure, the pineal gland is surgically removed and then plasma melatonin levels assayed. Thus, any melatonin detected must come from an alternative source. Melatonin is also produced by the retina, or even in the intestinal tract. However, these melatonin levels are not significant and will not produce a considerable effect upon the blood stream concentration (Delgado and Vivien-Roels, 1989; Zachmann, *et al.*, 1992c ; Ekström and Meissl, 1997).

Pinealectomy was first reported by Kezuka *et al.* (1989) using the goldfish (*Carassius auratus*) as a research model. The pinealectomized fish ovulated during the day. This was a surprising result, because in the goldfish, spawning never occurs during the day; spawning only ever occurs during the night. Thus, this investigation revealed that

the pineal gland is involved in determining ovulation time in goldfish. The melatonin levels of this species are strongly regulated by an endogenous rhythm or “internal clock” (Kezuka *et al.*, 1989).

Removal of the pineal gland in Atlantic salmon resulted in reduced night-time melatonin levels. Pinealectomized fish exhibited a night-time melatonin level of 96 ± 6.5 pg/ml. On the other hand, control fish showed far higher melatonin levels of 598 ± 19.3 pg/ml (Porter *et al.*, 1995, 1996). These results suggested that removal of the pineal gland may help to overcome the precocious maturation already reported in young salmon.

In rainbow trout, pinealectomy has delayed spawning activity. Fish, in which an incision was made, but the pineal gland was left intact (sham pinealectomy), spawned earlier compared with pinealectomised fish. Pinealectomized fish did not respond to changes in photoperiod, but spawned in advance of those fish held under long days or ambient photoperiods (Randall *et al.*, 1995b).

Pinealectomy and melatonin levels are also reported for a non-salmonid fish. The European sea bass (*Dicentrarchus labrax*) exhibited a similar pattern of melatonin production, with high levels during the night and low levels during the day; melatonin levels of 195 ± 5.7 pg/ml and 73.86 ± 5.7 pg/ml were reported for night and day respectively. Night-time melatonin levels were reduced in pinealectomised fish with values of 97.65 ± 4.4 pg/ml. No differences were found in melatonin levels during the day in both pinealectomised fish and control ones (Porter *et al.*, 2000a).

Table 4.1 Diurnal variation of melatonin concentration in a variety of fish species

Common name	Melatonin levels pg/ml	Culture conditions	References
Rainbow trout	750-550 (D) 50-60 (L) 750 -500 (D) 25 – 100 (L)	18L:6D F.W. 8L:16D F.W. <i>in vivo</i> 7.5 -8°C	Randall <i>et al.</i> , 1992
Rainbow trout	152.6 (D) 81.2 (L)	12L:12D F.W. <i>in vivo</i> 11°C	Gern <i>et al.</i> , 1978ab
Rainbow trout	204.6 (D) 48.4 (L)	10L:14D F.W <i>In vivo</i> sham operated	Mazurais <i>et al.</i> , 1992
Rainbow trout	120 – 230 (D) 20 – 40	12L:12D F.W. <i>in vivo</i>	Futter <i>et al.</i> , 2000
Atlantic salmon	200 – 350 (D) 125 – 175 (L)	Year round F.W. <i>in vivo</i>	Randall <i>et al.</i> , 1995a
Brook trout	600 – 1000 (D) 150 – 250 (L)	16L:8D F.W. <i>in vivo</i> 12°C	Zachmann <i>et al.</i> , 1992b
Atlantic salmon	600 (D) 60 (L)	12L:12D S.W. <i>in vivo</i>	Porter <i>et al.</i> , 1996
Atlantic salmon	580 – 650 (D) 100 – 280 (L) 300(D)(Additional light) 90 (L)	10L:14D S.W. <i>in vivo</i> 24L:0D S.W. <i>in vivo</i>	Porter <i>et al.</i> , 1999
Cherry salmon	700 (D) 100 (L)	16L:8D F.W. <i>in vivo</i>	Amano <i>et al.</i> , 2000
Catfish	20 - 40 (D) 5 – 20 (L)	12L:12D F.W. <i>in vivo</i> 22°C	Igo <i>et al.</i> , 1997
White sucker	10 - 40 ng/3h (D) 30 - 50 ng/3h (D)	12L:12D F.W <i>in vitro</i> 10°C 12L:12D F.W. <i>in vitro</i> 20°C	Zachmann <i>et al.</i> , 1992a
European sea bass	195(D) 73.8 (L)	12L:12D S.W <i>in vivo</i>	Porter <i>et al.</i> , 2000a
European sea bass	23 (D) 30 (D) 101 (D) 144 (D) 25 (L)	autumn S.W. <i>in vivo</i> winter spring summer year round	García-Allegue <i>et al.</i> , 2001
European sea bass	150 (D) 10-20 (L)	12L:12D S.W. <i>in vivo</i> 23°C	Bayarri <i>et al.</i> , 2002
Common dentex	384.3 (D) 54.4 (L)	S.W. <i>in vitro</i>	Pavlidis <i>et al.</i> , 1999

F.W =Fresh water; S.W. = Salt water; (D) = Dark phase; (L) = Light phase

The effects of light intensity and temperature *in vivo* have also been reported. In the Atlantic salmon, melatonin levels respond in different ways to variations in light intensity and temperature. Melatonin secretion was lower in fish held at 4°C (253.3 ± 16.8 pg/ml) compared with fish reared at 12°C (566 ± 34.8 pg/ml). There is a negative relationship between blood melatonin levels and light intensity, in which, melatonin levels decrease as light intensity increases during the night time. Therefore, high light intensities are recommended in order to reduce the melatonin levels of Atlantic salmon (Porter *et al.*, 2001).

Plasma melatonin levels have also been described in the river lamprey, (*Lampetra fluviatilis*). This species even when this is not a fish, exhibited a similar pattern of melatonin secretion of that already described in other species of fish; high levels (80 pg/ml) were found just 1.5 hours after the onset of the dark period. Base melatonin levels during day time were 12 pg/ml (Mayer *et al.*, 1998).

Melatonin profiles *in vivo* and *in vitro* have been described in several fish species. *In vivo* profiles are reported for rainbow trout (Randall *et al.*, 1991; Gern *et al.*, 1978ab; Mazurais *et al.*, 1992; Futter *et al.*, 2000), Atlantic salmon (Randall *et al.*, 1995ab; Porter *et al.*, 1996, 1999), brook trout (Zachmann *et al.*, 1992b), cherry salmon (Amano *et al.*, 2000), European river lamprey (Mayer *et al.*, 1998), European sea bass (Porter *et al.*, 2000a; García-Allegue *et al.*, 2001; Bayarri *et al.*, 2002), and catfish (Iigo *et al.*, 1997). *In vitro* studies are described for the white sucker (Zachmann *et al.*, 1992a), the goldfish (Kezuka *et al.*, 1989), the lamprey (Bolliet *et al.*, 1993), the rainbow trout (Yañez and Meissl, 1996), the ayu (Mizusawa *et al.*, 2001), and the common dentex (Pavlidis *et al.*, 1999)

In vitro studies describing melatonin production by the pineal gland has been reported in the white sucker (*Catostomus commersoni*). In this investigation it was reported

that both photoperiod and temperature controlled melatonin production. Melatonin production was lower at 10°C; higher melatonin levels were found in pineal culture at 20°C. In the white sucker, the secretion of melatonin *in vitro* showed a free-running circadian rhythm under constant darkness at 20°C, but a pineal culture at 10°C failed to release melatonin in a rhythmic manner. Thus, this investigation suggested that temperature is involved in the control of pineal melatonin production (Zachmann *et al.*, 1992a). Temperature has a strong influence on melatonin rhythm in the lamprey (*Petromyzon marinus*). The rhythm of melatonin secretion by pineal glands incubated at 10°C decreased in comparison with those pineals kept at 20°C. In both cases melatonin was released in a rhythmic way, even without any input from the central nervous system. Pineal glands cultured under constant darkness failed to show any pattern in melatonin release. Thus, this investigation reported that light and temperature have a strong effect on melatonin secretion (Bolliet *et al.*, 1993).

The effect of light intensity on *in vitro* experiments has also been reported. In rainbow trout, pineal glands were responded by light intensities and the highest production of melatonin was observed at low light intensity (Yañez and Meissl, 1996). Meissl *et al.* (1996) reported that *in vitro*, rainbow trout pineal glands have a robust irradiance-dependent manner, in which, low light irradiance enhanced melatonin production, whilst an increase in light intensity resulted in a clear reduction in melatonin production.

Investigations in the ayu (*Plecoglossus altivelis*), have helped to confirm that melatonin secretion by the pineal gland is regulated and entrained entirely by the diurnal rhythms of light. In ayu, light pulses in pineal culture maintained under constant darkness were able to suppress melatonin release (Mizusawa *et al.*, 2001). Even in tropical teleosts, the pineal organ is able to maintain a rhythmic production of melatonin. The sailfin molly (*Poecilia velifera*) is able to maintain at least four multiple circadian oscillations. It has

also been demonstrated that these circadian oscillations are fully functional in new born animals (Okimato and Stetson, 1999). Similar rhythmic production was reported in the zebrafish, in which a self-sustaining circadian oscillator is present, and this oscillator regulates melatonin production in the pineal gland (Cahill, 1996).

Melatonin was originally thought to be synthesised only in the pineal gland, but several recent studies have demonstrated that melatonin is present and probably also synthesised, in areas other than the pineal (Delgado and Vivien-Roels, 1989; Zachmann *et al.*, 1992c; Ekström and Meissl, 1997). High melatonin levels have been reported in ocular tissue in the common frog (*Rana perezi*). These melatonin levels exhibit diurnal variation like that reported for the pineal gland. This ocular melatonin production was regulated by the environmental temperature (Delgado and Vivien-Roels, 1989). In a similar manner, high melatonin levels have been reported in the gastrointestinal tract in several organisms including fish, amphibians and reptiles. The highest melatonin levels were found in the reptile (*Thamnophis sirtralis*) with a concentration of 2284 pg/g of stomach tissue. The lowest value was reported in the axolotl (*Ambystoma mexicanum*) with just 30 to 33pg/g of tissue. Fish melatonin levels lie within a range that is intermediate between reptiles and amphibians (Bubenik and Pang, 1997).

4.1.4 The role of melatonin in reproduction

4.1.4.1 Mammals

The role of melatonin in mammalian reproduction has been partially elucidated with seasonal rhythms of fertility driven by changes in the production and release of gonadotropin-releasing hormone (GnRH). This GnRH regulates the release of gonadotropic hormones from the pituitary gland, and thus controls function in the reproductive organs. In all mammals, the adaptation to seasonal changes in ambient conditions depends on the presence of intact pineal glands. Melatonin has the ability to

inhibit leuteinizing hormone (LH) and follicle stimulating hormone (FSH) release from the pituitary gland (Venecek, 1998). Thus, the major role of melatonin is the regulation of seasonal reproduction through alteration of the release of gonadotropins (Nakazawa *et al.*, 1991; Venecek, 1998).

4.1.4.2 Fish

The only studies suggesting that melatonin could be involved in fish reproduction were reported by Khan and Thomas (1996). These authors suggested that melatonin enhanced the stimulation of gonadotropin II (GtH II) secretion via the preoptic anterior hypothalamic area (POAH), and the pituitary itself to stimulate GtH II release in the Atlantic croaker (*Micropogonias undulates*). Melatonin inhibits the production of the leuteinizing hormone-releasing hormone analogue (LHRHa), thus, resulting in GtH II release during the night in a dose-dependent manner (Khan and Thomas, 1996). In Masu salmon, melatonin is responsible for the transduction of photoperiodic information and this information is then sent to the brain-pituitary-gonadal axis, resulting in the onset of fish reproduction (Amano *et al.*, 2000).

Although there has been much research into how melatonin and the pineal gland might influence reproduction in fish, there is still much speculation as to whether melatonin plays a pivotal role in reproduction. Indeed, Mayer, (2000) suggested that the pineal and circulating melatonin may not play an important role in the seasonal reproduction in fish.

Photoperiodic manipulation is known to have a positive effect upon Nile tilapia reproduction (Campos-Mendoza *et al.*, 2003, 2004); egg production can be improved and inter spawning interval (ISI) reduced under a photoperiod involving long daylength. The reproductive response to long day photoperiod strongly suggested that tilapia responds to light manipulation, even though this species is naturally distributed in tropical and

subtropical regions. This is the first evidence that light manipulation could be applied to the management of tilapia broodstock. To further investigate the relationship between photoperiod and tilapia reproduction, the hormone melatonin (N-acetyl-5-methoxytryptamine) was evaluated in blood samples taken from tilapia held under experimental conditions.

The use of environmental factors such as photoperiod could improve spawning synchrony as well as seed production in several species. The relationship between photoperiod, melatonin and the control of reproduction could become useful tools in the control of tilapia reproduction. Tilapia reproduction suffers from low fecundity as well as low spawning synchrony. This disadvantage is a major problem in the supply of seed for on-growing farms, which require a year-round supply of fry to maintain continuous production of market sized fish. This experiment was therefore designed to further investigate the findings of the previous chapter (Chapter Three), in which photoperiod manipulation was shown to improve egg production rate in tilapias. In this chapter, experiments have been undertaken to determine whether photoperiodic manipulation imparts its effect over reproduction in tilapias by melatonin. To answer these questions, the blood melatonin levels of *Oreochromis niloticus* reared under controlled conditions of light intensity, photoperiod and temperature were measured and analysed.

4.2 Materials and methods

4.2.1 Fish

One year old *O. niloticus* females were used in this experiment for the 24 h plasma melatonin profile (Section 4.3.2) and night-time melatonin levels (Section 4.3.3) mean average fish weight was 216.22 ± 4.70 g. However, comparison between day and night blood melatonin levels, and melatonin profiles under different photoperiods, were performed in those fish used and already described in Chapter Three.

A detailed description of fish husbandry and blood sampling is presented in Chapter Two (Section 2.3 – 2.4). Briefly, blood samples were collected at different intervals of time during the light phase and dark phase. Blood was sampled from the caudal aorta of fish anaesthetised in 1:10,000 ethyl 4-aminobenzoate (Sigma Chemicals, Ltd U.K.). In order to carry out the blood sampling during the dark phase, a dim red light (> 650 nm; Kodak Ltd.) was used to aid vision. Blood samples were collected using 1 ml syringes that had been previously rinsed with 4 mg/ml heparin (Sigma Chemicals, Ltd U.K.).

4.2.2 Diel profile of plasma melatonin

Five fish were sampled at hourly intervals in order to characterize the diel (24 hr) profile of plasma melatonin. Thirteen sample points were performed at the beginning of the experiment (29/08/02). After that, fish were allowed to recover and 11 sample points were taken a week after the first sampling (4/09/02).

4.2.3 Plasma melatonin levels during the night phase

To confirm the night phase plasma melatonin levels obtained in the diel profile, 45 fish were sampled at nine sampling points including two light base levels at 11:00 and 17:00 h. The night-time sample points were collected as followed: 20:00, 21:00, 23:00,

01:00, 02:00, 03:00, 05:00, 07:00, and 08:00 h, respectively. Each sample point consisted of five fish.

4.2.4 Comparison of day and night levels of plasma melatonin

In this experiment, melatonin levels of plasma samples taken at midday and midnight were compared, using fish exposed to different light regimes. Samples were collected at midday ($n = 4$) and midnight ($n = 4$) in each light regime (4 light regimes in total). In all photoperiods, the light went on at 08:00 and was turned off at different times according to the photoperiod regime. Sample points are described in Figure 4.3.

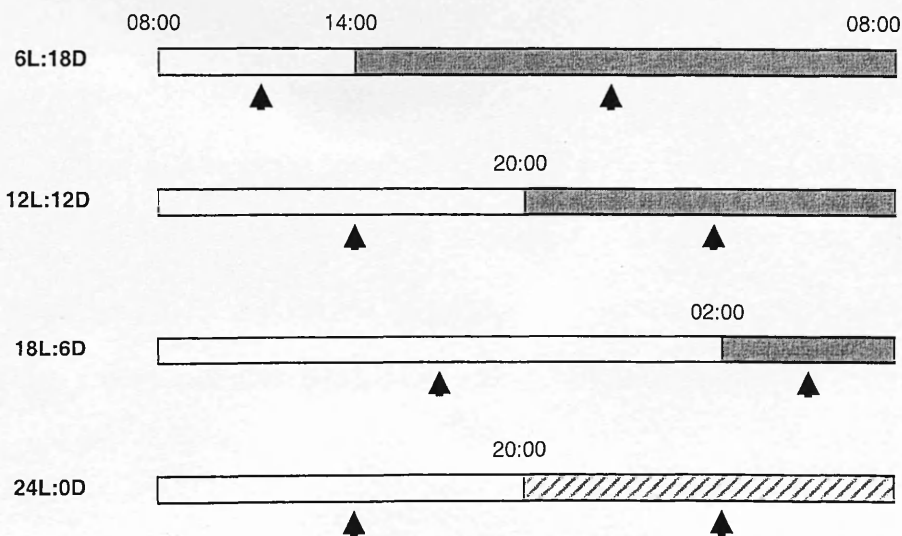


Figure 4.3 Sample points for melatonin comparison between day and night using fish held at different light regimes. (Bold numbers in the left side indicates the treatments, arrow heads indicate the sample point, open bars indicate day-time, grey bars indicate the night-time, and lined bar indicates the subjective night-time, numbers on the middle of the bars indicate the onset of the night-time)

4.2.5 Melatonin profiles of fish exposed to different light regimes

This experiment was designed to investigate how photoperiod manipulation could influence the levels of plasma melatonin. Four photoperiods were designed and have been previously described in Chapter Three: Briefly, four photoperiods were evaluated in this experiment: 6L:18D, 12L:12D, 18L:6D and 24L:0D. Fish were exposed to these light regimes for one year. In this particular case, samples were limited at each sampling point (n = 2 or n =3 individuals) due to the small number of fish available in each treatment (eight fish).

4.3 Results

4.3.1 Melatonin validation

An established melatonin assay (previously used for salmonid species) was validated for its use in tilapias following an RIA method according to Randall (1992), (Figure 4.4). Figure 4.5 shows parallelism of a pool of plasma extracted from fish during the middle of the night and a standard curve made up from a well known melatonin concentration. The intra-assay coefficient of variation was 5.67% and the inter-assay coefficient of variation was 7.34%.

4.3.2 Diel profile of plasma melatonin

Plasma melatonin levels were observed during a 24 h cycle. Significant differences were found between day and night levels of melatonin ($P < 0.05$), in which the highest level was detected at 21:00 (dark phase) with levels of 74.5 ± 10.7 pg/ml. The lowest level was found at the middle of the light phase at 16:00 with a basal level of 5.0 ± 1.4 pg/ml. Figure 4.6 shows the melatonin profile throughout a 24 h cycle; those sample points marked with an asterisk indicate that no melatonin levels were detected. This is likely to be due to very low concentration levels of melatonin, which were below the detection limit of this assay.

4.3.3 Plasma melatonin levels during the night phase

Night-time melatonin levels are shown in Figure 4.7. Significant differences were found between day and night plasma melatonin levels ($P < 0.05$). Day melatonin levels ranged between 22.2 ± 3.7 pg/ml at 11:00 and 13.7 ± 3.2 pg/ml at 17:00 h. However, melatonin levels increased soon after lights were turned off. Night-time melatonin levels varied between 99.4 ± 6.6 pg/ml at 23:00 h and 62.2 ± 4.7 pg/ml at 07:00 h.

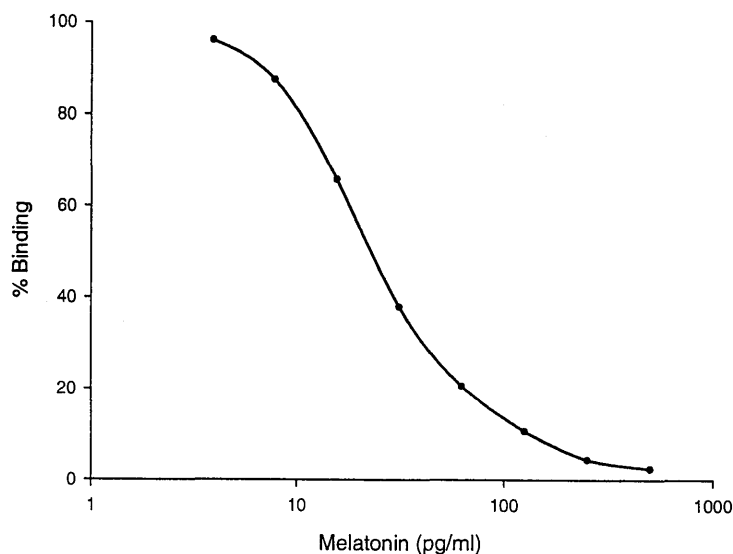


Figure 4.4 Typical standard curve from a radioimmunoassay (this example is from a melatonin assay performed during validation of the RIA for tilapia. The concentration of hormone in a sample is obtained by intersecting the standard curve at the point corresponding to the percentage binding (percentage of radiolabel bound to antibody) in the sample.

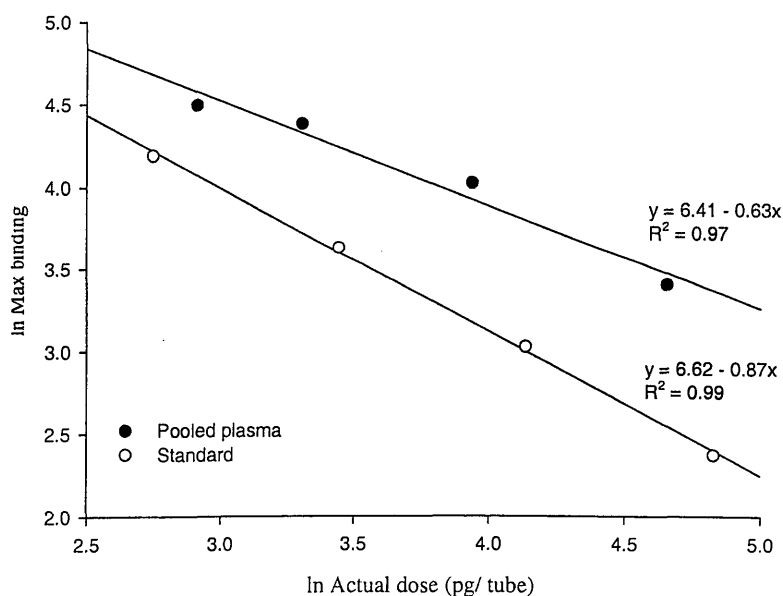


Figure 4.5 Parallelism of an inhibition curve obtained from a dilution curve (1:2) of 500 μ l aliquots of pooled tilapia plasma (collected during the dark phase) with the melatonin assay standard curve. The two curves have been linearised by logit transformation (see Rodbard and Lewald, 1970): $\text{logit } b = \ln(b/100-b)$ where b represents the proportion of the tritiated hormone bound to antibody expressed as a percentage of that in zero standard (% maximum binding). Each point represents the mean of duplicated samples. The scale on the x-axis denotes the natural log (ln) of the melatonin content in the standards.

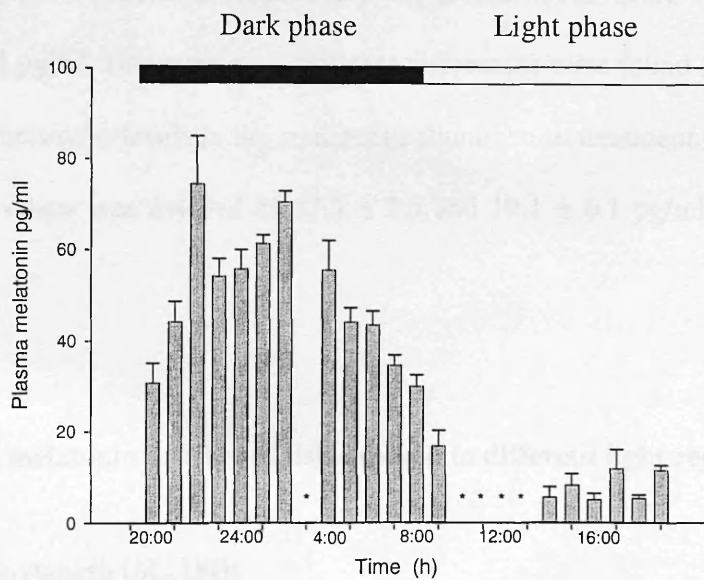


Figure 4.6 Diel plasma melatonin profile of *Oreochromis niloticus* reared under (12L:12D) photoperiod. Times are given in 24 hr format. Values are expressed as mean \pm S.E.M., (n = 5). (13 samples pints were initially taken (20:00 – 08:00) after a week, a second sampe was carried out, this consisted on 11 sample ponts (09:00 – 19:00).

4.3.4 Comparison between day and night levels of plasma melatonin

Comparisons of midday and midnight plasma melatonin levels of fish exposed to different light regimes are shown in Figure 4.8. Significant differences were found between day and night melatonin levels in all photoperiod treatments ($P < 0.05$); levels during the night were consistently higher than those during the light phase. Day-time melatonin levels of 20.7 ± 3.3 , 14.2 ± 1.1 , 14.7 ± 1.1 pg/ml were recorded in the 6L:18D, 12L:12D and 18L:6D photoperiod treatments respectively, night-time levels were 65.5 ± 6.1 , 69.7 ± 12.3 , 35.0 ± 3.4 pg/ml. However, no significant differences were found ($P > 0.05$) between night and day melatonin levels in the continuous illumination treatment (24L:0D) in which day and night values was reached as 17.5 ± 2.0 and 19.1 ± 6.1 pg/ml respectively ($P > 0.05$).

4.3.5 Plasma melatonin profiles of fish exposed to different light regimes

4.3.5.1 Short daylength (6L:18D)

The melatonin profile exhibited by fish held under short daylength is described in Figure 4.9 A. The highest plasma melatonin levels were recorded at 23:00 h with a mean value of 77.4 pg/ml. The lowest value was obtained at 13:00 h with a value of 15.7 pg/ml. (Note: there was a considerable variation in these values, this was due to the low number of fish sampled in every sample point ($n = 2 - 3$ fish)).

4.3.5.2 Normal daylength (12L:12D)

The melatonin profile of fish experiencing a normal daylength is given in Figure 4.9 B. Under this light regime the highest melatonin concentration was found at 03:00 h with 47.0 pg/ml. However, the lowest value of 13.2 pg/ml was detected at 10:00 h.

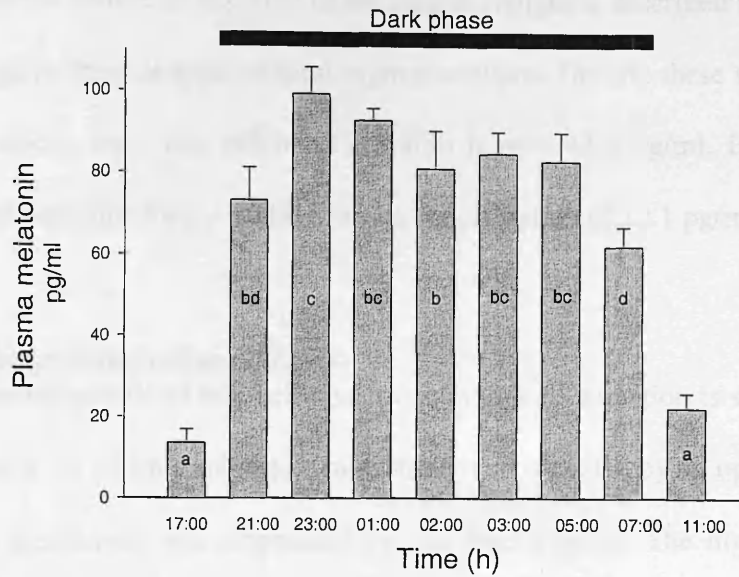


Figure 4.7 Night-time plasma melatonin profile of *O. niloticus* reared under normal daylength (12L:12D). Values are expressed as mean \pm S.E.M (n = 5). Bars annotated with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$), (n = 5).

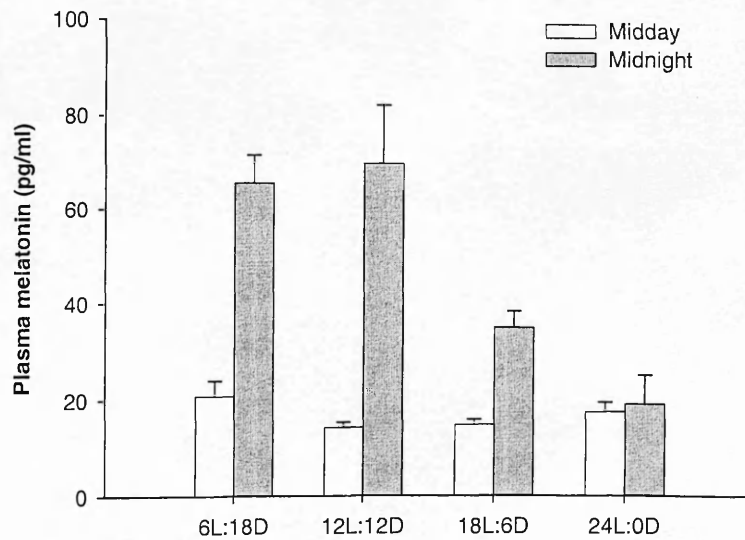


Figure 4.8 Midday and midnight plasma melatonin levels in fish held under different light regimes. Values are expressed as mean \pm S.E.M., (n = 4).

4.3.5.3 Long daylength (18L:6D)

The melatonin profile of fish held under long daylength is described in Figure 4.9 C. This light regime is characterized by short night conditions. Despite these short nights, the maximum melatonin level was exhibited at 14:30 h with 48.9 pg/ml. Baseline plasma melatonin levels were obtained at 10:00 h with a concentration of 12.1 pg/ml.

4.3.5.4 Continuous illumination (24L:0D)

The melatonin profile of fish held under continuous illumination is shown in Figure 4.9 D. This profile, in which a subjective night-time is represented by an open bar, showed that melatonin production was suppressed by this light regime. The highest melatonin concentration was obtained at 08:00 h with 19.5 pg/ml. Baseline melatonin levels were observed at 14:00 and 16:00 h with 10.2 pg/ml respectively.

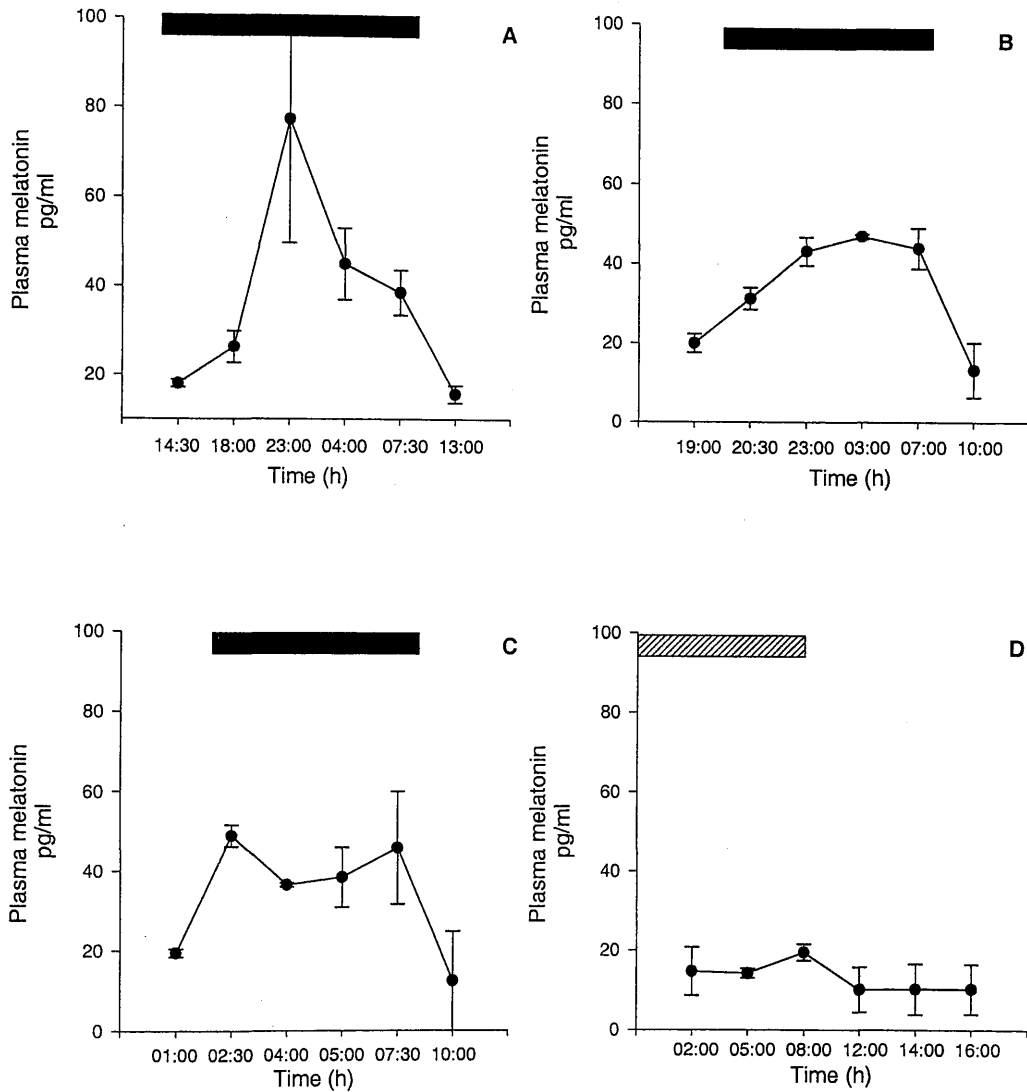


Figure 4.9 Plasma melatonin profile of *O. niloticus* reared under different light regimes: A) Short daylength (6L:18D); B) Normal daylength (12L:12D); C) Long daylength (18L:6D); D) Continuous illumination (24L:0D). Solid bars represent the dark phase and the lined bar represents the subjective night phase. Values are expressed as mean \pm S.E.M., (n = 2 -3). (Note: Scale in the (X) axes shows different sampling times).

4.4 Discussion and conclusion

In the present study, an established RIA for melatonin in salmonid species was adapted for use in tilapia. Subsequently, it was possible to demonstrate, for the first time in tilapias, how melatonin varies in the blood throughout a 24 h period. Furthermore, this allowed measurement of melatonin in plasma samples from the experiment described in Chapter Three, and thus gave an indication of how photoperiod alters melatonin in this species.

Melatonin assays have been successfully performed and described with high accuracy in temperate species (Bromage *et al.*, 2001). However, when this well established assay was applied to tropical species, such as tilapias a series of difficulties arose. Firstly, circulating melatonin levels are lower in tilapia compared with other temperate species such as salmon or rainbow trout (Bromage *et al.*, 2001). The low concentration of circulating melatonin in tilapias meant that a larger volume of plasma (500 μ l) was required in the melatonin assays rather than the 250 μ l typically used in rainbow trout or salmon (Randall, 1992). Reduced melatonin levels bring further complications in the sense that replication and statistical analysis becomes difficult, especially when low number of individuals is available for sampling. Secondly, tilapias possess highly pigmented plasma, due to the presence of pigments in the diet, as well as high concentrations of blood vitellogenin, which both cause interference in the RIA evaluation (Randall, personal communication).

These problems were solved with the production of melatonin-free plasma, which was used in the standard curve of the RIA protocol. Melatonin free-plasma allowed us to successfully evaluate melatonin levels in tilapia. However, other problems were encountered, for example, the high variability and quick degradation of radiolabel ([O-methyl- 3 H] melatonin), that produced some variation between assays in the melatonin profile, especially those profiles carried out on fish exposed to different light regimes, even

when the inter or intra assay coefficients of variations showed that the assay was reliable and reproducible. However, the major problem found in this study was the low number of fish sampled per sampling point in those profiles made with fish held under different light regimes.

Despite these problems found during the melatonin assay evaluation, the results showed that tilapia melatonin levels clearly responded to light regimes. In tilapia, plasma melatonin levels reached their maximum point just after the onset of the night phase, these levels remain high throughout the night and then just after the onset of light phase, melatonin levels were suppressed. These results were in total accordance with Randall (1992), who, using a non validated melatonin radio-immunoassay reported night-time melatonin levels of ~ 80 pg/ml, however, day-time samples were undetectable. This was the first indication suggesting that melatonin levels vary significantly in tilapia. In a subsequent analysis, night-time melatonin levels dropped to 25 pg/ml. This reduction in melatonin concentration was attributed to radiolabel degradation, as shown by changes in the slope of the standard curve (Randall, 1992). Tilapia exhibited typical diel changing melatonin levels, as broadly reported in most species investigated so far (Zachmann *et al.*, 1992c; Mayer *et al.*, 1997a; Bromage *et al.*, 2001).

Reiter (1991) classified mammalian melatonin profiles into three categories. In Type A (or I) melatonin levels remain low for several hours after the dark phase, and then increase to reach a low peak during the second part of the dark phase, then melatonin levels decrease to base levels just before the onset of the light phase. In Type B (or II), melatonin levels start to increase just after the onset of darkness and reach maximum levels during the middle of the night; however, melatonin secretion then gradually decreases to base levels by the onset of the light phase. In Type C (or III), melatonin levels increase as soon as the dark phase begins to reach a maximum level which remains constant through

the entire dark period; melatonin subsequently decreases to basal levels just before the onset of the light period. If we apply this mammalian classification to fish species, then a typical example for a Type A profile is given by the Atlantic cod (*Gadus morhua*), the Type B has not been clearly identified yet in fish species, and finally, the Type C which is well represented by the rainbow trout (*Oncorhynchus mykiss*), and the Atlantic salmon (*Salmo salar*), (Randall, 1992; Randall *et al.*, 1995a Bromage *et al.*, 2001; Porter *et al.*, 2001).

Melatonin profiles in tilapia are more likely to follow the type C pattern, characterised by a rapid increase in melatonin production just after the onset of the dark phase, then this melatonin levels remain high throughout the night and then decrease as soon as the light phase is reached. Thus, plasma melatonin profiles in tilapia are similar to those ones reported for the rainbow trout and Atlantic salmon (Randall, 1992; Randall *et al.*, 1995a; Bromage *et al.*, 2001). Nevertheless, the pattern of plasma melatonin exhibited by fish held under different light regimes may suggest in a preliminary way, that a possible type A profile could be present in tilapia, however, further investigations are needed in order to confirm these findings.

Melatonin levels in tilapia ranged from 5.0 pg/ml during the light phase to a maximum of 99.4 pg/ml during the dark phase and large variability was observed between fish held under the same photoperiod. Several hypotheses could explain this variability. First, these results might have been affected by tilapia size and age as it was indicated that rainbow trout broodstock displayed lower plasma melatonin levels in comparison to fingerlings (Guerrero-Tortolero *et al.*, 2003).

Gern *et al.* (1978a, b) also reported that melatonin levels were affected by age in rainbow trout; one year old fish exhibited 596 pg/ml at night in comparison to only 254 pg/ml in two year old fish. However a marked reduction was observed in four year old

rainbow trout with 153 pg/ml. In this study, tilapia were of different size and age, the fish used in the diel melatonin profile and in the night melatonin profile were one year old. Those fish exposed to different light regimes were two and a half year old.

Secondly, although plasma melatonin levels secreted by the pineal gland are similar in all fish from the same species at a given time (season and stage of development), Randall (1992) suggested that it is likely that there is a negative relationship between plasma melatonin concentration and the amount of blood in the fish in relation to fish size. This means that the lower the blood volume the higher the melatonin concentration in the fish would be (Randall, 1992). Another possible influence upon melatonin concentration is the amount of vitellogenin present in the blood, as this may cause interference with the antibody used in the RIA (Randall, personal communication).

In tilapia, melatonin was secreted soon after lights were turned off, then a plateau was reached. These high plasma melatonin levels seemed to be constant throughout the night, then melatonin concentration decreased and reached basal levels just after the onset of the light phase. In the particular case of those fish held under different light regimes, the maximum plasma melatonin levels during the dark phase could be attributed to differences in night duration. In the short daylength trial (6L:18D), midnight occurred nine hours after lights went off. However, for the normal daylength (12L:12D), midnight was reached six hours after lights turned off, and for the long daylength trial (18L:6D), midnight occurred just three hours after light was turned off. Thus, the highest melatonin levels were exhibited in those fish held under the short daylength (6L:18D) and the normal daylength trials (12L:12D). These two trials exhibited the longest dark periods, thereby, allowing melatonin levels to reach maximum levels, by simply allowing longer secretion and longer accumulation times in the blood stream. In the long daylength trial (18L:6D) (the shortest dark period), melatonin concentration was significantly lower because the dark period was

short and hence melatonin levels did not reach levels equivalent to those observed during a long dark phase.

Bromage *et al.* (2001) reported that the duration of the nocturnal increase in plasma melatonin levels is directly proportional to the length of the night and hence provides a direct transduction of night duration. This may explain why these variations in melatonin levels were found in those fish exposed to different light regimes. These results indicate that in tilapia, as well as most fish species, melatonin production by the pineal gland is under the direct control of photoperiod (Zachmann *et al.*, 1992c; Bromage *et al.*, 2001).

The continuous illumination treatment (24L:0D) showed a suppression in plasma melatonin levels. There was no variation between melatonin concentrations detected at midday and midnight in this particular treatment. However, fish spawned and produced the second best egg production and shortest ISI. These findings may indicate that melatonin is not directly involved in the control of tilapia reproduction and suppression of daily melatonin rhythms did not affect the timing of spawning. These results are in accordance with Bornestaf *et al.* (2001) and Mayer *et al.* (1997b) in which melatonin was given to three-spined stickleback (*Gasterosteus aculeatus*) in two different doses (20 – 80 µg of melatonin / l of water). These doses had no effect upon fish reproduction, thus these authors concluded that melatonin plays a relatively minor role in the control of the stickleback reproduction.

The results presented herein are similar to those reported for tilapia by Randall (1992), however on this occasion the assay was fully validated in order to obtain reliable and reproducible information. Melatonin levels in tilapia were similar to those values reported by Mayer *et al.* (1998) for the European river lamprey (non teleost) with maximum melatonin levels of 80 pg/ml using a light regime of 16L:8D in fresh water. However, the temperature used by Mayer *et al.* (1998) was very different (4°C) from the

temperature used in the present experiment (27°C). Similar melatonin levels were reported by Iigo *et al.* (1997) for the catfish (*Silurus asotus*), with maximum melatonin levels around 30 or 40 pg/ml under a 12L:12D photoperiod in fresh water culture. García-Allegue *et al.* (2001) reported similar melatonin levels in the European sea bass (*Dicentrarchus labrax*). These values varied little during the winter at around 30 pg/ml, a similar value was found in the present experiment in those tilapia reared under normal daylength (12L:12D) where maximal melatonin level was around 48 pg/ml. The melatonin concentration reported for spring time in sea bass was around 100 pg/ml, this value is similar to our night-time melatonin levels of tilapias reared under the normal photoperiod (12L:12D) in the diel cycle (99.4 pg/ml). Similar values were reported by Bayarri *et al.*, (2004) in which night melatonin levels in sea bass were found to be 85 pg/ml and 10 pg/ml during the day.

In the three-spined stickleback, some plasma melatonin has been reported. In fish under short daylength (8L:16D), plasma melatonin levels were high at the middle of the dark phase, reaching 311 ± 23 pg/ml and melatonin levels were below the assay detection limit at midday (Mayer *et al.*, 1997b). These levels are much higher compared with those reported in this present study in the middle of the dark phase. However, Bornestaf *et al.* (2001), reported daytime melatonin levels ranging from 11 – 30 pg/ml from stickleback females held under short (8L:16D), long daylength (16L:8D) and continuous illumination (24L:0D), the values were similar in all the light regimes with values ranging from 11 – 30 pg/ml. Thus, daytime plasma melatonin levels observed in the present study in tilapia seems to be in accordance with those already reported in the literature for the three-spined stickleback.

Through examination of the existing literature and the present results, there seems to be a tendency for some temperate fish species to produce more melatonin in the pineal

gland than tropical species. This was clear when tilapia melatonin levels were compared with those produced by the rainbow trout or salmon, in which melatonin concentration was six to tenfold higher than tilapia (Randall, 1992; Randall *et al.*, 1995a; Bromage *et al.*, 2001). There would be also strong indication that pineal melatonin production has changed in fish species and adapted to the environment. As Menaker *et al.* (1997), mentioned, pineal activity dramatically changed during phylogeny and the 500 millions years of evolution to the many environments occupied by the vertebrates during that time (Menaker *et al.*, 1997).

Temperate fish species experience large seasonal photoperiodic variations in comparison to tropical species and it is not surprising that they produce higher quantities of melatonin. Melatonin secretion is characterized by two characteristics according to García-Allegue *et al.* (2001); the duration of the nocturnal increase, and the amplitude of the night-time rhythm. In tilapias, duration of melatonin secretion (duration and amplitude) is clearly regulated by photoperiod. As for the night time amplitude, these results suggested that night time melatonin levels in tilapia were higher in those fish exposed to short daylength photoperiods (6L:18D) rather than long daylength photoperiods (18L:6D). In the case of the European sea bass, melatonin profiles are also controlled by the duration of the photoperiod; however, amplitude was related to a combination of water temperature and photoperiod. In sea bass, higher melatonin concentrations were found during long photoperiods (spring and summer) and lower melatonin concentrations found during short photoperiods (autumn and winter); it was clear that increased melatonin concentration was related to water temperature and photoperiod. However, in the present study fish were reared under constant temperature, thus the only variable was the changes in photoperiod.

Melatonin production is modulated by water temperature in such a way that melatonin is secreted in high concentration at higher temperatures and secretion is reduced

by low temperatures (Randall *et al.*, 1995a; Bromage *et al.*, 2001; Porter *et al.*, 2001; García-Allegue *et al.*, 2001). The melatonin profiles reported in the present work were all generated under controlled conditions with a water temperature of $27 \pm 1^\circ\text{C}$. However, further investigations are required in order to assess the relationship of between melatonin production and water temperature in this tropical species.

The results obtained in Chapter Three suggested that long daylength is the most suitable to improve tilapia reproduction; under this photoperiod fecundity was increased and ISI decreased. However, the melatonin levels of those fish reared under long daylength (18L:6D) were the lowest. This may suggest that a negative relationship between melatonin concentration and reproductive performances in tilapias is present. However further investigations are needed to elucidate this findings. It appears that melatonin levels would have to remain below certain “threshold” level in order to stimulate reproductive activity in tilapia. From these results, the threshold could be around 30 or 40 pg/ml. The opposite effect was observed in those fish reared under short photoperiods (6L:18D) and (12L:12D), which produced the highest levels of melatonin concomitant with reduced reproductive performance. A similar result has been reported for the three-spined stickleback. This species has a better spawning activity when it is reared under continuous illumination in captivity, and under natural conditions this fish shows a better reproductive performance in longer days. This species matures under long days rather than short days. However, no effect of melatonin was observed when fish were treated with melatonin, suggesting that melatonin is not playing any functional role in stickleback reproduction and that these effects shown for photoperiod are largely mediated by mechanisms other than circulating melatonin levels (Mayer *et al.*, 1997b; Bornestaf *et al.*, 2001). There is a possibility that the same mechanism might have evolved in tilapia. However, this requires further investigation to confirm this preliminary hypothesis.

However, the real mechanism behind the control of reproduction by photoperiod in fish reproduction remains to be elucidated. There are some important factors which might be playing a key role in the link between melatonin and reproduction: the environmental conditions (temperature, salinity, etc) and the geographical origin of the species under study (temperate or tropical). It is evident that melatonin secretion differs significantly between species and these differences appear to be regulated by the environment. For instance, high melatonin levels are reported for the rainbow trout. This species has a particular preference for clean, low turbidity waters, thus this may have an effect on light penetration, causing with this a different answer on melatonin production by the pineal gland.

The strain of *O. niloticus* used in the present study was originally collected from Lake Manzala, Egypt in 1979 (McAndrew, personal communication). The reproductive biology of this species shows that there is a tendency for seasonality in the breeding season. Thus, reproduction occurs all year round in equatorial populations, but as the distribution of this species moved from the tropics, a marked variation in reproductive seasonality was observed, with higher reproductive peaks during the longer days of the summer months, this is also related with high water temperature. The photoperiodic variation in Lake Manzala (31.5° N) is considerable in comparison to that of the equator. The photoperiod variation reported for the Manzala region is 14L:10D during the summer and 10L:14D during the winter. This photoperiodic variation induces seasonality in the breeding behaviour in the natural populations of tilapia within this region, but this may be also related to changes in water temperature. However, it is highly possible that those fish used in the present experiment still possess genetic information inside their internal clock, and this could be the reason for similarities found in the natural conditions in Lake Manzala and the longer photoperiod investigated here.

To fully understand and explain our results, further investigations are required in which a similar experiment is carried out with this species from a different geographical origin, for example a tilapia strain collected at the equator and a further strain from a very different latitude.

Although, more studies are required to fully understand the relationship between melatonin and reproduction in tilapia, possible clues could be discovered using other sources or tools such as the molecular technology that is now readily available to further investigate and correlate these two important physiological aspects of fish reproduction. Here, we have detected melatonin in Nile tilapia using a validated assay and shown how this hormone varies according to photoperiodic manipulation. This findings could have huge importance for the efficiency of future efficient culture of this species, but important key questions still remain to be asked: These involve the precise role of melatonin in tilapia reproduction; the precise cellular location of the melatonin receptor; the precise functional role of the melatonin receptor, and finally, how the melatonin receptors are related to fish reproduction.

5 Chapter Five Isolation of the Melatonin Receptor Gene from the Nile Tilapia, *Oreochromis niloticus* and its Specific Tissue Expression

5.1 Introduction

5.1.1 Melatonin receptors

The first melatonin receptor was cloned from *Xenopus laevis* melanophores by Ebisawa *et al.* (1994). Since then, melatonin receptors have been isolated from several vertebrate species (Reppert *et al.*, 1995a; Reppert, 1997). However, initially these melatonin receptors were named without any strict standardization. It was only in 1995 that the first classification scheme was proposed by Dubocovich (1995), with subsequent reviews (Dubocovich *et al.*, 1998, 2000). Nevertheless, in this new classification only the mammalian melatonin receptors were dealt with and the other melatonin receptors (non-mammalian) kept their original names. The melatonin receptors now belong to a new subfamily of seven transmembrane domain G protein-coupled receptor (GPCR's) (Dubocovich, 1995; Dubocovich *et al.*, 1998) (see below for further explanation). In the mammalian classification, two of these melatonin receptors (MT₁ and MT₂) are members of the G protein-coupled receptor super-family. However, a third subtype was found, MT₃, which does not fit in the GPCRs super family. The MT₃ belongs to the quinine reductase enzyme family (Witt-Enderby *et al.*, 2003). To avoid confusion with early nomenclature, the melatonin receptors cloned thus far are described individually herein, according to the relevant taxonomic grouping.

5.1.2 Melatonin receptors in mammals

MT₁ (formerly called Mel_{1a}, MEL_{1A} or ML_{1A}) is expressed in the suprachiasmatic nuclei of the hypothalamus (SCN), the hypophyseal pars tuberalis, cerebral artery, leptomeninges and was also found in the dorsal and ventral horn of the spinal cord and cardiac vessels (Reppert *et al.*, 1995a; Reppert, 1997; Drew *et al.*, 1997; Dubocovich *et al.*, 1998; Gauer *et al.*, 1998; Sugden *et al.*, 1999; Hunt *et al.*, 2001; Chucharoen *et al.*, 2003;

Dardente *et al.*, 2003; Zahn *et al.*, 2003). The role of MT₁ essentially involves the modulation of circadian rhythm and MT₁ mediates reproductive responses to melatonin in mammals (Reppert, 1997; Weaver *et al.*, 1996). The MT₁ receptor is involved in the reduction and inhibition of the cyclic adenosine 3' 5' monophosphate (cAMP) transduction cascade, which results in decreased levels of protein kinase A (PKA), a reduction of cAMP responsive element binding-protein (CREB) phosphorylation and potentiation of phospholipase activation (Godson and Reppert, 1997; Barrett *et al.*, 2003). The protein structure of the human MT₁ receptor is shown in Figure 5.1A.

The MT₂ gene (formerly called Mel_{1b}, MEL_{1B} or ML_{1B}) is expressed in the cerebellum, the SCN, retina, kidney, ovary, cardiac vessels and spinal cord in hamster. This receptor has not been found to be expressed in rat brain or pituitary. However, using RT-PCR a transcription factor has been found in human retina and brain (Reppert, 1997). MT₂ is involved in retinal physiology as well as modulation of circadian rhythms. It may also play an important role in the neurobiological effects of melatonin, works as a dilator in the cardiac vessels (Dubocovich, 1995; Dubocovich *et al.*, 1998; Reppert *et al.*, 1995b; Reppert, 1997; Hunt *et al.*, 2001; Witt-Enderby *et al.*, 2003; Zahn *et al.*, 2003). The protein structure of the human MT₂ receptor is shown in Figure 5.1B.

The MT₃ gene (formerly called ML₂) is expressed in the hamster brain, kidney, testes and mouse brain (Dubocovich, 1995; Dubocovich *et al.*, 1998) but has not been cloned yet. However, MT₃ has been pharmacologically characterised in mammals (Dubocovich, personal communication).

A similar gene has been identified and shows strong similarity in amino acid sequence (40 – 57 %) to the melatonin receptor, but the receptor does not bind with the hormone melatonin. This gene has been named 'melatonin-related receptor (MRR)' and is also known as H9. This receptor has been characterised and isolated in human, rat and

sheep. The expression sites of MRR are located in the hypothalamus, preoptic area, peribranchial nuclei, olfactory bulb, pituitary, retina and retinal pigment epithelium, kidney, adrenal gland, intestine, stomach, heart, lungs skin, testis and ovary suggesting that this receptor is involved in some neuroendocrinological process. Some expression sites are similar to those already reported for the MT₁ gene (Reppert *et al.*, 1996a; Reppert, 1997; Drew *et al.*, 1998, 2001; Conway *et al.*, 2000; Barrett *et al.*, 2003).

5.1.3 Melatonin receptors in amphibians and birds

In the African clawed frog *X. laevis*, three different melatonin receptor genes have been cloned and identified as Mella, Mellb and Mellc. The first two receptors are homologous to mammalian MT₁ and MT₂. Although, Mellc has not been cloned in mammals yet, it does have pharmacological and functional characteristics similar to those of mammalian MT₁ and MT₂. The Mellc was the first melatonin receptor cloned, marking the beginning of investigations in this field (Ebisawa *et al.*, 1994). The Mellc protein structure is graphically represented in Figure 5.1C. These three melatonin receptors are expressed in brain, neural retina and retinal pigment epithelium (RPE), and also non-pigmented ciliary epithelium and retina photoreceptors (Ebisawa *et al.*, 1994; Reppert *et al.*, 1995a; Wiechmann *et al.*, 1999; Wiechmann and Smith 2001, Wiechmann and Wirsig-Wiechmann 2001). A high number of melatonin binding sites were observed in the common frog (*Rana perezi*), especially in the neural retina and brain areas such as the telencephalon, diencephalon and optic tectum. These binding sites were confirmed to be part of the Mella subtype (Isorna *et al.*, 2004).

Two melatonin receptors were cloned in chicken (*Gallus domesticus*) by Reppert *et al.* (1995a) and named CKA and CKB respectively. Chicken CKA has strong homology (80% at the amino acid level) with human MT₁. However, chicken CKB shares similarities

with the *Xenopus* Mel1c receptor and they exhibit 80% homology in their amino acid sequence. It appears that in birds, the homologue of the mammalian MT₂ receptor is absent or it has yet to be cloned (Reppert *et al.*, 1995a). The CKA receptor is expressed in the optic tectum and retina, but has also been found in reduced levels in the neostriatum, hypothalamus, and thalamus. The CKB receptor is expressed in the optic tectum, neostriatum, hypothalamus, thalamus and pineal gland, low numbers have also been reported in the cerebellum and retina (Reppert *et al.*, 1995a).

5.1.4 Melatonin receptors in fish

After the isolation of the first melatonin receptor by Ebisawa *et al.* (1994) in *Xenopus*, a series of attempts were made to isolate a melatonin receptor in fish. In 1995 Reppert and co-workers isolated three partial sequences of melatonin receptor in the zebrafish (*Danio rerio*). These partial sequences were named as Z1.4, Z1.7 and Z2.6 (Reppert *et al.*, 1995a). Four years later Mazurais *et al.* (1999) cloned three partial sequences in rainbow trout (*O. mykiss*); these were designated RT1.4, RT1.7 and RT2.6. These rainbow trout partial sequences showed high amino acid homology (84%) with those already reported in zebrafish by Reppert *et al.* (1995a).

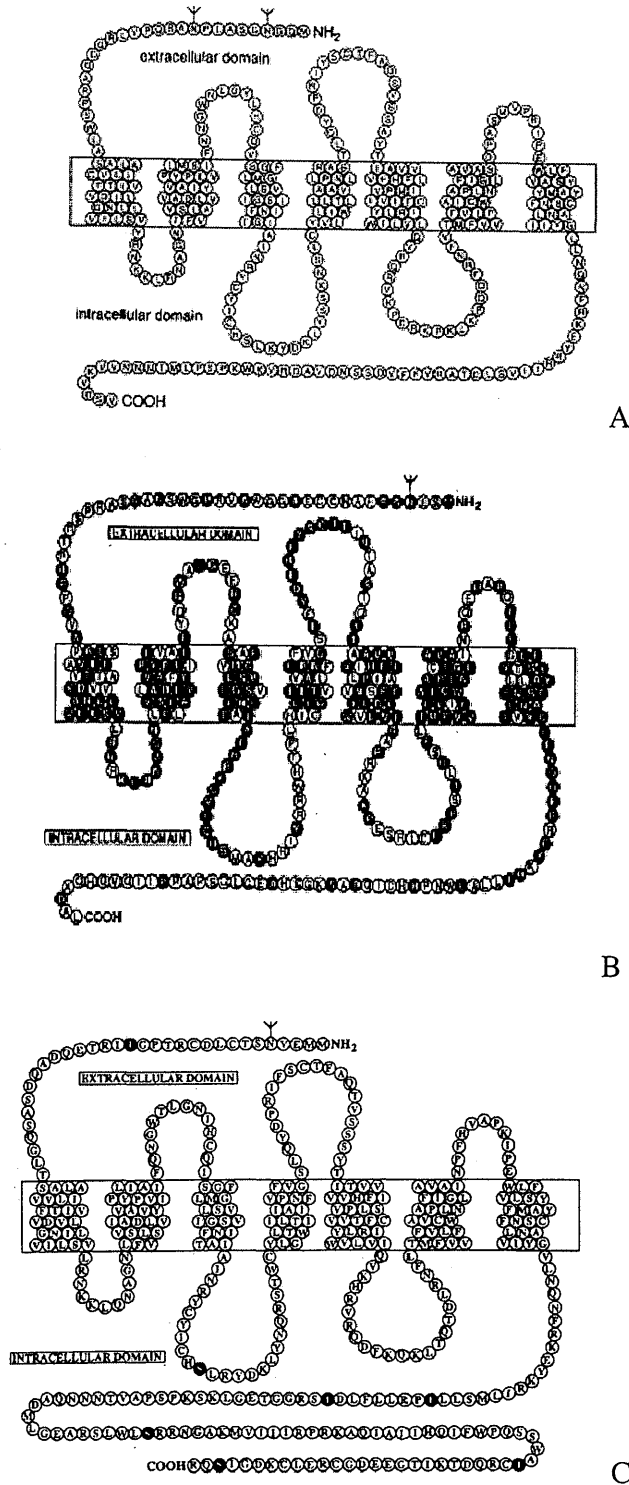


Figure 5.1 A) Amino acid sequence and proposed membrane structure of the human melatonin MT₁ receptor. B) Human MT₂ receptor, amino acids that are shaded are identical between MT₂ and MT₁. C) Mel1c from *Xenopus*. Ψ = potential linked N-linked glycosylation site. Solid circle = consensus sites from protein C phosphorylation. Taken from Reppert *et al.* (1995b, 1996b) and Ebisawa *et al.* (1994).

The rainbow trout partial sequences RT1.4 and RT1.7 are both members of the Mel1a subtype (i.e. are homologous to the mammalian MT₁), whilst RT2.6 forms part of the Mel1b (homologous to the mammalian MT₂) subtype (Mazurais *et al.*, 1999). Both of these melatonin receptors have similar patterns of expression in rainbow trout brain, as demonstrated by in situ hybridization. These receptors were highly expressed in the stratum periventriculare of the optic tectum, the posterior pretectal nucleus, as well as the molecular and granular layer of both corpus and valvula in the cerebelli (Mazurais *et al.*, 1999; 2000). Gaildrat and Falcon (2000) reported two partial cDNA sequences in pike (*Esox lucius*). These sequences were designated P1.4 and P2.6. The names of these sequences arose due to the high homology between these pike sequences and the partial sequences already reported for rainbow trout and zebrafish (Reppert *et al.*, 1995a; Mazurais *et al.*, 1999). Northern blots and RT-PCR analysis demonstrated high levels of P1.4 and P2.6 in the optic tectum. However, lower levels were detected in the pituitary using RT-PCR. This was the first time in which receptors of this kind had been detected in the pituitary, suggested that melatonin may have a direct effect upon this tissue and may therefore be involved in reproductive function (Gaildrat and Falcon, 2000).

Gaildrat *et al.* (2002) subsequently cloned the whole sequence of the previous partial clone P2.6 from pike using RACE (Rapid amplification of cDNA ends) and brain cDNA (Optic tectum) as a template. This finding allowed full characterization of a novel melatonin receptor. These interesting results suggested that this full-length melatonin receptor P2.6 sequence (1101 bp) belonged to a different group of melatonin receptors, to that previous proposal (i.e. Mel1b thought to be homologous to the mammalian MT₂) (Gaildrat and Falcon, 1999). According to phylogenetic analysis, the new P2.6 (full sequence) was allocated to the second lineage within the vertebrate melatonin receptor family, in a branch near to the Mel1a and Mel1c receptors. The mRNA distribution of pike

P2.6 was evaluated using Northern blot analyses; the results demonstrated the presence of one transcript (5.4kb) which was expressed in the optic tectum. Expression was not detected in the pituitary gland, liver or other brain areas. This was further investigated by RT-PCR and Southern blot analyses; expression was confirmed in the optic tectum, but also in pituitary gland (as reported before by Gaildrat and Falcon, 2000), olfactory bulbs, telencephalon, diencephalon, cerebellum and retina. However, no expression was detected in the ovaries, liver and intestine.

The final work available in the literature concerning gene expression of melatonin receptors in fish came from Shi *et al.* (2004), who demonstrated expression of the Mel1a and Mel1b receptors using RT-PCR and real time PCR. They cloned Mel1a and Mel1b in the chum salmon (*Oncorhynchus keta*), using the methodology of Mazurais *et al.* (1999). Expression sites for these two chum salmon receptors were the optic tectum, thalamus, hypothalamus, and cerebellum. Expression of the Mel1b was also detected in the eyes using RT-PCR.

5.1.5 Melatonin binding sites in fish

$2[^{125}\text{I}]$ Iodomelatonin is a melatonin analogue, which allows autoradiography and radioligand studies on melatonin receptors in fresh or fixed tissues (Pang *et al.*, 1998). In fish, several melatonin binding sites have been demonstrated using this technique. These binding sites are widely distributed in the brain, however, the highest concentration was found in the visual region located in the optic tectum, nucleus rotundus, pretectum and dorsal thalamus. Binding sites were also found in the gustatory regions in the hypothalamus, preoptic area and cerebellum. Melatonin receptors have now been found in a variety of fish species including rainbow trout, masu salmon, pike, goldfish, catfish and

zebrafish, (Davies *et al.*, 1994; Iigo *et al.*, 1994, 1997; Ekstrom and Meissl, 1997; Gaildrat *et al.*, 1998; Mazurais *et al.*, 1999, 2000; Amano *et al.*, 2003a, 2003b).

5.1.6 G Protein-coupled receptors

G Protein-coupled receptors (GPCRs) are a super family of integral membrane proteins. These receptors are formed from seven membrane spanning helices (transmembrane domains), three periplasmic loops (extra-cellular), three cytosolic loops (intra-cellular), a periplasmic N- terminal and a cytosolic C- terminal domain. The extra-cellular loops and the N-terminal domain are glycosylated. However, the C terminal domain generally contains a cysteine residue attached to a lipid group (Horn *et al.*, 2000; Barrett *et al.*, 2003; Figures 5.1 A-C.). GPCRs are involved in the communication between the cell and its surrounding; they detect signals on the outside of the cell. These receptors essentially respond to different signals, which might be a protein, a peptide, a small organic molecule, an ion or even a photon that causes a structural change in a retinal cell. Once the signal is received it is transmitted by the seven transmembrane helices to the inner part of the cell (cytosolic side), where the trimeric G protein becomes activated and triggers the relevant or appropriate response in the cell.

Any malfunction of these GPCRs could trigger disease such as Alzheimer's, Parkinson's, diabetes, dwarfism, colour blindness, retina pigmentosa, and asthma (Horn *et al.*, 2000). Furthermore, these GPCRs are also involved in depression, schizophrenia, sleeplessness, hypertension, impotence, anxiety, stress, renal failure, cardiovascular disorders and inflammation (Horn *et al.*, 2000). For these reasons, the GPCR family has been the subject of much research and remains a valuable target for pharmaceutical companies (Horn *et al.*, 2000).

To further investigate the specific location at which melatonin is acting, and its precise function in reproduction, it was first necessary to investigate the possible existence of tilapia melatonin receptors. The aims of this chapter were to attempt to isolate a melatonin receptor in *O. niloticus*, and to evaluate the specific expression of the melatonin receptor gene in several tissues from this species.

5.2 Materials and methods

5.2.1 Fish tissue

For tissue specific expression of the melatonin receptor, fish were killed by an overdose of Ethyl 4-aminobenzoate (Sigma Chemicals, Ltd U.K.) with a working concentration of 1:10000 in fresh water. The liver, spleen, intestine, gonad, kidney, gills, heart, eyes, brain, red muscle and white muscle were carefully dissected out. On one occasion, one fish was dissected and samples were kept on ice for immediate RNA extraction. When a second fish was culled, tissue samples were taken and immediately frozen in liquid nitrogen and kept at -70° C for further RNA extractions.

5.2.2 DNA extraction

DNA was extracted from blood samples of *O. niloticus* using the protocol described by Taggart *et al.* (1992). Briefly, blood samples were spun at 2500 rpm for 15 minutes. The supernatant plasma was discarded and the remaining pellet (red cells) was retained in a 1.5 ml Eppendorf tube. Then, 375 μ l of a solution composed of 0.2 M EDTA (pH 8.0) and 0.5% sodium lauroylsarcosine was added along with 10 μ l proteinase K (20 mg/ml). Samples were mixed and incubated overnight at 55 °C in a hybridization cylinder in a hybridization oven. After that, 10 μ l of RNase was added and the samples were vigorously shaken and then incubated for 60 min at 37°C. Then, 400 μ l of phenol was added and the samples were vigorously shaken for 10 sec. Then, samples were gently mixed for 15 to 20 minutes in a rotary mixer. 400 μ l of chloroform was then added to the samples and they were vigorously shaken for 10 sec followed by gentle mixing in a rotary mixer for 15 to 20 min. Samples were then centrifuged at 10,000 g for 5 min. Then, 300 μ l was carefully removed from the aqueous layer and transferred to a fresh Eppendorf tube. 900 μ l of 92 % ethanol was then added and the samples were vigorously shaken 5 to 6 times in order to

precipitate the DNA. Samples were left on the bench for a few minutes and then the ethanol was carefully discarded. The DNA pellet was then washed with 1 ml of 70% ethanol and the samples were gently mixed for 30 minutes (or overnight) at room temperature in a rotary mixer. Ethanol was then carefully discarded and samples were allowed to dry at room temperature. DNA pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA) (pH 8.0). Then, DNA concentration was estimated by using optical density measured by spectrophotometry. DNA concentration was double checked by agarose gel electrophoresis (1 %).

5.2.3 RNA extraction

Total RNA was extracted using the TRIzol® Reagent (GIBCO-BRL®) which is based upon the method proposed by Chomczynski and Sacchi (1987). Fresh tissue was homogenized using a mechanical tissue homogeniser in 1 ml Trizol solution per 100 mg of tissue. After that, a phase separation was carried out, in which the samples were incubated at room temperature (15 - 25°C) for 5 minutes and then 0.2 ml of chloroform was added for each ml of Trizol. Samples were carefully capped and shaken vigorously for 15 seconds and then incubated at room temperature for two or three minutes. Samples were then centrifuged at 12,000 g for 15 minutes at 2 - 8°C. The upper layer (aqueous phase) was transferred to fresh tube. For RNA precipitation, 0.5 ml of isopropanol was added for each 1 ml of Trizol used. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 g for 10 minutes at 2 - 8°C. After centrifugation, the supernatant was discarded and the RNA pellet washed once with 75% ethanol. Samples were then vortexed and centrifuged at 7,500 g for 5 minutes at 2 - 8°C. Finally, the supernatant was discarded and the RNA pellet allowed to air dry. For RNA precipitation, RNase-free water was added and the samples mixed and incubated for 10 minutes at 55 to

60°C. At the end of this procedure, RNA concentration was estimated by spectrophotometry and samples checked by agarose gel electrophoresis (1 %).

5.2.4 cDNA synthesis

To generate first strand cDNA, 5 µg of total RNA was transferred to a sterile RNase-free tube, along with 0.5 µg of Oligo (dT)₁₅ and made up to a total volume of 14 µl with RNase-free water. Samples were heated to 70°C for 5 minutes then quickly transferred to ice for 5 minutes. After that, 5 µl of M-MLV-RT 5X buffer (250mM Tris-HCL pH 8.3, 375 mM KCL, 15mM MgCl₂, 50mM DTT), 1.25 µl dNTP mixture (dATP, dCTP, dGTP, dTTP, 10mM each), and 0.5 µl (100 units) of M-MLV RT (H-) and 4.25 µl of RNase free-water were added. The reagents were gently mixed and incubated at 40°C for 10 minutes, then the temperature was increased to 55°C and the samples were incubated for 50 minutes. After that, the reaction was inactivated by heating to 70°C for 15 minutes. At this point, the new cDNA could be used for PCR amplification and RT-PCR. All the reagents used for this protocol were from Promega (Madison, WI. USA).

5.2.5 Polymerase Chain Reaction (PCR)

In order to use PCR to amplify the melatonin receptor gene a search was done on the NCBI homepage (<http://www.ncbi.nlm.nih.gov>), in order to get the most complete and up-to-date information in this gene. Once the sequences were identified, they were aligned and then primers were designed from the most conservative regions using the Primer3 programme (V0.2) (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>).

5.2.5.1 Melatonin receptor

The following melatonin receptors sequences were aligned: rainbow trout Mella mRNA (accession number AF156262) and pike P2.6 Mel1b mRNA (accession number AF188871). A set of degenerate primers were designed based on this alignment. The forward primer sequence was as follows: 5'- ATG GG(C/T) (C/G)TC AGC GTA ATC GC -3' and the reverse primer sequence was: 5'-GAA GTT CTG GTT GAG CA(G/C) (G/C)C-3'. This pair of primers generated a PCR product of ~ 520 bp using tilapia genomic DNA.

Another set of primers previously reported by Mazurais *et al.* (1999), for the rainbow trout melatonin receptor 1.4 (accession number AF178538), were also used in this experiment. The sequence of these degenerate primers was as follow: 5'-TG(C/T) CAC AGC CT(C/T) AAG TA(C/T) GAC AAG CT -3' in the forward sense and 5'-ATG AAG TT(C/T) AA(C/T) GG(A/T) GCC CAG CA(A/C/T) -3' for the reverse. This set of primers allowed the amplification of ~ 300 bp PCR product using genomic tilapia DNA.

These two sets of primers were used to produce two PCR products (520 and 300 bp). These PCR products were sequenced and the resultant sequences were aligned to allow the design of a further set of primers. The resultant forward primer was: 5'-TAG CTG GCC ACG AAC AAC CAC -3' and the reverse primer: 5'-AGC GTA ATC GGC TCC ATC TTC-3'. This set of primers allowed the amplification of a PCR product of around 520 bp.

5.2.6 Cloning of desired PCR products

Once the desired PCR products had been obtained, they were first purified using a glass fibre matrix column (PCR purification columns, Amersham Pharmacia). The clean PCR products were then sub-cloned into a T vector, which was modified from pBluescript II+ vector (Stratagene): the vector was opened by digestion with EcoR V to blunt the ends of the plasmid and then dTTP added to produce 3' overhanging ends. Transformation of the ligated vector-PCR insert was performed using *E. coli* XL-Gold Ultracompetent cells

(Stratagene). The transformed cells were plated onto LB agar previously treated with ampicillin. These plates were further treated with IPTG (Isopropyl-1-thio- β -D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) to enhance blue and white colour selection. The white colonies were picked up and screened by PCR with gene specific primers or M13 primers. The resultant positive clones were further inoculated into 4 ml of LB broth medium in order to generate a large scale plasmid preparation. After that, the plasmid DNA was extracted using the GFXTM Micro Plasmid Prep. Kit (Amersham Biosciences, Piscataway, N.J. USA). The final plasmid DNA was used as a template for further PCR amplification and sequencing.

5.2.7 DNA Sequencing

DNA sequencing was carried out using the ABI Prism Dye-Deoxy terminator sequencing kit (Perkin Elmer). Samples were run and analysed using an Applied Biosystems DNA Automated Sequencer (Model 377, Applied Biosystems, Foster City, CA). The resultant sequences were analysed and aligned using a BLAST search from the National Centre for Biotechnology Information home page (<http://www.ncbi.nlm.nih.gov>). Sequences were aligned and a phylogenetic tree was prepared using GeneBee (http://www.genebee.msu.su/services/malign_reduce.html) multi-sequences alignment and ClustalW (<http://www.clustalw.genome.ad.jp>) programs.

5.3 Results

5.3.1 Identification of the melatonin receptor gene and tissue specific gene expression

5.3.1.1 Identification of the melatonin receptor gene in tilapia

PCR amplification from genomic tilapia DNA produced an appropriate fragment of around 520 bp (Figure 5.2). This PCR product was sub-cloned into pBluescript II and sequenced (For more details see Chapter Two). In order to confirm these findings, total RNA was extracted from tilapia brain and reverse transcribed. This new single strand brain cDNA was then used as a template in a PCR reaction and the resultant products sequenced directly. The tilapia brain cDNA PCR product had 100% homology with the tilapia genomic DNA sequence. The resultant brain cDNA sequence presented a total length of 492 bp. The amino acid and nucleotide sequence is shown in Figure 5.4. Four transmembrane domains were found in the partial sequence obtained from tilapia brain cDNA. These transmembrane domains correspond to the IVth, Vth, VIth and VIIth conservative regions of the G protein structure (Figure 5.4). The highest level of homology between these transmembrane domains (80%) was found in the VIth transmembrane region when all the sequences were aligned.

In order to compare the phylogenetic relationship of the *O. niloticus* brain melatonin receptor with other melatonin receptor sequences already reported for vertebrates, a phylogenetic tree was constructed using the Neighbour-Joining method of Clustal W and GeneBee programmes (Figure 5.5). The information generated by this phylogenetic tree indicated that the tilapia brain cDNA sequence belongs to the MT₁ melatonin receptor subtype in the mammalian classification, or the Mella subtype in the non-mammalian classification. This was deduced because the tilapia partial sequence was clustered in the same lineage as the rainbow trout Mella sequence. Furthermore, all the

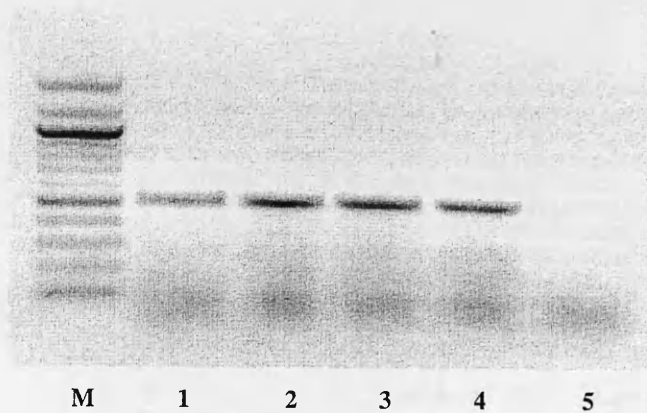


Figure 5.2 PCR product of melatonin receptor (520 bp) using tilapia genomic DNA (Lines 1 and 2) and cDNA (Lines 3 and 4), line 5: negative control, M: 100 bp DNA marker.

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T W Y S I N R Y C Y I C H S L K Y D K L 20
ACACCTGGTATAGCATCAACCGATACTGTTACATCTGCCACAGCCTCAAATACGATAAAC 60
Y S D K N S V C Y V L L I W A L T V V A 40
TGTACAGCGACAAAACTCCGTCTGCTATGTGTTGTTAATCTGGGCGCTGACTGTTGTTG 120
I V P N L F V G S L Q Y D P R V Y S C T 60
CCATCGTGCCCAACCTGTTTGTGGGCTCGCTTACAGTACGACCCGCGGGTTTATTCCCTGCA 180
F V Q S A S S A Y T I A V V F F H F I L 80
CATTGTTTCAGTCAGCCAGCTCCGCGTACACCATCGCCGTGGTCTTCTTCCACTTCATTT 240
P I M I V T Y C Y L R I W I L V I Q V R 100
TACCCATCATGATTGTACCTACTGCTACCTGCGCATTTGGATATTGGTCATACAGGTAA 300
R R V K P D N R P K L T P H D V R N F V 120
GGAGACGGGTCAAGCCAGACAATCGACCCAAGCTGACGCCACATGACGTTAGGAACTTTG 360
T M F V V F V L F A V C W A P L N F I G 140
TTACCATGTTTGTGGTGTGTTGTGCTCTTTGCCGTGTGCTGGGCACCGCTAAACTTCATCG 420
L A V A I K P E V V I P L I P E W L F V 160
GACTGGCTGTAGCGATCAAACCGGAGGTTGTGATTCCCTCATCCCTGAGTGGTTGTTCG 480
A P K 163
TGGCCCCATAAA 492

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Figure 5.3 Partial amino acid and nucleotide sequence of tilapia brain melatonin receptor cDNA.

IV

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Tilapia          CHSLKYDKLYSDKNSVCYVLLIWALT VVAIVPNL FVGS LQYDPRVY SCTF VQSASSAYTI
Zebrafish 1.4   CHSLKYDKLFSNKNTVCYVILVWALT VLAIVPNW FVESLQYDPRVFSCTFAQSVSSLYTI
Zebrafish 1.7   CHSLKYDKLYSDKNSVCYVLLIWALT VLAIVPNL FVGS LQYDPRVY SCTFEQSASSAYTI
Zebrafish 2.6   CHSFAYGR LCSFRNTLL LVALIWALT VLA I L P N F F V G S L S Y D P R V Y S C T F T Q T A S S S Y T V
Zebrafish 2.3   CHSLRYDR LYSRRNTCLYLLLTWMLT ALATVP NLFV GSLKYDPRVFSCTFTQTASSSYTV
Human MT1       CHSLKYDKLYSSKNSLCYVLLIWLLT LAAVLP NLRAGTLQYDPR IY S C T F A Q S V S S A Y T I
Xenopus Mel1c  CHSLRYDKLYNQ RSTWCYLG L TWIL T I I A I V P N F F V G S L Q Y D P R I Y S C T F A Q T V S S S Y T I
Chicken Mel1c  CHSLRYDKL FNLKNTCCYICL TWLT VVAIVPN F F V G S L Q Y D P R I Y S C T F A Q T V S T S Y T I
Chicken MT1     CHSLKYDKLYSDKNSLCYVGLI WVLTVVAIVPN L FVGS LQYDPR IY S C T F A Q S V S S A Y T I
Rainbow trout 2.6 CHNLKYDKL FSNQNTVCYVILVWSL TVLAI V P N W F M E S L Q Y D P R V Y S C T F A Q S V S S S Y T I
Rainbow trout Mella CHSLKYDKLYSDKNSVCYVLLIWALT I V A I V P N L F V G S L Q Y G P R V Y S C T F E Q S A S S A Y T I
Pike 2.6        CHSF SYDKFYSYRNTLL LVALIWLLT I L A I I P N F F V G S L Q Y D P R V Y S C T F A Q A V S T S Y T I

Consensus      CH  Y              L  W  L  T  A  P  N      L  Q  Y  P  R  S  C  T  F  Q  S  Y  T

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V

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Tilapia          AVVFFHFILPIMIVTYCYLR I W I L V I Q V R R R V K P D N R P K L T P H D V R N F V T M F V V F V L F A V
Zebrafish 1.4   MVVVVHFIVPIGIVTYCYLR I W I L V I Q V P R R V K P D S R P K I K P H D F R N F L T M F V V F V L F A V
Zebrafish 1.7   AVVFFHFILPIMIVTYCYLR I W L V I Q V R R R V K N D N R P K I T P H D V R N F V T M F V V F V L F A V
Zebrafish 2.6   VVVVHFIVPIAVVTFCYLR I W L V I Q V R R K V K S E E R S R V R P S D L R N F V T M F V V F V L F A I
Zebrafish 2.3   CVVLIHFLVPLGVVSPCYLR I W T L V I R V K G R V R P -- N P K V R A A D L R N F L T M F V V F V L F A V
Human MT1       AVVVFHFIVPMI I V I F C Y L R I W I L V L Q V R Q R V K P D R K P K L Q D F R N F V T M F V V F V L F A I
Xenopus Mel1c  TVVVVHFIVPLSVVTFCYLR I W L V I Q V K H R V R Q D F K Q K L T Q T D L R N F L T M F V V F V L F A V
Chicken Mel1c  TVVVVHFIVPLSIVTFCYLR I W I L V I Q V K H R V R Q D C K Q K I R A A D I R N F L T M F V V F V L F A V
Chicken MT1     AVVFFHFILPIAIVTYCYLR I W I L V I Q V R R R V K P D N N P R L K P H D F R N F V T M F V V F V L F A V
Rainbow trout 2.6 TVVVIHFVPIAVVTFCYLR I W I L V I Q V R R K V K S E V K S R L K P S D M R N F I T M F V V F V L F A I
Rainbow trout Mella AVVFFHFILPIMIVTYCYLR I W I L V I Q V R R R V K P D N R P K L T P H D V R N F V T M F A V F V L F A I
Pike 2.6        TVVVIHFIVPIAVVTFCYLR I W I L V I Q V R R K V K S E V R P R L K P S D M R N F V T M F V V F V L F A I

Consensus      VV  HF  P  V  CYLR I W L V I Q V  V              D  RNF  TMF  V F V L F A

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	VI		VII
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Tilapia	CWAPLNFIGLAVAIKPEVVIPLIPEWLFVA--		
Zebrafish 1.4	CWAPLNFIGLAVAIHP-RLGQSIPEWLFFTA--		
Zebrafish 1.7	CWAPLNFIGLAVAISPERVVPLIPEWLFVA--		
Zebrafish 2.6	CWAPLNLI GLVVAINPEVMAPRVPEWLFV--		
Zebrafish 2.3	CWAPLNFIGLAVAINPAKVAPNIPEWLFVT--		
Human MT1	CWAPLNFIGLAVASDPASMVPRIPEWLFV--		
Xenopus Mel1c	CWAPLNFIGLAVAINPFHVA PKIPEWLFVL--		
Chicken Mel1c	CWGLPNFIGLAVSINPSKVQPHIPEWLFVL--		
Chicken MT1	CWAPLNFIGLAVAVDPETII PRIPEWLFVS--		
Rainbow trout 2.6	CWAPLNFIGLAV AIDPETVAPRIPEWL-----		
Rainbow trout Mella	CWAPLNFIGLAVAINPEVVVPLIPEWLFVA--		
Pike 2.6	CWGLPNFIGLAV AIDPERVAPRIPEWLFV--		
Consensus	CW PLNFIGL V P		PEWL V

Figure 5.4 Multiple alignment of tilapia *O. niloticus* brain melatonin receptor amino acid sequence with sequences of other melatonin receptor subtypes for different vertebrates. The four transmembrane domains (IVth – VIIth) are dash lined.

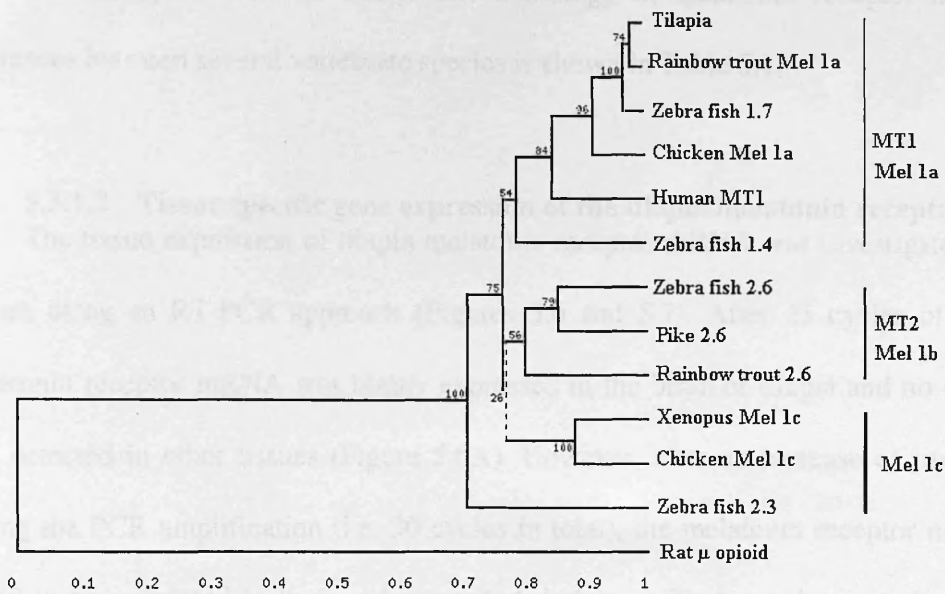


Figure 5.5 Phylogenetic analyses of vertebrate melatonin receptors (Accession numbers used are as follows [from top to bottom] AY569971; AF156262; U31822; U31820; U14108; U31823; U31824; AF188871; AF178929; U09561; U31821; U31825; Rat μ opioid provides an out group for the tree)

Table 5.1 Homology of melatonin receptor amino acid sequences of different vertebrates by pair-wise comparison (Values expressed as percentage)

	ZF1.4	ZF1.7	ZF2.6	ZF2.3	H(MT ₁)	X(Mel _{1c})	C(Mel _{1c})	C(Mel _{1a})	RT2.6	RT(Mel _{1a})	P2.6
T	77.3	94.6	68.0	64.0	76.0	69.3	70.0	88.6	73.4	96.0	72.0
ZF1.4		78.0	62.6	62.0	68.0	68.0	70.6	75.3	76.1	75.3	68.0
ZF1.7			68.6	64.0	74.6	69.3	69.3	85.3	72.8	94.0	72.0
ZF2.6				66.6	63.3	65.3	62.6	66.6	73.4	66.6	79.3
ZF2.3					60.6	72.0	69.3	60.6	63.3	63.3	63.3
H(MT ₁)						66.0	66.0	80.0	70.7	75.3	70.0
X(Mel _{1c})							82.6	69.3	70.1	69.3	70.0
C(Mel _{1c})								70.0	69.4	68.7	68.7
C(MT ₁)									76.2	86.0	74.7
RT2.6										71.4	82.3
RT(MT ₁)											71.3

T: *Tilapia Oreochromis niloticus*, ZF: Zebrafish *Danio rerio*, H: Human *Homo sapiens*, X: *Xenopus Xenopus laevis*, C: Chicken, *Gallus gallus*, RT: Rainbow trout *Oncorhynchus mykiss*.

sequences surrounding the tilapia partial sequence in the phylogenetic analysis belong to the same subtype (Mella or MT₁). The homology of melatonin receptor amino acid sequences between several vertebrate species is shown in Table 5.1.

5.3.1.2 Tissue specific gene expression of the tilapia melatonin receptor

The tissue expression of tilapia melatonin receptor mRNA was investigated in adult tissues using an RT-PCR approach (Figures 5.6 and 5.7). After 25 cycles of PCR, the melatonin receptor mRNA was highly expressed in the brain of tilapia and no expression was detected in other tissues (Figure 5.6A). However, after an increase of just 5 cycles during the PCR amplification (i.e. 30 cycles in total), the melatonin receptor mRNA was found to be expressed in liver, spleen, gonad, kidney, gills, heart, brain, red muscle and white muscle. No expression was detected in the intestine or eye cups (Figure 5.6). In this experiment, the highest expression was found in the brain tissue, confirming the results found in the previous experiment (25 cycles of PCR). In both experiments β -actin primers were used as a control for gene expression. β -actin mRNA was expressed in most of the tissues evaluated in both experiment, with the exception of the eye cups (Figures 5.6B and 5.7B). This lack of expression in the eyes suggested that the resultant cDNA generated by reverse transcription was not enough to express the expected melatonin receptor. This was confirmed by the lack of expression with β -actin primers.

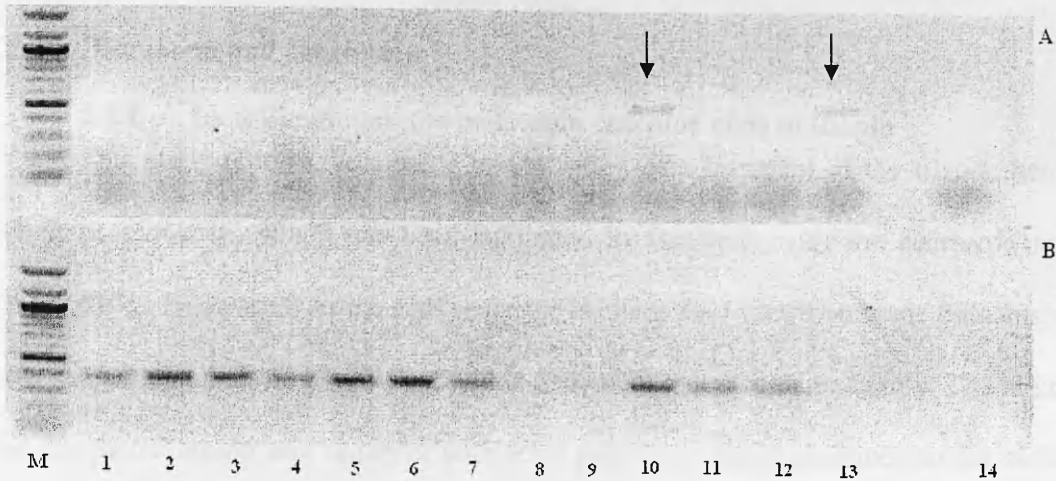


Figure 5.6 Tissue specific melatonin receptor gene expression in several tissues of *O. niloticus* exposed to 25 cycles of PCR. A) Melatonin receptor products. B) β -actin products. Samples number are: 1 liver, 2 spleen, 3 intestine, 4 gonad, 5 kidney, 6 gills, 7 heart, 8 eye (1), 9 eye (2), 10 brain (1), 11 red muscle, 12 white muscle, 13 brain (2), 14 negative control, M size marker 100 bp, arrows shows the PCR product band. (Note: no brain cDNA was loaded in the β -actin gel in line B13)

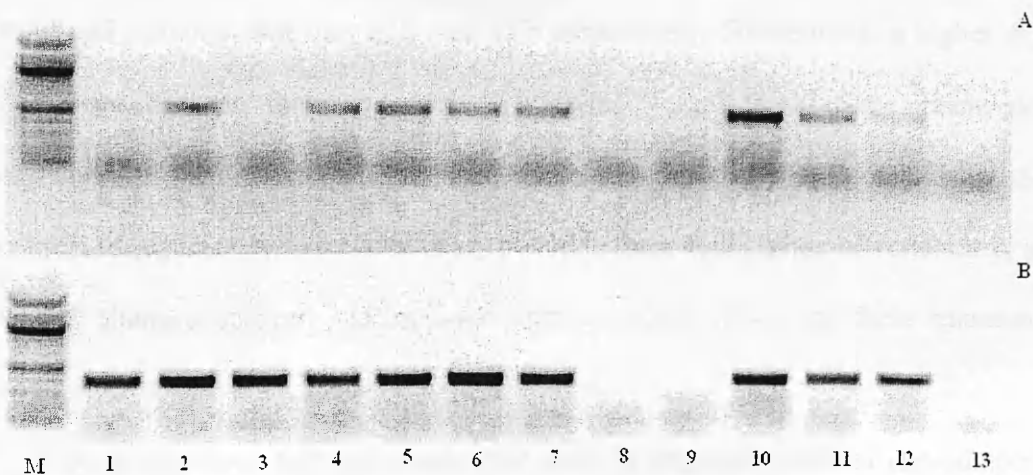


Figure 5.7 Tissue specific melatonin receptor gene expression in several tissues of *O. niloticus* exposed to 30 cycles of PCR. A) Melatonin receptor products. B) β -actin products. Samples number are: 1 liver, 2 spleen, 3 intestine, 4 gonad, 5 kidney, 6 gills, 7 heart, 8 eye (1), 9 eye (2), 10 brain, 11 red muscle, 12 white muscle, 13 negative control, M size marker 100 bp.

5.4 Discussion and conclusion

5.4.1 Identification of the melatonin receptor gene in tilapia

This study reports the cloning and isolation of a fragment of the tilapia melatonin receptor sequence, which has been submitted to GenBank with the accession number AY569971. This partial amino acid sequence contains four transmembrane domains which are characteristic of the G-protein coupled melatonin receptor super-family. The alignment of this partial amino acid sequence with other previously reported sequences for melatonin receptors (Ebisawa *et al.*, 1994; Reppert *et al.*, 1995a; Mazurais *et al.*, 1999; Gaildrat and Falcon, 2002) suggested that this partial amino acid sequence exhibits high homology to the rainbow trout (Mell1a) mRNA and the zebrafish Z1.7 mRNA with 96% similarity. The degree of homology between the human MT₁ and the new tilapia fragment was 76%; a higher degree of homology was found with the chicken (88.6%). However, at the nucleotide level, the similarity of the tilapia fragment to those already reported for rainbow trout and zebrafish was only 82% and 83% respectively. Furthermore, a higher degree of homology between these sequences was found within the transmembrane domains, especially in the Vth and VIth domain, which suggests that these domains are the most conserved regions over the evolutionary period in these four classes of vertebrates, and that further changes between species have since occurred outside of these transmembrane domains (Reppert *et al.*, 1995a)

A phylogenetic tree was constructed with 12 sequences (partial and completed) of melatonin receptors already reported for at least four different classes of vertebrates, and it clearly suggested that the partial sequence of the tilapia melatonin receptor belongs to the Mell1a receptor subtype. This was confirmed by the high level of homology found between this sequence and the Mell1a sequence already reported for zebrafish and rainbow trout (Reppert *et al.*, 1995a; Mazurais *et al.*, 1999). Furthermore, this tilapia fragment was

located in the same branch of those sequences previously reported for chicken and human within the MT₁ and Mel1a subtypes (Reppert, 1995a). The degree of homology found between the tilapia and the human is low, however, there is a better location of the chicken melatonin receptor, and this may suggest that there has been considerable change in this gene in humans but not so much divergence between birds, fish and amphibians.

The fragment of the tilapia sequence reported herein contains four transmembrane domains. This was obtained because the primers used for the PCR and RT-PCR were designed using sequences from these transmembrane domains in which the first intracellular loop was avoided. According to the protein structure of previously reported melatonin receptors, these genes are formed by two exons separated by an intron. The intron is located in the first intracellular loop and could be as long as 8 Kb in length (Reppert *et al.*, 1995a, 1996b; Reppert, 1997).

5.4.2 Tissue specific gene expression of tilapia melatonin receptor

Expression of the novel tilapia melatonin receptor cloned herein was observed in most of the tissues evaluated, with the exception of the eyes. In the first experiment (after 25 cycles of PCR), expression was only noted in the brain; this was in accordance with the literature, which indicates that strong transcription appears to occur in the brain, with particularly high levels in the suprachiasmatic nucleus, optic tectum, thalamus, pretectal area and cerebellum (Mazurais *et al.*, 1999, 2000a; Gaildrat and Falcon, 2000, Gaildrat *et al.*, 2002; Shi *et al.*, 2004). These expression areas in the brain were also reported using the 2-[¹²⁵I] iodomelatonin binding site method in several fish species such as the rainbow trout, the goldfish, salmon, lamprey, catfish and skate (Iigo *et al.*, 1994; Davies *et al.*, 1994, 1995; Mazurais *et al.*, 1999, 2000a; Iigo *et al.*, 1997; Vernadakis *et al.*, 1998; Amano *et al.*, 2003a,b). In the present investigation, whole brain was dissected for RNA isolation, as

it was difficult to elucidate which region of the brain contributed the highest amount of mRNA expression. To further investigate the precise location of mRNA expression of this particular gene, *in situ* hybridization methodology should be applied in further studies, as well as detailed dissections of the brain for more precise RNA extraction. However, this experiment has shown the way for future investigations on tilapia melatonin receptors: many questions remain to be elucidated.

In the second experiment, using a higher number of amplification cycles in the PCR (i.e. 30 cycles), it was possible to obtain positive mRNA signals for tilapia Mel1a in most of the tissues tested, with the exception of the eyes. This experiment confirms that with a large number of cycles it was possible to detect better expression in a wider range of tissues. However, no expression was found in tilapia eyes, even under high cycle amplification. It may have been that the RNA obtained from the eye cups was insufficient to generate good quality cDNA. However, all RNA samples were previously assayed in order to confirm RNA quality. Besides these quality controls, these experiments failed to demonstrate expression in eye tissue, despite a variety of experimental conditions (25 and 30 cycles using both set of primers, specific primers for the melatonin receptor and control primers for β -actin). This suggests that the eye cDNA was not capable of amplifying expression of the transcript. The retina is one of the tissues exhibiting the highest expression of melatonin receptor mRNA in other vertebrate species, such as the claw frog *X. laevis* and the zebrafish *D. rerio*, chicken *G. gallus* and human *H. sapiens* (Reppert *et al.*, 1995a, b; 1997; Wiechmann *et al.*, 1999; Wiechmann and Smith, 2001). However, most of the information reported in which melatonin receptors are highly expressed in the retina correspond to the MT₂ (Mel1b) subtype, which is 60% similar to the human MT₁ (Mel1a). This may suggest that, in contrast to other animal groups, the melatonin receptor in tilapia is not playing a fundamental role in the retina. In the case of the amphibians (i.e.

Xenopus), melatonin receptors have been detected in both the neural retina and retinal pigment epithelium (REP), however, the expression of these melatonin receptors corresponded to the Mel1b and Mel1c subtypes; Mel1a was not detected. The lack of detection of Mel1a using RT-PCR in *Xenopus* suggests that Mel1a is not expressed in these tissues (Wiechmann *et al.*, 1999; Wiechmann and Smith, 2001). Similar results were found in tilapia in the present study, and this may suggest that limitations of the PCR technique may be involved in the failure to detect Mel1a subtype expression. However, after a second attempt to detect the expression of Mel1a in *Xenopus*, Wiechmann and Smith (2001) reported, for the first time, the expression of Mel1a in the neural retina and pigment epithelium (REP). A higher number of melatonin binding sites were observed in the common frog (*Rana perezii*) especially in the neural retina and brain areas including telencephalon, diencephalon and optic tectum. These binding sites were confirmed to be part of the Mel1a subtype (Isorna *et al.*, 2004).

Wiechmann and colleagues have demonstrated that melatonin receptors in *Xenopus* (Mel1b and Mel1c) have rhythmic oscillations and the mRNA expression was higher during the light phase and considerably lower in the dark phase (Wiechmann *et al.*, 1999; Wiechmann and Smith, 2001). This may indicate that both receptors are controlled by a circadian clock. On the other hand, Sugden *et al.* (1999) reported a lack of melatonin receptor (MT₁) oscillation in the SCN in rats, and suggested that in this particular case, MT₁ was not regulated by an internal clock, at least in rats. Nevertheless, a controversial result was reported by Neu and Niles (1997), who reported a strong rhythmic oscillation in the expression of the MT₁ mRNA in rats. This discrepancy in results was attributed to the different techniques used in these investigations, even though both teams have used the same RT-PCR approach. The detection of melatonin receptor expression in retina is difficult and some techniques have failed to achieve the required sensitivity. This problem

was previously reported by Wiechmann and Smith (2001), when they failed to detect any difference in expression levels of melatonin receptors in *Xenopus* retina using Northern blots.

There are some reports in which the numbers of melatonin receptors have been evaluated in terms of ontogeny. The results suggest that higher numbers of melatonin receptors are found in younger stages, and the levels of mRNA decrease in advanced stages. This has been confirmed in fetal human brain in which high expression of MT₁ was detected in the leptomeninges; these results were further confirmed by investigating melatonin binding sites in which the concentration of binding sites was higher in younger stages (Drew *et al.*, 1997). Similar results were reported by Gauer *et al.* (1998) working with Syrian hamsters. They reported higher levels of MT₁ mRNA expression and larger numbers of melatonin binding sites in the pars tuberalis (PT) and suprachiasmatic nucleus (SCN) in newborn hamsters than in eight days postnatal animals. On the other hand, Masana *et al.* (2000) demonstrated the expression of the MT₁ receptor in the C3H/HeN mouse SCN is strongly regulated by an internal biological clock and by the direct effect of light. This investigation reported a higher amount of MT₁ mRNA expression in the dark phase and lower levels in the light phase, in mice exposed to a light:dark regime. Mice exposed to continuous darkness expressed higher levels of mRNA in the subjective dark phase and lower levels in the light phase. This suggested that the expression of MT₁ mRNA was regulated by an internal biological clock. These results were correlated with the secretion of the melatonin hormone, which had higher levels during the night and lower concentrations during the day. However, a different pattern of expression was found in the *in situ* hybridization, in which low levels were reported during the dark period and high levels during the light period. Similar results were found by Poirel *et al.* (2002), who also reported a rhythmic oscillation of the MT₁ mRNA in the SCN in rats and that this mRNA

oscillation was not totally correlated with the melatonin binding sites. This also suggested that the expression of the MT₁ receptor in rat is controlled by the circadian activity in the SCN or by an internal biological clock. This also indicates that the expression of MT₁ mRNA is not playing a functional role upon the amount of melatonin binding sites. All the above investigations may suggest that melatonin was playing an important role in the early growth and development of mammalian brains.

Regarding the expression of the melatonin receptors in fish eyes, Shi *et al.* (2004) were able to detect mRNA expression of the Mel1a receptor in chum salmon. They used real time PCR and their results suggested that levels of mRNA expression were high in small embryos (9 to 2 days before hatch), but low levels were found in small fry (180 day post hatch). However, no significant rhythm was detected in ocular mRNA expression (Shi *et al.*, 2004). Using a different approach (reverse transcription), mRNA expression was detected in salmon eyes and this time the Mel1b receptor was identified (Shi *et al.*, 2004). On this occasion, a clear rhythm in the mRNA expression was observed in fish 180 days post hatch. This may indicate that in the case of Mel1a in tilapias, the expression of this receptor in eyes might be lost in the development of the fish (in this experiment adult fish were dissected for RNA extraction). Further investigation is therefore required in order to confirm this finding and clarify our results. Currently, more advanced technology is available, in which more sensitive assays may be used in order to detect small variations in mRNA expression. This investigation has only just marked the beginning of a broad research area in melatonin receptor research in tilapia and more sophisticated approaches should be adopted in order to fully understand the precise spatial and temporal expression of these receptors in tilapia.

The expression of the tilapia Mel1a subtype in peripheral tissues suggested that this receptor might be transcribed but perhaps not translated in these structures or it maybe

possible that this receptor has been translated into a non functional truncated protein as previously suggested by Reppert *et al.* (1995a) for birds and fish. A similar process may have occurred in tilapia in which some expression was detected in peripheral tissues; however, expression in these tissues was not found in other species reported so far (Reppert *et al.*, 1995a). However, Naji *et al.* (2004) reported the expression of melatonin receptors in different peripheral tissues, brain, liver and kidney; these results were confirmed using Southern blot hybridization along with weak expression in heart and lung tissue. Conway *et al.* (1997) reported expression and localizations of binding sites of the MT₁ subtype in embryonic kidney cells. Vera *et al.* (1997) reported melatonin receptor binding sites in immature rat testis. Furthermore, high levels of melatonin receptor mRNA were found in human fetal kidney by Drew *et al.* (1998). The expression of melatonin receptors was analysed by RT-PCR. High levels of expression were obtained for the MT₁ receptor subtype. Nevertheless, the MT₂ receptor subtype was expressed in lower concentrations and detected only by Southern blot. These findings were confirmed by in situ hybridization and melatonin binding sites.

It may be that the peripheral tissue Mella expression in tilapia is real and that these melatonin receptors are playing some function in these tissues. Alternatively, it may be that the expression of the Mella in tilapia peripheral tissues was an artefact due to the high number of amplification cycles used for the PCR, in which a considerable amount of DNA was produced. This might have served as a template for further amplification and resulted in non-specific results.

The melatonin receptor MT₁, or the non-mammalian Mella, is the only melatonin receptor found in all vertebrates studied so far. The expression sites of this melatonin receptor mRNA are wide ranging and has been discussed earlier in this chapter.

The precise role of these melatonin receptors in mammals has been widely discussed, and it is argued that MT₁ is playing an important role in the endocrine systems within the pituitary gland, particularly in the pars tuberalis, in which the secretion of pituitary gonadotropic hormones are regulated by melatonin. The literature suggests that the melatonin receptor MT₁ is directly involved in the function of the hypothalamic-pituitary-gonadal axis in mammals, as well as in the circadian response to melatonin (Weaver *et al.*, 1996; Reppert *et al.*, 1996b; Dubocovich *et al.*, 1996; Pang *et al.*, 1998; Goldman, 1999; Johnston *et al.*, 2003).

Nevertheless, the same suggestion cannot be applied in fish, in which the highest levels of melatonin receptor expression and melatonin binding sites occurred in areas totally related with vision, rather than reproduction. These results therefore suggest that melatonin is directly involved in the processing of visual information (Davies *et al.*, 1994; Ekstrom and Meissl, 1997; Mazurais *et al.*, 1999, 2000). The only evidence of the expression of melatonin receptors in fish pituitary have come from the pike in which transcription of the Mel1a and Mel1b subtypes was demonstrated by RT-PCR and Southern blot hybridization. These results may suggest possible interaction between the melatonin receptor and the reproductive physiology of fish (Gaildrat and Falcon, 2000, 2002).

In conclusion, the present study has demonstrated that the melatonin receptor subtype Mel1a is present in tilapia, with high levels of mRNA expression in the brain. However, further investigation will be required to elucidate with more accuracy the specific spatial distribution of the Mel1a receptor in tilapia. More sophisticated techniques are required for the characterisation of this receptor, such as real time PCR, in situ hybridization or melatonin binding site assays. Another question which remains unsolved is the identification of any rhythm in the expression of Mel1a in tilapia. This may help

elucidate the role of the hormone melatonin in the reproduction of this tropical species. This investigation has opened a whole world of future investigations and many questions remain to be addressed, such as: Do tilapia contain Mel1b and Mel1c receptors or just contain the Mel1a subtype? Where are the precise expression sites of Mel1b and Mel1c receptors in tilapia? Do these exhibit a rhythmic expression throughout the day, or do they remain constant during the day? Finally, where is the precise spatial location of the melatonin receptor genes in the tilapia chromosomes?

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6 Chapter Six Mechanism of Egg Activation in Cultured Species of Finfish

6.1 Introduction

Many species of farmed fish exhibit problems associated with reproduction. In females for example, there are often problems associated with final oocyte maturation, ovulation and final spawning (oviposition). As far as males are concerned, problems are usually involved with spermiation, low milt quality, or poor sperm production (Bromage, 1995; Zahor and Mylonas, 2001).

The Nile tilapia (*Oreochromis niloticus*) has become an important species for aquaculture; it is also an important species for research (i.e. as a research model) (Maclean *et al.*, 2002), in a similar manner to zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Tilapia exhibit certain reproductive traits that collectively make them ideal candidates for research into reproductive physiology; for example short spawning cycles, relatively large eggs, and resistance to disease (see Coward and Bromage, 2000, Coward *et al.*, 2002). Above all, tilapias are able to breed all year round under controlled conditions, allowing the production of significant numbers of eggs and larvae. This is an important issue, especially in genetics research; it allows the production of several generations in small periods of time and easily allows family comparison (genotyping) (Coward and Bromage, 2000, Coward *et al.*, 2002).

Unfortunately, under farmed and hatchery conditions, tilapias tend to breed in an asynchronous manner. This is considered to be the most significant problem associated with tilapia aquaculture at present. To circumvent these problems, hatchery operators tend to utilize large numbers of broodstock, which undergo strict rotation on a time to time basis (i.e. every six months) in order to guarantee the production of a certain number of eggs and fry (Macintosh and Little, 1995; Little *et al.*, 2000, Bromage, 1995, 1992; Coward and Bromage, 2000, Coward *et al.*, 2002).

Tilapias are multiple spawners, with high degrees of parental care and reasonable fertilization rates; fertilization rates can often reach as high as 70 or 80% under controlled conditions according to some authors (Coward *et al.*, 2000), though we do not have accurate data from wild fish to allow comparison. The high fertilization capacity of tilapias, coupled with their role as reliable research models, could be utilised in order to further investigate and perhaps help alleviate known problems encountered in the reproduction efficiency of other species with high potential for aquaculture such as gilt-head sea bream (*Sparus aurata*) (Zohar *et al.*, 1995), red sea bream (*Pagrus major*) (Watanabe and Kiron, 1995), sea bass (*Dicentrarchus labrax*) (Carrillo *et al.*, 1995), turbot (*Scophthalmus maximus*) (Suquet *et al.*, 1998), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) (Kjørsvik and Holmefjord, 1995), and red drum (*Sciaenops ocellatus*) (Thomas *et al.*, 1995). All of these species exhibit some kind of problem in their reproductive activity under culture conditions (Bromage, 1995; Carrillo *et al.*, 1995; Kjørsvik and Holmefjord, 1995; Zanuy *et al.*, 1999, 2001; Dahle *et al.*, 2003; Vermeirssen *et al.*, 2004), which unfortunately might have severe consequences upon their success as aquaculture species. Many of the problems encountered with these species relate to fertilization and early embryonic development (Coward *et al.*, 2002), this is particularly so in marine species.

In the present study, we used tilapia as a research model to undertake preliminary investigations into the process of egg activation at fertilization to further understand the mechanisms involved with this vital developmental process. At present we know little about the precise mechanisms involved in the activation of fish eggs at fertilization. It might be possible to apply lessons learnt with this model to other finfish species that are more difficult to work with but exhibit known problems associated with fertilization, seed production, and low survival under culture conditions. Such strategies might also help all-

year-round production, as well as help producers to avoid the collection of wild seed from natural stocks, thus damaging natural populations (Bromage, 1992). Presently, the commercial culture of many important freshwater, but especially marine, teleosts is beset by problems associated with fertilization, hatching and early embryonic development. These problems have been particularly acute in certain species leading to the application of spawning induction technologies in an effort to optimize production. Increased knowledge of the processes of egg activation and fertilization in these groups of fish is likely to make a significant contribution to commercial aquaculture (Coward *et al.*, 2002).

6.1.1 Fertilization and egg activation in teleost fish

Problems related to reproduction often arise in cultured fish. These problems are sometimes caused by asynchrony between male and female gonadal development reproductive behaviour, but are sometimes unexplained. The most common problem encountered appears to be the low quality of gametes (sperm and eggs), the lack of synchrony between gamete productions, or the inability to achieve oocyte final maturation (OFM) and ovulation. A problem associated with any of these processes would inevitably result in low fertility rates and poor hatching rates (Bromage, 1995, Bromage and Cumaranatunga, 1988). Such problems are often caused by environmental, nutritional or culture conditions. Furthermore, increases in mortality are often found during embryonic development and larval rearing. Presently, to overcome these problems, farmers rely on the use of high numbers of broodstock such that the production of a specific number of fish suitable for on-growing farms or market-demand is guaranteed (Bromage, 1995).

Certain commercially important fish species, notably marine species, suffer from low fertilization and hatching rates; examples include Atlantic halibut *H. hippoglossus* (Norberg *et al.*, 1991; Holmefjord *et al.*, 1993; Bromage *et al.*, 1994), sole *S. solea*

(Houghton *et al.*, 1985), turbot *S. maximus* (Bromley *et al.*, 1986), gilthead seabream, *S. auratus* (Carrillo *et al.*, 1989) and some salmonids (Bromage *et al.*, 1992). Furthermore, similar problems often arise when fish are held captive in artificial environmentally controlled conditions. These problems have serious ramifications for successful and profitable culture. There are numerous possible explanations for these observations.

The success of fertilization is totally dependent on the quality of spermatozoa and oocytes. In fish, the egg and sperm quality is determined by several factors such as seasonal variation, the nutritional and endocrinological status of the fish, and also genetic make up; these are discussed at length elsewhere (Billard, 1990a; Billard *et al.*, 1995; Brooks *et al.*, 1997; Pustowka *et al.*, 2000). In terms of sperm quality, which is defined as being the ability of sperm to successfully fertilise an egg (Rurangwa *et al.*, 2004), there are two principal factors that should be considered: those variables involving sperm motility, and those concerning duration of movement (Billard *et al.*, 1995; Kime *et al.*, 2001). However, there are several other variables that should also be considered such as spermatocrit, sperm density, osmolarity and pH of seminal plasma, chemical composition of seminal plasma, enzymatic activity, adenosinetriphosphate (ATP) concentration, motility, morphology and ultrastructure, and fertilising capacity (Rurangwa *et al.*, 2004).

One of the major problems encountered at fertilization time is the short life-span of spermatozoa, which is less than a minute in freshwater. In fish, egg activation is enhanced by the seminal and ovarian media, which accompanies the gametes. This fluid plays an important role in sperm motility and also stabilises osmotic pressure and pH (Billard, 1990b, Kime *et al.*, 2001; He *et al.*, 2004; Rurangwa *et al.*, 2004). In fish, spermatozoa remain immotile within the seminal plasma in those species that have external fertilization. The spermatozoa become motile at spawning when they are expelled into the surrounding media (water, sea water and ovarian fluid). Changes in the ionic and osmotic environment

of the sperm cells have been identified as two critical external factors that may be responsible for initiating motility in fish spermatozoa (Billard, 1990b; He *et al.*, 2004). In the Pacific herring (*Clupea pallasii*), sperm is activated when it reaches the chorion surface, particularly near the micropyle (Griffin *et al.*, 1996). In Arctic charr (*Salvelinus alpinus*), sperm exhibit a short activation period of less than 24 seconds; during the first 30 seconds post-activation, swimming speed varied between 106 to 21 $\mu\text{m/s}$. Ovarian fluid enhances sperm motility in this species and increases the chances of fertilization success and the outcome of sperm competition (Turner and Montgomerie, 2000). In the black bream (*Acanthopagrus butcheri*), fertility and motility rates decrease in freshwater, and these rates tend to gradually increase concurrent with increasing salinity (Haddy and Pankhurst, 2000). Teleost fish exhibit an extremely diverse array of reproductive strategies ranging from mass spawning in open water to pair-mating in enclosed spaces, as well as internal fertilisation (Kime *et al.*, 2001). Although the sperm of externally-fertilizing fishes usually exhibits a brief life span of up to a few minutes, in the particular case of the three-spined stickleback (*Gasterosteus aculeatus*), the spermatozoa move for several hours in brackish water, and up to at least 10 hours in the presence of ovarian fluid (Elofsson *et al.*, 2003). In brackish water, sperm motility in the three-spined stickleback lasted for 165–270 minutes; in seawater however, sperm motility lasted for only up to 65 minutes. In the presence of ovarian fluid some stickleback sperm remained active for up to 24 hours (Elofsson *et al.*, 2003).

In the majority of teleosts, eggs are fertilized externally; this process requires large numbers of spermatozoa. The spermatozoa must travel long distances to find the micropyle of the egg. Sperm have low swimming capacity in the external environment (~ 2 mm at 55 $\mu\text{m/s}$ on average). In the African catfish (*Clarias gariepinus*), sperm swimming velocity can reach 120 - 140 $\mu\text{m/s}$ and last for 30 seconds in water, and 40 seconds in NaCl

solutions (Mansour *et al.*, 2002). In sea bass, sperm motility lasts for only 40 seconds and the required number of sperm was estimated to be around 66,000 spermatozoa per egg to guarantee a maximum fertilization rate (Fauvel *et al.*, 1999). In the common carp (*Cyprinus carpio*), sperm motility lasts for more than one minute, with a swimming velocity ranging from 70 – 90 $\mu\text{m/s}$ (Warnecke and Pluta, 2003). In the Atlantic salmon (*Salmo salar*), mean sperm swimming speed varied between 18 - 127 $\mu\text{m/s}$ and was active for 18 to 78 seconds (Gage *et al.*, 2002). In the red porgy (*Pagrus pagrus*), a species with protogynous hermaphroditism, sperm motility lasted from 2 to 4 minutes (Mylonas *et al.*, 2003). In turbot, sperm motility in fresh sperm lasted 60 seconds with a swimming speed of 190 $\mu\text{m/s}$ at 10 seconds post-activation, and 120 $\mu\text{m/s}$ at 60 seconds post-activation (Suquet *et al.*, 1998).

In fish with large eggs (i.e. salmonids), the duration of sperm motility is so low that the sperm is not able to swim round even half the circumference of the egg (Kime *et al.*, 2001). Even minor reductions in sperm motility, whatever the cause, could therefore have a profound effect on its ability to fertilise the egg (Kime *et al.*, 2001). These factors are thought to be the underlying reason for fish species producing large numbers of spermatozoa (Billard, 1990b), without such large numbers, fertilization success would be reduced dramatically.

Studies on the composition of fish spermatozoa indicate that there is large intra and inter-specific variation in sperm concentration, as well as seminal plasma concentration (Billard *et al.*, 1995; Rana, 1995; Suquet *et al.*, 2000; Casselman and Montgomerie, 2004). This variation in sperm concentration is thought to be due to genetic variability, seasonality, breeding status and reproductive strategy, sampling location (testes or genital pore), contamination with urine, or fish age (Scott and Baynes, 1980; McAndrew *et al.*, 1983; Fauvel *et al.*, 1999; Suquet *et al.*, 2000). Changes in sperm quality, motility and

seminal plasma composition have been reported during a spawning season in some species such as the sea bass and some salmonids (Fauvel *et al.*, 1999; He *et al.*, 2004). In rainbow trout, the source of dietary lipids has been shown to have considerable effect on sperm viability and quality after cryopreservation. High levels of cholesterol and monounsaturated fatty acids (MUFA) appeared to provide the spermatozoa with greater resistance to damage during the freeze-thaw process (Pustowka *et al.*, 2000). The nutritional status of sea bass and its effect on reproductive performance has been described, in which broodstock growth, duration of spermiation, milt production, milt spermatozoa density, sperm motility, milt lipid composition, and fertilization rates, were compared during the reproductive season (Navas *et al.*, 1998; Bruce *et al.*, 1999; Asturiano *et al.*, 2000, 2001).

In the European sea bass, fish fed with highly unsaturated fatty acid diets exhibited long spermiation periods, producing higher milt volumes and milt spermatozoa densities as compared with fish fed with un-enriched diets. Although, no differences were found in sperm motility and quality, the results from this study demonstrate that male European sea bass fed two commercial PUFA-enriched diets exhibited enhanced reproductive performance as compared to fish fed a wet diet.

Variations in sperm concentration generally involve a gradual reduction as the breeding season progresses, although spermatocrit in rainbow trout has been reported to increase over the spawning season (Scott and Baynes, 1980). There is a significant variation in sperm quality between virgin- and repeat-spawning cod (Trippel and Neilson, 1992); these authors also report that hatching success was more variable in virgin spawners.

One of the major concerns about marine aquaculture is the “capacity to fertilize” of certain species (Bromley *et al.*, 1986; Carrillo *et al.*, 1989; Norberg *et al.*, 1991; Bromage

et al., 1994). In captivity, fertilization and hatching success rates are often lower than those observed under natural conditions. This problem increases when eggs and sperm are stripped from broodstock to achieve artificial fertilization. Similar problems are observed in freshwater species. In catfish for example, there is a limitation to the hand stripping of male gametes, in which spontaneous semen release does not occur and hand-stripping becomes practically impossible. To overcome this problem, testicular semen must be used. There are some reports of sperm viability in different catfish species such as the African catfish (*Clarias gariepinus*) (Viveiros *et al.*, 2000, 2003; Mansour *et al.*, 2003; Rurangwa *et al.*, 2004), the bagrid catfish (*Mystus nemurus*) (Muchlisin *et al.*, 2004), the Asian catfish (*Clarias macrocephalus*) (Tan-Fermin *et al.*, 1997), the blue catfish (*Ictalurus furcatus*), and the channel catfish (*Ictalurus punctatus*) (Bart and Dunham, 1996). In these species, sperm concentration and hatching rates obtained with stripped fluid were very low compared to those obtained with intra-testicular semen (Bart and Dunham, 1996; Tan-Fermin *et al.*, 1997; Viveiros *et al.*, 2000, 2003; Mansour *et al.*, 2003; Rurangwa *et al.*, 2004)

Atlantic cod and halibut both inhabit North Atlantic waters, and are both commercially important species of fish. However, both species suffer from problems associated with fertilization and embryonic development. In cod, spawning activity occurs naturally and fertilization rates are reasonably high, but increased fish handling and stress results in irregular spawning patterns and low fertilization rates, concomitant with a high number of deformities and abnormal embryos (Kjesbu, 1989). Production of Atlantic halibut fry is currently based upon artificial fertilization, which involves the manual stripping of eggs and milt from mature broodstock. The stripping time in relation to ovulation time is important to guarantee a maximum egg yield and ensure good fertilization rates (Norberg *et al.*, 1991; Bromage *et al.*, 1992; Holmefjord *et al.*, 1993).

Even considering ovulatory rhythms from individual broodstock, fertilization rates and egg viability remain highly variable in hand stripped halibut (Norberg *et al.*, 1991; Bromage *et al.*, 1994; Holmefjord *et al.*, 1993); hatching rates under these circumstances can be as low as 1% (Norberg *et al.*, 1991). Similar variation in fertility has been reported for sole and turbot reared under captive conditions, under these conditions, fertilization rates for these species can be just 50% of the annual egg production (Houghton *et al.*, 1985; Bromley *et al.*, 1986).

In sea bass and gilthead seabream, hatching rates are often just only 10 – 15% of the total number of eggs spawned (Carrillo *et al.*, 1989). In salmonids, losses of up to 50% are common (Bromage *et al.*, 1992). However, it is important to remember that wild stocks of fish also demonstrate high variability in fertilization rates. In the Baltic herring (*Clupea harengus membras*), there is variation from one year to the next one. This may be due to variations in the food supply available and other environmental conditions (Laine and Rajasilta, 1999). There are also age-related effects upon fertility and sperm quality in some teleosts. For example, in the striped bass (*Morone saxatilis*), a three years old fish exhibited far superior sperm quality to that 1 or 2 year old fish, both in terms of higher sperm production and increased sperm longevity (Vuthiphandchai and Zahor, 1999).

In view of the problems associated with reproduction in many cultured species of fish, manipulative techniques are often used to induce spawning, especially in those species which exhibit reduced spawning activity under culture conditions, or demonstrate pronounced asynchronous spawning periodicity. In some species, the induction of spawning is becoming a vital part of their management and it is usually necessary to artificially induce ovulation in females by use of hormones. In males, it is also often advantageous to stimulate spermiation by hormonal manipulation so that sperm is available in plentiful supply at the same time as the eggs. It is also often beneficial to store sperm,

either refrigerated or frozen, until the female can be stripped, or for transport to other establishments (Kime *et al.*, 2001).

The silver perch (*Bidyanus bidyanus*) is a freshwater fish native to Australia with high potential for aquaculture due to its rapid growth under diverse conditions. This species does not spawn naturally under the captive culture conditions encountered in Israel. As a result, hormone manipulation is the only viable alternative to allow the completion of the life cycle of this species under culture conditions, thereby allowing the constant production of eggs and fry (Levavi-Sivan *et al.*, 2004). In the case of the Asian catfish (*C. macrocephalus*) females complete vitellogenesis within the first year under culture condition. However, final maturation and ovulation does not occur unless, hormone manipulation is applied (Tan-Fermin *et al.*, 1997).

The dusky grouper (*Epinephelus marginatus*) is a commercially and recreationally important species in many areas of the Mediterranean Sea. This fish exhibits protogynous hermaphroditism, which makes it difficult to obtain a sexually balanced broodstock. There is also some reproductive dysfunction in wild dusky grouper maintained in captivity; females fail to complete vitellogenesis, resulting in the failure to undergo final oocyte maturation (FOM), ovulation and spawning (Marino *et al.*, 2001, 2003).

The implantation of testosterone pellets into tissues of the European sea bass results in an advancement in male gonad maturation, thus indicating the possible participation of sex steroids in the acceleration of gonadal differentiation and the stimulation of spermatogenesis in pre-pubertal sea bass (Zanuy *et al.*, 1999). In this species, spawning is especially problematic under winter temperatures and changing photoperiods (Carrillo *et al.*, 1995; Zanuy *et al.*, 1995, 2001). As a consequence, it is important to develop methods to control reproductive processes in European sea bass, especially those that concern oocyte maturation, ovulation and spawning. Another example of hormone manipulation is

that reported for the European eel (*Anguilla anguilla*). This species cannot be bred under farming conditions, thus farms base their annual production on the capture of eels from river mouths during autumn and winter months. Thus, hormone manipulation studies are warranted to develop techniques to induce European eel reproduction for the reliable supply of larvae (Perez, 2000).

The failure of cultured fishes to undergo oocyte maturation, ovulation and spawning is predominantly due to lack of leuteinizing hormone (LH) release from the pituitary during the spawning season. Therefore, exogenous hormone manipulations have proven very effective in controlling the reproductive processes of fish in captivity and contributed significantly to the efficiency of the aquaculture industry (Zohar and Mylonas, 2001). One of the most commonly used treatments includes synthetic agonists of gonadotropin-releasing hormone (GnRHa), which stimulate pituitary synthesis and release of LH, thus inducing ovarian steroidogenesis and oocyte maturation (Nagahama, 1994). Since GnRHa is rapidly cleared from the bloodstream with a half-life of only 10–23 min (Gothilf and Zohar, 1991), the elevation of plasma LH is short-lived after a single GnRHa injection. Consequently, GnRHa injections are often administered at multiple times over the course of a few hours (Prat *et al.*, 2001) or days (Mylonas *et al.*, 1992; Dabrowski *et al.*, 1994). Sustained-release GnRHa-delivery systems, that eliminate the need for multiple GnRHa injections, have been developed over the last two decades, and have been successfully used to induce oocyte maturation, spermiation and spawning in various cultured fishes (Fornies *et al.*, 2001; Zanuy *et al.*, 2001; Mylonas *et al.*, 2003).

Spawning induction techniques involve temperature and photoperiod manipulation, injection or implantation of exogenous hormones. These techniques, although useful, tend to have detrimental effects upon subsequent fertilization, for example, in brown trout (Mylonas *et al.*, 1992), sea bass (Carrillo *et al.*, 1995), Japanese eel (*Anguilla japonica*)

(Ohta *et al.*, 1996) and the red bellied tilapia *Tilapia zillii* (Coward *et al.*, 2000). In brown trout injected with gonadotropin releasing hormone analogue (GnRHa), premature ovulation occurred with a significant reduction of fertility thought to be due to a disruption of final oocyte maturation and ovulation (Mylonas *et al.*, 1992).

Problems associated with sperm and egg quality contribute greatly to the difficulties encountered in the culture of some commercially important fish. Many of the factors contributing to these problems are being investigated and addressed. Perhaps one of the greatest unknowns in fish reproductive biology is the precise nature of fertilization, the union of egg and sperm. We know from the literature that many fish species exhibit problems associated with low fertilization rates; in some cases these problems can be explained, in other cases not. An increased knowledge of the processes involved in egg activation at fertilization in these groups of fish is likely to make a significant contribution to commercial aquaculture. Studies of a wide variety of animal species has demonstrated that development at fertilization is triggered by an increase in intracellular calcium (Ca^{2+}) concentration within the egg that occurs as either a single transient or a series of distinctive oscillations depending upon the species under investigation. This increase in intracellular Ca^{2+} activates the egg and also appears to play an important role in later embryonic development. Currently, studies of egg activation in teleosts are confined to laboratory species such as medaka (*O. latipes*) and zebrafish (*D. rerio*). In zebrafish, the egg is activated by the presence of spawning fluid; when mature eggs are discharged from the ovarian stroma and come in contact with the spawning medium, they are spontaneously activated. Even in the absence of sperm, these activated eggs undergo a programmed series of developmental steps. These parthenogenetically activated eggs then proceed to elevate their chorions, and undergo normal cytoplasmic segregation. After several abortive cleavages, however, eggs stop further development (Lee *et al.*, 1999). In medaka, egg

activation is achieved by the simple union between gametes. When a sperm reaches the egg, both cells fuse and the egg starts a series of biochemical and structural changes in both the cytoplasm and chorion. These changes include the resumption of meiotic division, exocytosis of cortical alveoli (vesicles) changes in the egg envelope (chorion), oscillatory contractions with the accumulation of cortical cytoplasm and the migration of oil droplets and pronuclei (Iwamatsu *et al.*, 1995; Iwamatsu, 1998). These findings may suggest that a sperm-specific mechanism is involved in medaka egg activation, but not in zebrafish eggs. In both cases (zebrafish and medaka), at the moment of egg activation, a calcium transient was observed and it appears that this might be the key element in fish egg activation, as it is in other animal groups (Gilkey *et al.*, 1978; Fluck *et al.*, 1991; Lee *et al.*, 1999). The present study makes a preliminary investigation into the processes that might occur at egg activation in teleost fish, using tilapia as a research model, and using findings and theories emerging from other animal groups.

6.1.2 Calcium and its role in egg activation

The calcium ion (Ca^{2+}) is an important component in the physiology of all organisms. It has an important role in cell signalling and general metabolism (Parrington, 2001). Ca^{2+} controls and regulates most of the vital functions in the body. It is required for the maintenance of cell structures, control of ion permeability (i.e. Na^+ and K^+), and plays an important role in the regulation of cell mobility and contraction. One of the most vital roles of Ca^{2+} is as a second messenger; a molecule that relays signals received at the cell surface such as the arrival of a protein hormones, or growth factor at cell surface receptors, to target molecules in the cytosol or nucleus. In this way, second messengers control a significant portion of the changes in biochemical activity within cells (Morgan, 1989).

In all species studied thus far, the egg activation is characterised by a remarkable increase in Ca^{2+} in the egg at the moment of cell fusion. This Ca^{2+} increase is considered to be the activator of cell division and further embryonic development (Whitaker and Swann, 1993; Miyazaki *et al.*, 1993; Stricker, 1999; Parrington, 2001; Saunder *et al.*, 2002) in that it re-activates the oocyte, which has previously been arrested in meiosis. It is broadly accepted that this Ca^{2+} is released by intracellular egg stores (i.e. endoplasmic reticulum, ER) (Jones *et al.*, 1998a, 1998b, 2000; Rice *et al.*, 2000; Parrington 2001; Howell *et al.*, 2003; Swann *et al.*, 2004). The abolition of such Ca^{2+} increases within the egg by the use of chelators (substances that block the passage of Ca^{2+}) results in the inhibition of egg activation, and the abolition of cortical granule release, which prevents polyspermy; which then causes a failure in the meiotic resumption and subsequent embryonic development (Parrington, 2001).

The increase of Ca^{2+} within the egg is a universal observation during egg activation in all living organisms studied thus far. However, the patterns of these Ca^{2+} signals are different between species. In sea urchins, starfish, frogs, and fish (zebrafish), just a single Ca^{2+} wave (or transient) is observed crossing the egg from one pole to the other (Gilkey *et al.*, 1978; Fluck *et al.*, 1991; Deguchi and Osanai, 1994; Busa and Nuccitelli, 1995; Creton and Jaffe, 1995; Stricker, 1996; Fontanilla and Nuceitelli, 1998; Sardet *et al.*, 1998; Lee *et al.*, 1999; Stricker 1999; Jaffe *et al.*, 2001; Bugrim *et al.*, 2003;). On the other hand, in mammals, nemertean worms, annelids and ascidians, the Ca^{2+} release is characterised by a series of periodic increments known as “ Ca^{2+} oscillations” (Miyazaki *et al.*, 1993; Whitaker and Swann, 1993; Swann and Ozil, 1994; Eckberg and Miller, 1995; Stricker, 1996, 1997, 1999; Kyosuka *et al.*, 1998; Sardet *et al.*, 1998; Stricker *et al.*, 1998; Dumollard *et al.*, 2002, 2004). The duration of these oscillations is variable. In mammals they last between two and six hours post-fertilization (Swann and Ozil, 1994; Jones *et al.*,

1995). These oscillations are suggested to be an essential part of ongoing egg development, although the precise nature of their role in development has yet to be fully elucidated. Activation rates were improved when multiple Ca^{2+} oscillations were artificially triggered in eggs by means of electroporation (Ozil, 1990; Vitullo and Ozil, 1992; Ozil and Swann, 1995; Jones, 1998b). To demonstrate the efficacy of Ca^{2+} oscillations at egg activation, Lawrence *et al.* (1998) used the heavy metal chelator N,N,N1,N1-Tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) to reduce the number of oscillations, and demonstrated that just a few Ca^{2+} transients were required to activate the eggs. Hence, it was demonstrated that these Ca^{2+} oscillations were indeed important for the egg activation. However, the role of these Ca^{2+} oscillations in embryonic development remains uncertain (Ozil and Swann, 1995; Jones, 1998b; Ozil, 1998). Saunders *et al.* (2002) further demonstrated that Ca^{2+} oscillations were indeed necessary for egg activation and subsequent embryonic development in mouse eggs.

The role of Ca^{2+} in embryonic development has been investigated by several authors and findings suggest that gene activation is highly sensitive to Ca^{2+} levels and Ca^{2+} signalling patterns within the cell. This suggested that various transcription mechanisms are also sensitive to Ca^{2+} oscillations in terms of amplitude and frequency, and that these mechanisms could help to translate Ca^{2+} signals into gene expression, with further consequences on the developing embryo (Dolmetsch *et al.*, 1997, 1998; Leung *et al.*, 1998; Gilland *et al.*, 1999).

6.1.3 Models of egg activation

For several decades it has been well accepted that Ca^{2+} has a vital role in egg activation. However, the precise mechanism by which sperm trigger this Ca^{2+} release was only discovered very recently. Prior to this discovery, three main theories were originally

proposed: the “Ca²⁺ bomb” or “conduit” theory, the “membrane receptor” or “contact” theory, and the “sperm factor” or “content” theory (Jaffe, 1991).

6.1.3.1 “Ca²⁺ Bomb” or “conduit” model

This theory suggested that Ca²⁺ is introduced into the egg by the sperm. The sperm was thought to work as a transporter or channel, which allows the introduction of extracellular Ca²⁺ into the egg (Jaffe 1991; Creton and Jaffe, 1995; Jones *et al.*, 1998b) as shown in Figure 6.1A. This theory was supported by the fact that the fusion of sperm and egg is followed by the release of intracellular calcium in the egg; this might be caused by Ca²⁺-induced-Ca²⁺ release (CICR) inside the egg caused by a “calcium bomb” initiated by the sperm (Jaffe, 1991). These findings have been demonstrated in sea urchin and mouse eggs (McCulloch and Chambers, 1992; Lawrence *et al.*, 1997). This theory has major limitations, however, such as the fact that an injection of Ca²⁺ into sea urchin, ascidian and mouse eggs failed to cause any Ca²⁺ oscillations within the these eggs (Whitaker and Swann, 1993; Swan and Ozil, 1994). Furthermore, the removal of extracellular Ca²⁺ failed to block Ca²⁺ release in the mouse egg (Jones *et al.*, 1998b). In view of these findings, this theory is largely dismissed by the research community.

6.1.3.2 “Membrane receptor” or “contact” model

This model suggested that a ligand on the sperm surface binds to an egg membrane receptor and that this receptor then activates a signal cascade by the activation of an egg phospholipase C (PLC). This PLC then generates inositol 1,4,5 trisphosphate (IP₃) by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂). The IP₃ released then triggers Ca²⁺ release through the inositol trisphosphate receptor (IP₃R) on Ca²⁺-containing intracellular organelles such as the endoplasmic reticulum (Jaffe, 1991; Myles, 1993; Lawrence *et al.*, 1997; Jones *et al.*, 1998b; Parrington, 2001; Coward *et al.*, 2002). Figure

6.1B shows a diagrammatic representation of this model. Some studies have supported this theory, although to date despite the advances in molecular and genomic biology, a receptor of this nature has yet to be identified on the egg of any organism (Kevin Coward, personal communication). However, currently there is some convincing research evidence to support the existence of the receptor theory in sea urchins (Kevin Coward, personal communication), which might also be true of other non-mammalian organisms.

6.1.3.3 “Sperm factor” or “content” model

This theory proposed that a sperm component (“sperm factor”) is responsible for the Ca^{2+} release in the egg at fertilization (Whitaker and Swann, 1993; Swan and Ozil, 1994; Swan and Lai, 1997; Fissore *et al.*, 1998; Jones *et al.*, 1998b; Parrington *et al.*, 1998; Stricker, 1999; Swann and Parrington, 1999; Coward *et al.*, 2002, 2003). Figure 6.1C shows a graphic representation of this novel mechanism of egg activation. In the sperm factor model, the fusion of the sperm and the egg is followed by an increase of Ca^{2+} release. In the sperm factor model, it is suggested that a sperm-specific PLC enters the egg at fertilization and that this PLC hydrolyses PIP_2 inside the egg, which creates IP_3 , which in turn releases Ca^{2+} from intracellular egg stores.

Clinical studies support the “sperm factor” model through an indirect method. Using a clinical procedure known as intracytoplasmic sperm injection (ICSI; a common method used in human fertility treatments) in humans, sperm are introduced into the centre of an egg by a fine needle. Despite bypassing any egg surface interaction, this procedure causes normal egg activation in addition to Ca^{2+} release in the egg, followed by distinctive Ca^{2+} oscillations (Tesarik and Sousa, 1994; Tesarik *et al.*, 1994). Similar results have been observed in nemertean worms (Stricker, 1996; Stricker *et al.*, 2000) and in mice (Nakano *et al.*, 1997; Knott *et al.*, 2003). The Ca^{2+} oscillations created by ICSI are identical to those reported during normal fertilization.

A further indirect way to confirm and support the “sperm factor” model is through the injection of soluble sperm extracts prepared from boars, hamsters, or humans. Injection of these extracts into eggs produces a series of Ca^{2+} oscillations similar to those reported at fertilization in mouse, hamster, human, and cow eggs (Swann, 1990, 1992, 1994; Homa and Swann, 1994; Palermo *et al.*, 1997; Wu *et al.*, 1997; Fissore *et al.*, 1998; Knott *et al.*, 2002). The injection of sperm extracts into eggs obviously avoids membrane-bound receptors and thus supports the “sperm factor” model in a convincing manner.

Sperm extracts of hamster, human, boar, mouse, pig, cow, monkey, frog, chicken, and ascidian, and even a flowering plant, can trigger Ca^{2+} oscillations when injected into mouse eggs (Swann, 1990; Homa and Swann, 1994; Kono *et al.*, 1995; Parrington *et al.*, 1996; Wu *et al.*, 1997, 1998; Parrington *et al.*, 1998, 1999; Abbott *et al.*, 1999; Oda *et al.*, 1999; Swann and Parrington, 1999; Jones *et al.*, 2000; Dong *et al.*, 2000; Parrington *et al.*, 2000; Rice *et al.*, 2000; Stricker *et al.*, 2000; Tang *et al.*, 2000; Lee *et al.*, 2001; Li *et al.*, 2001; Ogonuki *et al.*, 2001; Parrington *et al.*, 2001; Cox *et al.*, 2002; Runft *et al.*, 2002; Saunders *et al.*, 2002; Knott *et al.*, 2003; Kim and Gye, 2003). In all these cases, injection of the sperm extract resulted in a pattern of Ca^{2+} release typically seen at fertilization (Swann and Ozil, 1994; Kyojuka *et al.*, 1998).

Two principal candidates were initially suggested as being the “sperm factor”. The first was a 33 kDa hamster sperm protein called oscillin (Parrington *et al.*, 1996). The second candidate was a truncated form of the c-kit receptor called tr-kit; this receptor was isolated in mouse (Albanesi *et al.*, 1996). Both of these candidates, however, exhibited certain limitations that proved that they could not be the “sperm factor”. Recombinant oscillin protein failed to cause Ca^{2+} oscillations in mouse eggs when injected (Shevchenko *et al.*, 1998; Wolosker *et al.*, 1998). Furthermore, tr-kit was located on the mid-piece of the

sperm; a “sperm factor” should theoretically be localised immediately behind the acrosome in the sperm head (Sette *et al.*, 1997)

In view of these findings, the most likely candidate responsible for egg activation in this model is a sperm-specific PLC (Parrington, 2001; Swann *et al.*, 2001). Several experiments, using a series of combinations of intact mouse eggs and a cell free sea urchin egg homogenate Ca^{2+} bioassay, have clearly demonstrated that the sperm factor mediated Ca^{2+} release via the IP_3 receptor (Parrington, 2001; Swann *et al.*, 2001), and was thus most likely to be a PLC of some description.

These findings suggest the intervention of a discrete PLC component (Jones *et al.*, 1998a, 2000). In other words, the sperm itself contains a PLC rather than being a protein which activates a PLC inside the egg (Jones *et al.*, 1998a; Rice *et al.*, 2000). The ability of the sperm factor to trigger Ca^{2+} oscillations was clearly tissue specific, because soluble extracts prepared from tissues other than sperm have consistently failed to cause any Ca^{2+} oscillations when injected into eggs (Swann, 1990; Stricker, 1997; Wu *et al.*, 1997; Jones *et al.*, 2000).

It thus appeared that the sperm introduce a specific PLC, which hydrolyses intracellular PIP_2 to produce IP_3 , which then binds to the IP_3 receptor located on the membrane of the endoplasmic reticulum, resulting in the release of Ca^{2+} from the endoplasmic reticulum (Miyasaki *et al.*, 1993; Swann and Ozil, 1994; Jones *et al.*, 1998a, b; Stricker *et al.*, 1998; Stricker, 1999; Jones *et al.*, 2000; Rice *et al.*, 2000; Parrington 2001; Swann *et al.*, 2001; Saunders *et al.*, 2002; Coward *et al.*, 2003; Howell *et al.*, 2003; Swann *et al.*, 2004) as shown in Figure 6.1C.

6.1.4 Sperm factor research; discovery of a candidate molecule

Prior to 2002, there were 11 different PLC isoforms: PLC β 1-4, PLC γ 1-2, PLC δ 1-4, and PLC ϵ (Dupont *et al.*, 1996; Katan *et al.*, 1998; Kurokawa *et al.*, 2004). However, chromatographic evaluation proved beyond doubt that none of these subtypes could have been the “sperm factor” (Wu *et al.*, 2001). Furthermore, the injection of purified or recombinant PLC β , PLC γ 1, γ 2 or PLC δ 1 proteins have failed to trigger Ca²⁺ release in the sea urchin egg homogenate, or when microinjected into mouse eggs (Jones *et al.*, 2000; Saunders *et al.*, 2002) and ascidians egg (Runft and Jaffe, 2000).

In 2002, a novel mouse PLC was isolated and described by Saunders *et al.* (2002). This new PLC was an entirely new isoform and was named PLC ζ . This novel PLC subtype exhibited all of the described properties that would be characteristic of the sperm factor. PLC ζ was 1941 bp in length and its protein sequence consisted of 647 amino acids with a molecular mass of 74 KDa. PLC ζ is the smallest of the entire PLC family and contains a series of domains characteristic of PLCs: X and Y domains, which are responsible for catalytic activity, an EF hand domain that binds to Ca²⁺, and a C2 domain which binds Ca²⁺ or phospholipids. Curiously and in contrast to many other PLCs, PLC ζ does not have a PH domain. These domains bind to polyphosphoinositides such as PIP₂ or other proteins (Katan *et al.*, 1998). However, this obviously does not compromise the ability of PLC ζ to bind to PIP₂ in the egg; it remains unknown at present how this mechanism occurs in the absence of a PH domain.

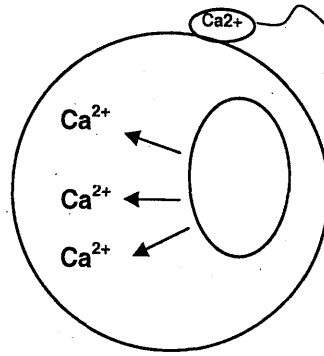
To confirm that the novel PLC ζ was indeed the “sperm factor”, Saunders *et al.* (2002) injected complementary RNA (cRNA) prepared from PLC ζ into mouse eggs and this indeed triggered Ca²⁺ oscillations, identical to these seen at fertilization. However, the injection of cRNA from PLC δ 1, which also lacks a PH domain, failed to trigger any Ca²⁺ oscillations in the egg. The injection of the PLC ζ cRNA into mouse eggs resulted in further egg development, in which the two cell stage was reached by two days post-injection.

Morula and blastocyst stages were reached after 4 to 5 days post-injection (Saunders *et al.*, 2002).

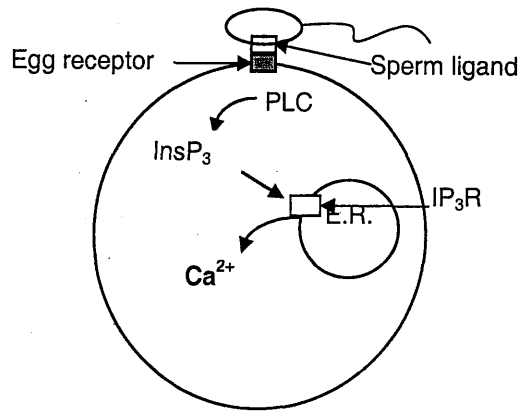
The precise amount of “sperm factor” required to trigger Ca^{2+} oscillations in an egg was previously suggested to be in the range of one to ten sperm (Swann, 1990; Wu *et al.*, 1997). However, after densitometry evaluation, Saunders *et al.* (2002) reported that a single mouse sperm contains 20 – 50 fg of PLC ζ protein. The minimum level required to produce Ca^{2+} oscillations in the egg similar to normal fertilization is around 4 – 75 fg, of protein. This work suggested that a single sperm is indeed sufficient to trigger Ca^{2+} oscillations similar to those reported at fertilization (Saunders *et al.*, 2002; Rice *et al.*, 2000). Therefore, these findings suggested that the novel PLC ζ is by far the most attractive candidate to be the long sought-after “sperm factor” (Saunders *et al.*, 2002; Cox *et al.*, 2002; Kurokawa *et al.*, 2004, Yoda *et al.*, 2004).

Recently, Cox *et al.* (2002) reported the isolation and identification of human and simian (monkey) homologues of PLC ζ . These sequences represent 608 amino acids and 641 amino acids for the human and simian PLC ζ respectively. Molecular weights are therefore 70 KDa for the human and 74 kDa for the simian. Both of these proteins have a similar molecular weight of that already reported for the mouse (74 KDa) (Saunders *et al.*, 2002). Injections of human and simian PLC ζ cRNA also produce Ca^{2+} oscillations in mouse eggs (Cox *et al.*, 2002). These oscillations were similar to those ones already reported for the mouse PLC ζ (Saunders *et al.*, 2002). In a similar way, the injection of human PLC ζ triggered cell division in the mouse egg; injected concentrations of 20, 2.0 and 0.2 $\mu\text{g ml}$ of human PLC were all successful. Cell division was achieved in all three concentrations, two-cell division was observed 24 hours post-injection. After that, morula and blastocyst stages were observed after 96 hours, but only in those mouse eggs injected with 2.0 and 0.2 $\mu\text{g ml}$ of human PLC ζ respectively (Cox *et al.*, 2002).

A



B



C

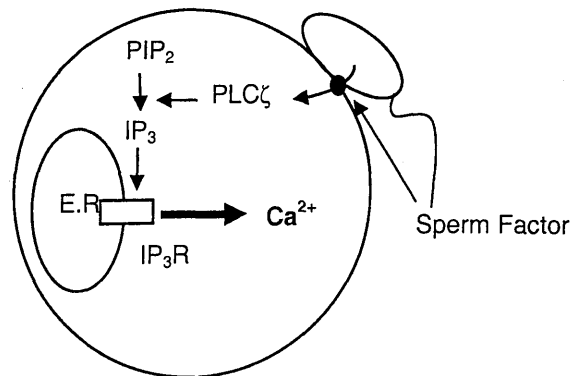


Figure 6.1 The three models of egg activation: (A) Ca^{2+} Bomb” or “conduit model”, (B) “membrane receptor” or “contact model” and, (C) “sperm factor” or “content model”. Ca^{2+} , calcium ion; PLC, phospholipase C or ζ ; PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_3 , inositol 1,4,5-trisphosphate, E.R., Endoplasmic reticulum; IP_3R , IP_3 receptor (modified from Swann, 1990; Parrington *et al.*, 2000; Parrington, 2001; Coward *et al.*, 2002)

Egg activation in mammals at least is triggered by a sperm-specific protein factor which we now know to be PLC ζ . It is presently unknown as to whether PLC ζ , or homologues to PLC ζ , exist in non-mammals. Investigations suggested that in ascidians, egg activation could indeed be triggered by a sperm factor, although in this case, the “sperm factor” is thought to activate an internal egg PLC. In the ascidian model, Ca²⁺ oscillations caused by normal fertilization and injection of ascidian sperm extracts are inhibited by injecting the SH2 domain of PLC γ (Runft and Jaffe, 2000), suggesting the involvement of an egg PLC γ .

A “sperm factor” might be involved in the activation of ascidian eggs, although only via an additional internal egg PLC. Similar mechanisms involving “sperm factors” might be operating in other non-mammalian-species. The injection of sperm extracts prepared from many different organisms often has the same effects when injected into the same kind of egg, mouse eggs for example. The sperm factor is not species-specific, because the injection of sperm extracts from different organisms such as hamster, human, boar, mouse, pig, cow, monkey, frog, chicken, ascidian, and even a species of flowering plant, can trigger Ca²⁺ oscillations when injected into mouse eggs. This might suggest that the sperm factor is a conserved protein across different species, or at least these organism share similar mechanisms and molecules that trigger Ca²⁺ release in the egg (Parrington, 2001). Some schools of thought believe that PLC ζ , or something very similar, will be present in the sperm of all sexually-reproducing organisms. The “sperm factor”, which triggers the initial Ca²⁺ release may be the same in all species; however the molecule responsible for subsequent Ca²⁺ oscillations might be a different PLC isoform (Whitaker and Swann, 1993). Sperm extracts from marine worms were highly effective in the hydrolysis of PIP₂ and are able to trigger Ca²⁺ release from intracellular stores; this was the first real evidence

that the sperm factor mechanism in invertebrates might be similar to that reported for mammals (Howell *et al.*, 2003).

The aim of this chapter was to analyse sperm extracts prepared from a commercially-important, model species of fish (the Nile tilapia), and three other commercially-important species, and investigate these extracts for their ability to release Ca^{2+} in an established bioassay, and upon injection into a living mouse oocyte. Further analyses involve an investigation of whether fish sperm extracts exhibit PLC activity. Findings when related to mammalian data, will provide insight into the precise mechanisms operating at fertilization in fish eggs.

6.2 Materials and methods

6.2.1 Fish supply

Tilapia (*Oreochromis niloticus*) and catfish (*Clarias gariepinus*) were obtained from the Tropical Aquarium (Institute of Aquaculture) rainbow trout (*Oncorhynchus mykiss*) were obtained from the “Niall Bromage” Freshwater Research Facility of the Institute of Aquaculture, University of Stirling. Halibut (*Hippoglossus hippoglossus*) samples were kindly provided by Dr. Carlos Mazorra de Quero at SEAFISH, Marine Farming Unit, Ardtoe, Argyll, Scotland.

6.2.2 Preparation of cytosolic sperm extracts

Fresh milt was collected from tilapia (n = 30, 8 ml in total), rainbow trout (n = 15, 15 ml in total), halibut (n = 2, 25 ml in total) and catfish (n = 1, 10 ml in total). Fish were anaesthetized with a solution of 100 g/l of ethyl 4-aminobenzoate dissolved in ethanol (working concentration in water 100 mg/l, with a final concentration of 1:10,000) in order to facilitate sperm collection and avoid stress. Milt was extracted by gentle abdominal pressure. Milt samples were collected with glass capillary tubes and assessed under a light microscope to detect urine contamination (for tilapia only). In tilapias, the collection of clean milt is very difficult. To get a clean sample, the bladder must be emptied before sample collection and milt must be assessed for sperm motility before use. Clean uncontaminated milt was collected in 1.5 ml Eppendorf tubes, (in the case of rainbow trout, and for halibut, a clean 50 ml Sterilin container was used) and kept on ice until further preparation. Milt samples were first washed in PBS (Phosphate Buffered Saline) pH 7.4, and then centrifuged at 800 g for 10 min. The resultant sperm pellet was resuspended and washed three times in PBS; in the final wash, 0.2 mM of PMSF (phenylmethylsulphonyl fluoride) was added to the PBS.

Next, the pellet was resuspended in 120 mM KCl (potassium chloride) 20 mM Hepes (N-[2-hydroxyethyl] piperazine-N¹[2-ethanesulfonic acid]) solution containing an excess of chelex 100 (to remove contaminating calcium). Samples were then mixed and transferred to a 1 ml cryovial. Samples were then carefully submerged in liquid nitrogen (N₂). Frozen samples were then thawed at room temperature, and the freeze-thaw process repeated 3 or 4 times in order to produce a sperm cell lysate. Sperm lysate was transferred to an ultracentrifuge tube and centrifuged at 100,000 g for 1 hour at 4°C. After that, supernatant was transferred to a centrifugal filter unit (Centricon®) and then micro-concentration device (Microcon®, Millipore, Corporation) to concentrate the samples to approximately 50 µg/µl. The centrifugal filter units were used according to the user guide. Sperm extracts were stored at -80°C until further analysis.

6.2.3 Protein concentration

Protein concentrations were measured using a BCA assay (Pierce Chemical Co., St Louis, MO, USA) with BSA standards.

6.2.4 The sea urchin egg homogenate bioassay of calcium release

Concentrated fish sperm extracts were first tested for sperm factor activity (which is the ability to cause Ca²⁺ release) in an established cell-free Ca²⁺ bioassay, the sea urchin egg homogenate. This assay preserves the structure of the sea urchin eggs' membrane bound Ca²⁺ stores and utilizes a fluorescent dye (Fluo-3), which in association with a fluorimeter, allows the detection of Ca²⁺ release from internal egg stores upon the addition of test substances.

The sea urchin homogenate system is well characterized and has been used extensively to study Ca²⁺ release patterns in eggs. Briefly, homogenates (2.5%) of

unfertilized sea urchin (*Lytechinus pictus*) eggs (Marinus, Inc., Long Beach, California, USA) were prepared using 250 mM potassium gluconate; 250 mM mannitol; 20 mM HEPES pH 7.2; 1 mM adenosine triphosphate (ATP); 10 mM phosphocreatine; 10 IU/ml creatine phosphokinase; 1 $\mu\text{g}/\text{ml}$ oligomycin; 1 mM sodium azide, and 3 mM fluo-3. The intracellular medium was treated with iminodiacetic acid chelating resin (1% v/v) to remove heavy metal contamination before the addition of 1 mM magnesium chloride (MgCl_2) (Galione *et al.*, 1997; Rice *et al.*, 2000). Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 and 535 nm using a Perkin-Elmer LS-50B fluorimeter. Fish extracts were injected at 17°C using 500 μl of sea urchin homogenate containing the appropriate amount of Fluo-3.

The homogenate system was first tested by injecting 2 μM inositol 1,4,5-triphosphate (IP_3). An additional control test involved the injection of 3 μl (~ 50 $\mu\text{g}/\mu\text{l}$) of boar sperm extract, which was previously shown to be extremely effective in inducing Ca^{2+} release in the sea urchin homogenate (Rice *et al.*, 2000). Boar sperm extract was prepared as described previously, using the same method used for the fish sperm extracts (Rice, *et al.*, 2000). Once we had confirmed that the homogenate was responding to positive controls (IP_3 and boar sperm extract) in an appropriate manner, the two tilapia sperm extracts (Extract No. 1 and Extract No. 2) could be tested.

In each experiment, 3 μl (40–70 $\mu\text{g}/\mu\text{l}$) of tilapia sperm extract could be injected directly into the sea urchin egg homogenate and monitored for a total time period of 500–800 sec. Then the concentrated tilapia sperm extracts were tested for PLC activity. Concentrated Nile tilapia sperm extract (2 μl , 40–70 $\mu\text{g}/\mu\text{l}$) was first incubated with 2 μl of 1 mM phosphatidyl inositol 4,5-bisphosphate (PIP_2) at room temperature for 1 minute. The cocktail was then injected into the sea urchin egg homogenate bioassay system (Jones *et al.*, 1998a; Rice *et al.*, 2000). Ca^{2+} levels were controlled in this assay by use of

Ca²⁺/EGTA buffers (Jones *et al.*, 1998a) such that levels remained at 160 mM. IP₃ generated via the hydrolysis of PIP₂ by a potential cytosolic factor in the fish sperm extract would thus induce the release of Ca²⁺ from internal egg stores, which could be readily assayed using the Ca²⁺ sensitive Fluo-3 dye present in the homogenate.

6.2.5 Microinjection of tilapia sperm extract into mouse oocytes and imaging of intracellular egg Ca²⁺

The ability of concentrated Nile tilapia sperm extract to cause Ca²⁺ release in a living mammalian oocyte was then investigated by micro-injecting small volumes of fish sperm extract directly into isolated living mouse oocytes and visualizing resultant changes in intracellular egg Ca²⁺ using laser confocal microscopy. In brief, female MF1 mice were super-ovulated by an injection of five international units (IU) of pregnant mares serum gonadotropin (PMSG; Intervet). This was followed by an injection of human chorionic gonadotropin (HCG; Intervet) 48 h later. Eggs were collected after a further 13.5–14.5 h, as previously described by Lawrence *et al* (1997), and maintained in 100 µl droplets of HEPES-buffered KSOM media under mineral oil at 37°C. Intracellular changes in egg Ca²⁺ were measured with Fura red-AM (Molecular Probes), and oocytes were injected with tilapia sperm extract (Extract No. 1, 40 µg/µl) as previously described (Saunders *et al.*, 2002 and Swann, 1990). The volume injected (1–3% of egg volume) was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. Control experiments involved the injection of a HEPES-buffered sperm-extract vehicle buffer (120 mM KCl, 20 mM HEPES).

6.2.6 Analysis of sperm extracts prepared from four fish species of high commercial value using the sea urchin egg homogenate bioassay

The sea urchin egg homogenate was used to test sperm extracts prepared from four different species of teleost fish (tilapia, rainbow trout, Atlantic halibut and African catfish) for calcium release properties. The methodology used in this investigation was the same as that detailed earlier (see 6.5.4.)

6.2.7 Use of PCR in an attempt to isolate a tilapiine homologue of mammalian PLC ζ

In order to isolate a tilapia PLC ζ homologue, a PCR (Polymerase Chain Reaction) approach was carried out, using a set of degenerative primers. The primer sequence was 5'-CCA GAG GCA CTA AAA TTC AAA ATA TTA GT - 3' (forward) and 5'-GTA TAA ATG ACA AGA TCA GAT AAG GCC A -3' (reverse). This set of primers was designed using the most conservative regions of the mouse, human and monkey PLC ζ (accession numbers AF435950, AF 532185 and AB 070108 respectively).

The PCR reaction mixture contained 1 μ l of 1.5 mM MgCl₂, 0.1 μ l Taq DNA Polymerase, 2.5 μ l 10X reaction buffer, 1 μ l of 10 mM dNTPs, 0.5 μ l each primer, 18 μ l water and 1 μ l tilapia testis cDNA as a template. PCR conditions were 94°C for 3 minutes for denaturation, 35 cycles of 94°C for 30 seconds, 50°C 30 seconds and 72°C for 90 seconds, then one cycle of 72°C for 5 minutes. When PCR was completed, samples were run on a 1% agarose gel electrophoresis.

6.2.8 Use of a tilapia BAC library screen to isolate a homologue of mammalian PLC ζ

Using the same set of degenerative primers above described, a PCR reaction mixture contained 1 μ l of 1.5 mM MgCl₂, 0.1 μ l Taq DNA Polymerase, 2.5 μ l 10X reaction buffer, 1 μ l of 10 mM dNTPs, 0.5 μ l each primer, 18 μ l water and 1 μ l mouse DNA as a template.

PCR conditions were 94°C for 3 minutes for denaturation, 35 cycles of 94°C for 30 seconds, 50°C 30 seconds and 72°C for 90 seconds, then one cycle of 72°C for 5 minutes.

When PCR was completed, samples were run on a 1% agarose gel. After a desired PCR product was obtained (2.2 kb for a full length PLC ζ probe), PCR samples were purified and the resultant DNA used as a probe to screen a tilapia Bacterial Artificial Chromosome (BAC) library.

6.2.8.1 Screening of tilapia BAC library

Tilapia BAC library was generated by Katagiri *et al.* (2001). Then a non-radioactive chemiluminescence method (DIG DNA labelling kit, Roche) was used. The 2.2 kb probe (mouse PLC ζ probe) was labelled with biotin and was denatured at 94°C for 5 minutes followed by a rapid cooling on ice, prior to use in the hybridization with BAC library membranes as follows

Briefly, BAC library membranes were initially washed twice with 0.1% sodium diethyl sulphate, (SDS) at 84°C in a hot water bath before the hybridization procedure. The SDS was discarded and membranes left at room temperature until used. Membranes were pre-hybridised with hybridization buffer for 30 minutes at 55°C. Then labelled probes was then added and allowed to hybridize overnight with the BAC library membranes at 45°C.

The next day, membranes were washed twice at 45°C for 15 minutes (2X sodium saline citrate (SSC), 0.1 % SDS). After that, the membranes were rinsed with washing buffer (0.1 M maleic acid, 0.15M NaCl₂, pH 7.5 and 0.3% (v/v) Tween 20) and then incubated for 30 minutes in 100 ml blocking solution (5X working solution was prepared by diluting the 10X blocking solution 1:1 in maleic acid buffer) at room temperature in a rotary mixer. Then 20 ml of streptavidin solution was added and incubated for 30 minutes. Membranes were then washed twice with washing buffer for 15 minutes. Membranes were then equilibrated with 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5).

Membranes were placed with the DNA facing up in a plastic bag and 1 ml dioxetane (CSPD) ready-to-use was added. The membrane was then covered and the CSPD spread over the whole membrane and incubated for 5 minutes. Excess liquid was then removed out with a plastic pipette, the plastic bag sealed, and the membranes incubated for 10 minutes at 37°C to enhance the luminescent reaction. Finally an X-ray film was exposed for 30 minutes to the membranes and the film developed. The film was analysed and positive clones picked from the library plates and cultured in LB broth medium previously treated with chloramphenicol (12.5 µg/ml) to allow preparation of plasmid DNA.

Tilapia BAC plasmid DNA was then used as a template in a PCR using the same degenerative primers and PCR conditions as Seccion 6.2.8. When PCR was completed, samples were run on a 1% agarose gel. After a ~650 – 700 bp PCR product was obtained, PCR samples were then purified, cloned and sequenced.

6.2.8.2 Plasmid preparation

Plasmid DNA was prepared using a QIAGEN plasmid mini kit (QIAGEN Ltd. West Sussex, UK) After overnight culture, the culture media was spun for 10 minutes at 10,000 g at room temperature. The bacteria pellet was resuspended in 0.3 ml of P1 buffer (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). Then, 0.3 ml of buffer P2 (200 mM NaOH, 1% SDS) was added followed by 0.3 ml of chilled buffer P3 (3.0 M potassium acetate, pH 5.5). Samples were mixed gently and cell debris removed by centrifugation at maximum speed for 10 minutes. The supernatant was then transferred to a fresh Eppendorf tube, and samples added to a previously equilibrated with 1 ml of buffer GBT (750 mM NaCl; 50 mM MOPS, pH 7.0) QIAGEN-tip 20. After that, samples were washed 4 times with 1 ml of buffer QC (1.0 M NaCl; 50 mM Tris-Cl, pH 7.0) and the DNA eluted with 0.8 ml of buffer QF (1.25 M NaCl; 50 mM Tris-Cl, pH 8.5) DNA was then precipitated by adding isopropanol (15% of the original QF volume). Finally, samples were centrifuged at

10,000 rpm for 30 minutes, the supernatant decanted, and the DNA pellet washed with 70% ethanol. The DNA was left to dry for 5 to 10 minutes and then eluted in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). DNA samples were left overnight at 4°C to enhance DNA elution. DNA concentration was estimated by optical density comparing the ratio at 260 and 280 nm using a spectrophotometer. Then DNA concentration was double checked by agarose gel electrophoresis (1%).

6.2.8.3 DNA sequencing

DNA sequencing was carried out using the ABI Prism Dye-Deoxy terminator sequencing kit (Perkin Elmer). Samples were run and analysed using an Applied Biosystems DNA Automated Sequencer (Model 377, Applied Biosystems, Foster City, CA). The resultant sequences were analysed and aligned using a BLAST search from the National Centre for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>).

6.3 Results

6.3.1 Validation of the sea urchin egg homogenate bioassay

The sea urchin egg homogenate bioassay was successfully generated and reacted to test samples in an appropriate manner. This bioassay has been used in many different investigations and has been validated on several occasions (Galione *et al.*, 1997; Jones *et al.*, 1998a, 2000; Parrington *et al.*, 1999; Parrington, 2001). In order to verify that the bioassay was working properly, the egg homogenate was first tested with boar sperm extracts and IP₃ injection. The sea urchin egg homogenate responded to injections of 3 μ l of boar sperm extract (50 μ g/ μ l of protein in a predictable manner). Injection of this sperm extract resulted in significant Ca²⁺ release within the bioassay as shown in Figure 6.2. Injection of IP₃ also caused similar Ca²⁺ release (data not shown). These results clearly demonstrated that the sea urchin egg homogenate was working properly and it was ready to be used with the experimental samples.

6.3.2 Sperm extracts from Nile tilapia induce Ca²⁺ release in the sea urchin egg homogenate bioassay

Cytosolic tilapia extracts were successfully prepared; samples were rich in protein (protein content ranged between 40 to 70 μ g/ μ l). Sperm extracts produced a remarkable release of intracellular Ca²⁺ when injected into the bioassay (Figure 6.3 A-B). The amount of calcium within the bioassay increased more than two fold just seconds after the injection of tilapia extracts and a peak was reached 50 or 60 seconds post-injection. This suggests that a sperm factor is present in the sperm of tilapia. This pattern of calcium release was highly consistent with those previously reported for other mammalian and worm species using the same bioassay (Galione *et al.*, 1997; Jones *et al.*, 1998a, 2000; Parrington *et al.*, 1999; Parrington, 2001; Rice *et al.*, 2000).

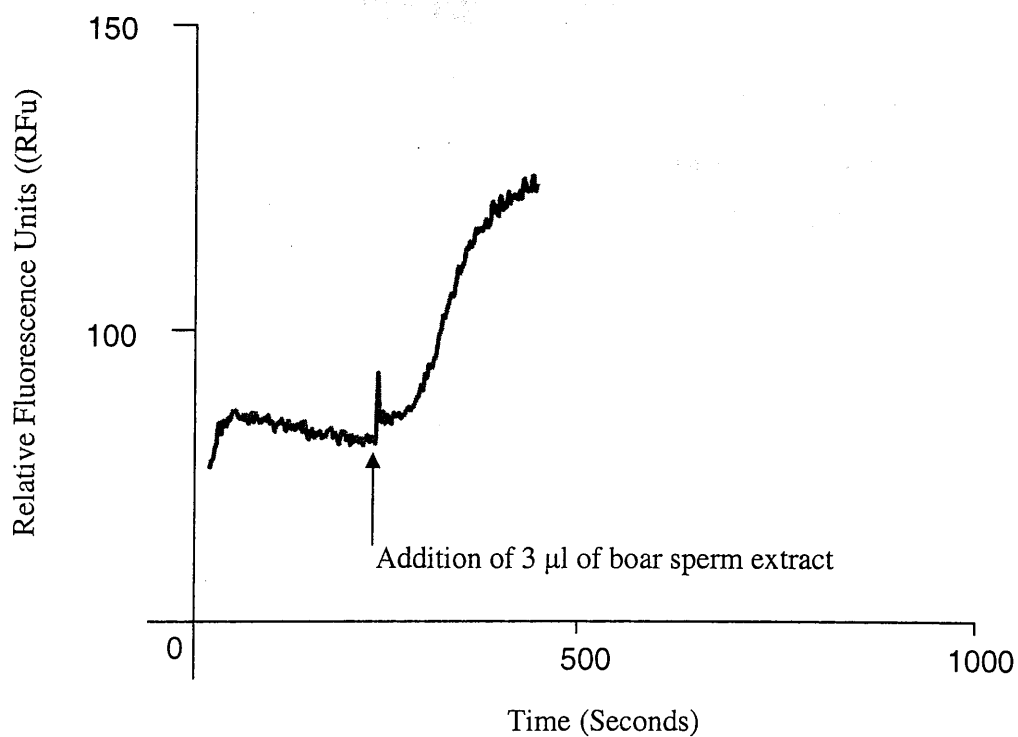


Figure 6.2 Calcium release in the sea urchin egg homogenate when boar sperm extract was injected. Arrow indicates injection time (From Coward *et al.*, 2003). (Injections were carried out by Dr. Kevin Coward).

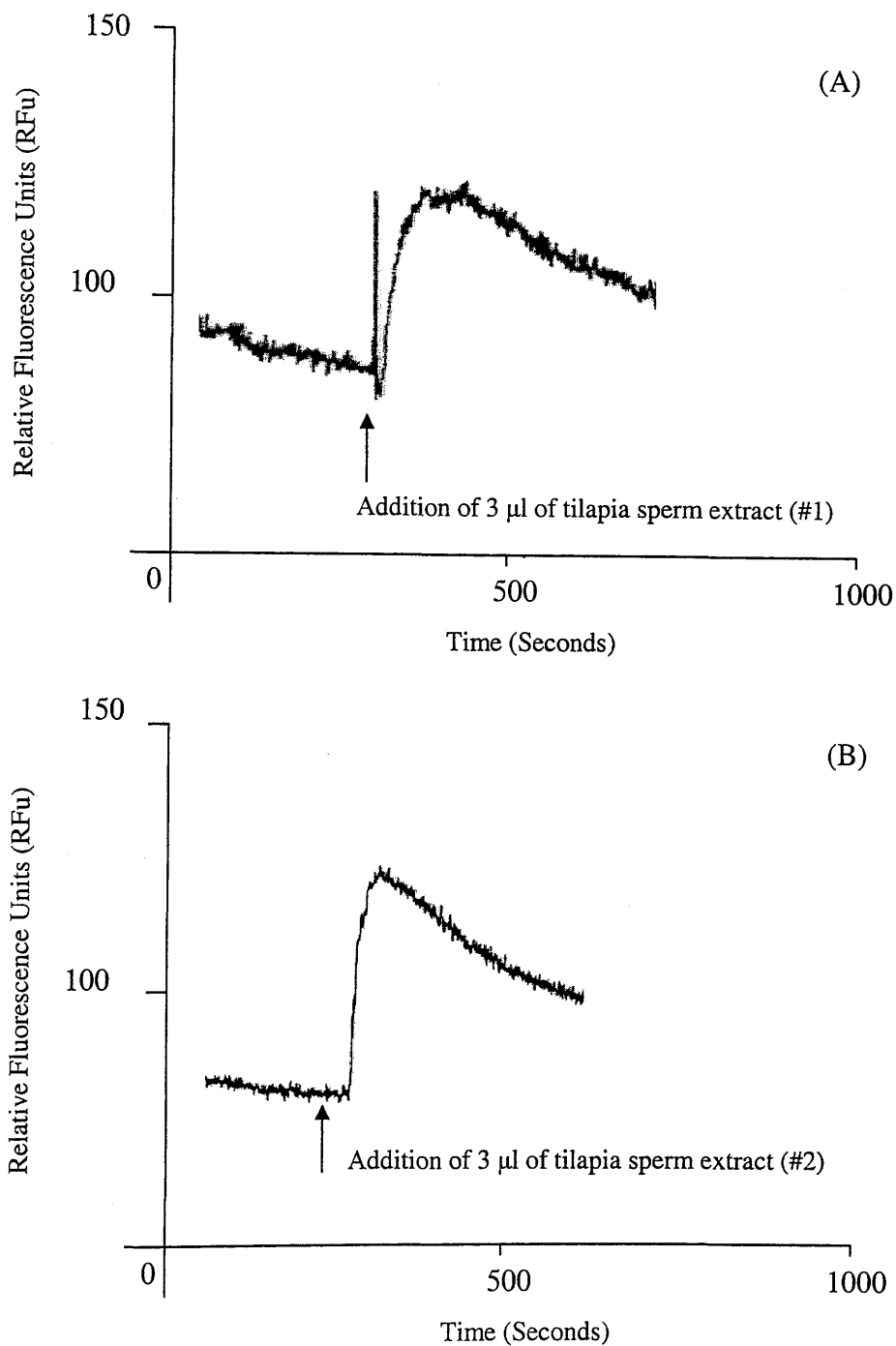


Figure 6.3 Calcium release in the sea urchin egg homogenate when tilapia sperm extract was injected. A) Sperm extract No. 1 B) Sperm extract No. 2. Arrows indicate injection time (From Coward *et al.*, 2003). (Injections were carried out by Dr. Kevin Coward).

6.3.3 Identification of PLC activity in sperm extracts from the Nile tilapia

Further utilization of the sea urchin egg homogenate bioassay allowed us to determine that Nile tilapia sperm extracts exhibit characteristic PLC activity. Tilapia sperm extract was able to hydrolyse PIP_2 and thus create IP_3 which caused release of calcium from the sea urchin intracellular Ca^{2+} stores when added to the sea urchin egg homogenate. Incubation of the tilapia sperm extracts with PIP_2 resulted in the generation of large amounts of IP_3 , which when added to the homogenate resulted in very high Ca^{2+} release (within 25 seconds of injection). The amount of Ca^{2+} released in the bioassay was two fold higher than the initial concentration, as clearly shown in Figure 6.4.

6.3.4 Intracellular Ca^{2+} oscillations caused by tilapia sperm extracts when injected into mouse oocytes

Injection of tilapia sperm extracts into living mouse oocytes ($n = 3$) caused an initial large Ca^{2+} transient, followed by a series of Ca^{2+} oscillations (4 – 5) within the egg (Figure 6.5). Free Ca^{2+} levels within the oocytes approximately doubled within 10 - 20 seconds post-injection. The initial transient lasted 150 - 200 seconds and was followed by 4 – 5 subsequent oscillations (with lower duration of 20 – 30 seconds). The initial calcium transient was higher in amplitude, and longer in duration than the subsequent oscillation. The frequency of subsequent Ca^{2+} oscillation declined with time. A similar pattern was observed in all of the injected egg ($n = 3$). Figure 6.6 shows that a control injection of 120 mM KCl and 20 mM HEPES was not able to induce Ca^{2+} release in the mouse oocyte.

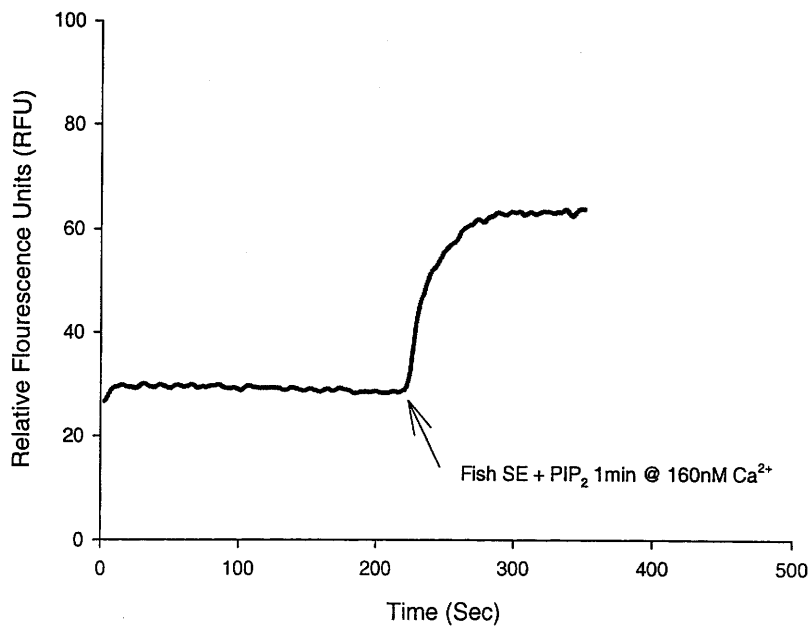


Figure 6.4 Confirmation of PLC activity in tilapia sperm extract. Incubation of fish sperm extracts with PIP₂ for one minute at low concentrations of calcium, allowed the production of large amounts of IP₃, which then resulted in a dramatic release of Ca²⁺ when added to the sea urchin egg homogenate bioassay (From Coward *et al.*, 2003). (Injection was carried out by Dr. Kevin Coward).

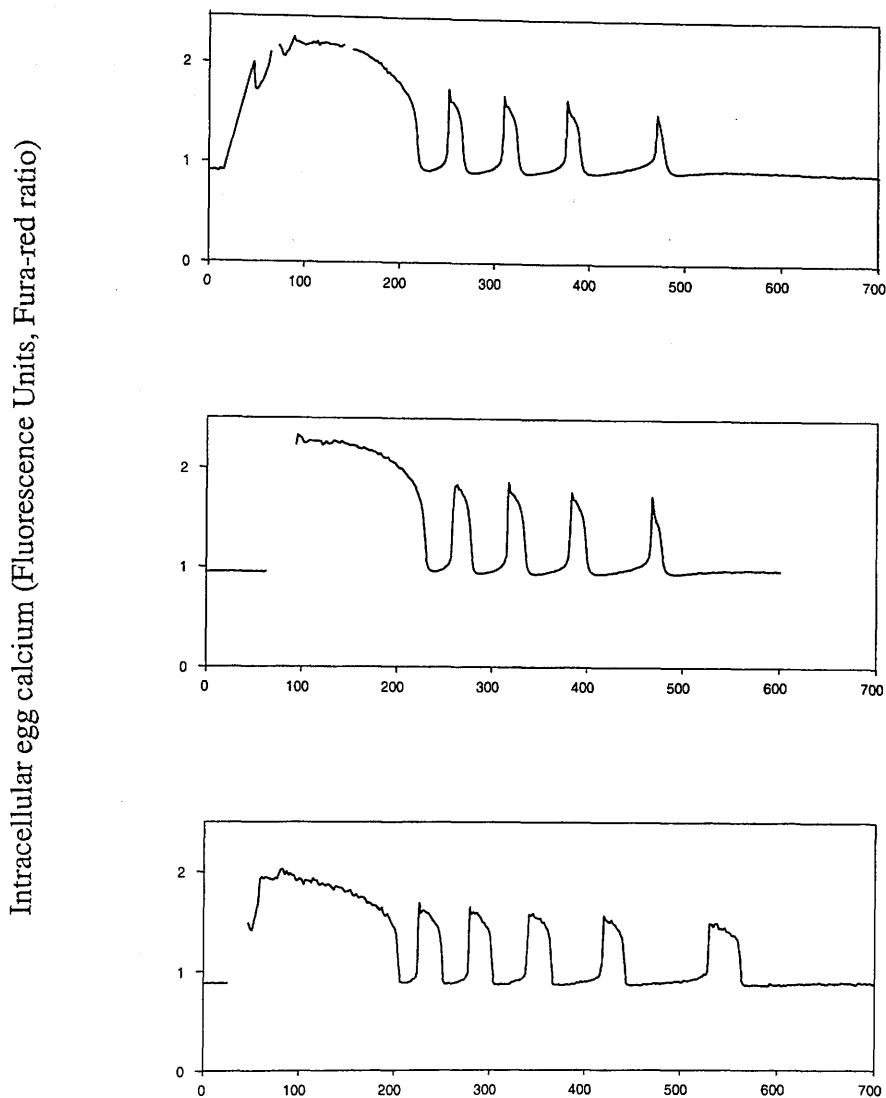


Figure 6.5 Oscillations of intracellular calcium in mouse oocytes after injection of concentrated tilapia sperm extract (each trace represent a different mouse oocyte) (From Coward *et al.*, 2003). (Injections were carried out by Dr. Mark Larman).

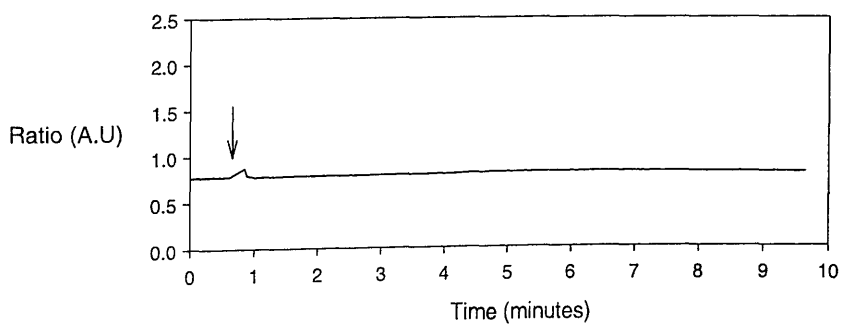


Figure 6.6 Control injection of Hepes-buffered sperm extract vehicle buffer, showing only a single artefact shortly after injection. Arrows indicate injection time (From Coward *et al.*, 2003). (Injections was carried out by Dr. Mark Larman).

6.3.5 Induced Ca^{2+} release in the sea urchin egg homogenate bioassay by sperm extracts prepared from a variety of species

The experiments described suggest the existence of a molecule similar to the mammalian “Sperm Factor” (PLC ζ) in tilapia sperm. Evidence for this include the fact that extracts were able to release Ca^{2+} in the sea urchin egg homogenate, that a molecule present in fish sperm extract was able to convert PIP_2 to IP_3 , and also that injection of sperm extract into mouse eggs caused Ca^{2+} oscillations. To further investigate the possible presence of sperm factor activity in other farmed fish species of high importance to aquaculture, sperm extracts were prepared and assayed in an identical manner from four different species: tilapia, catfish, rainbow trout and halibut. All of these species were able to generate Ca^{2+} release in the sea urchin egg homogenate. Figure 6.7 shows the different traces generated by the injection of 200 μg of protein extract of each species. The highest amplitude was generated by tilapia extracts followed by the catfish (Figure 6.7 a - c). The lowest amplitude was found in the rainbow trout samples (Figure 6.7 b - d). All four species contain a sperm specific molecule capable of generating calcium release in the sea urchin homogenate. This is the first time that potential sperm factor activity has been reported for these four different species of fish. These species represent different modes of life and very different reproductive strategies.

6.3.6 PCR amplification and screening tilapia BAC library

After several attempts to isolate a PCR product using degenerative primers and tilapia cDNA as a template, no fragment of predicted size was obtained (300 bp) (Data not shown). A 2.2 Kb probe was generated of the full length PLC ζ in mouse (Figure 6.8). Once this PCR product was purified, it was used as a probe to screen the tilapia BAC library.

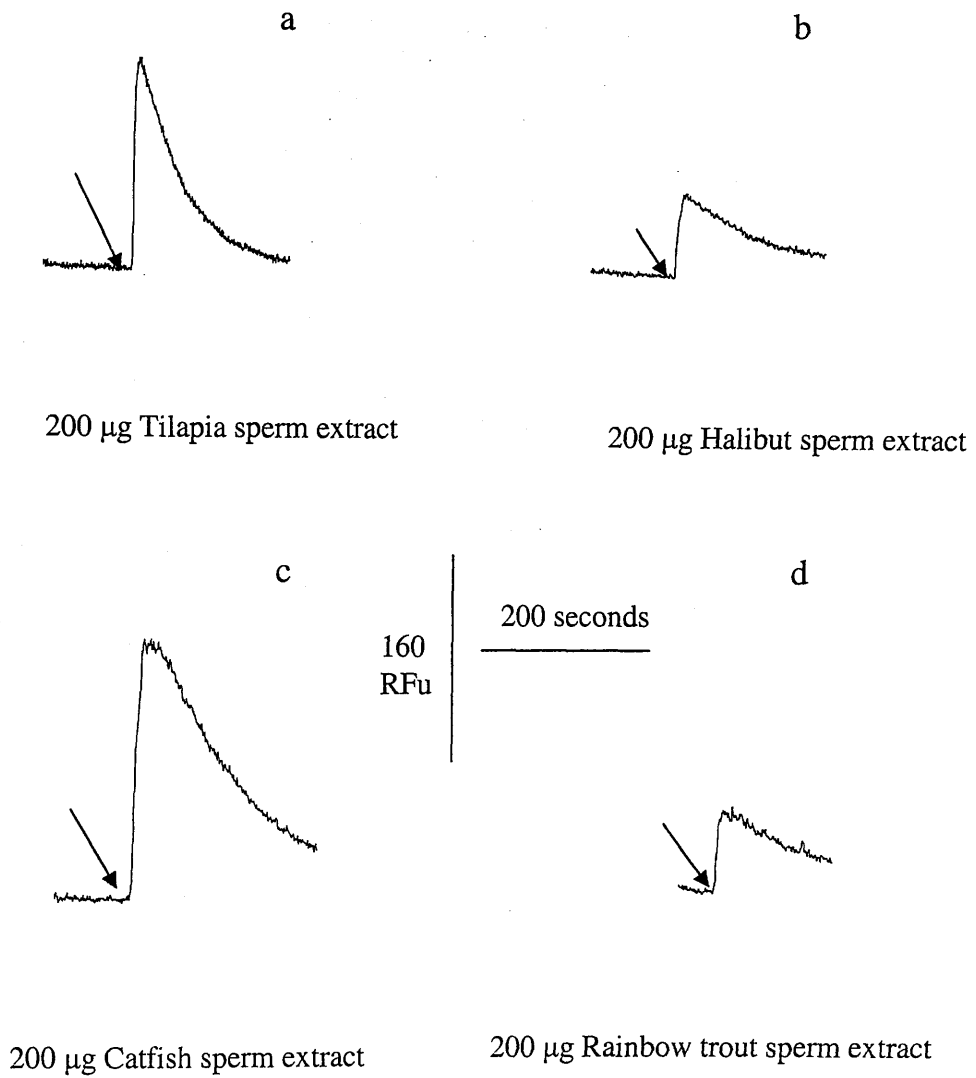


Figure 6.7 Calcium release in the sea urchin egg homogenate induced by different teleost sperm extracts: A) Tilapia, B) Halibut, C) Catfish, and D) Rainbow trout. Arrows indicate when the injections were made. (Injection was carried out by Dr. Kevin Coward).

After screening, three positive clones were found in the BAC library membranes (data not shown). These clones were picked from their respective BAC plates and then plasmid DNA prepared. The tilapia BAC plasmid was used as a template in a PCR. The PCR produced a product of around 650–700 bp. This PCR product was subcloned and sequenced. Unfortunately the sequences obtained from this investigation were not related to PLC ζ or any other signalling molecule. Indeed, sequences obtained appeared to be related only to cloning vector (data not shown). However a considerable amount of time and effort was spent in this approach and research efforts continue at the University of Oxford to attempt to isolate a fish PLC ζ homologue. Current strategies include cDNA library screening, screens of zebrafish (*Danio rerio*) and Fugu (*Fugu rubripes*) genomes and use of zebrafish testis EST (expressed sequence tags) database.

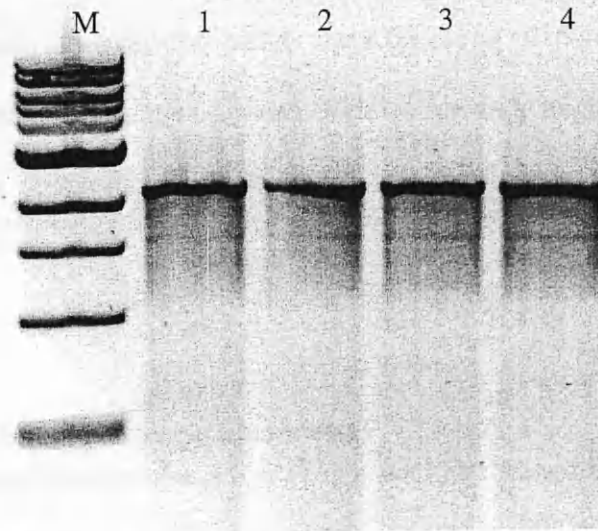


Figure 6.8 Gel electrophoresis showing the 2.2 Kb PCR product used for the tilapia BAC library screening. M = DNA ladder (1Kb). Lanes 1- 4 are mouse DNA and lane 5 is the negative control.

6.4 Discussion and conclusion

6.4.1 General overview

This investigation was a preliminary investigation of the possible mechanisms involved during egg activation in fish. Egg activation has been extensively researched in mammals. However, this investigation allowed the partial elucidation of some of the events which take part in the egg activation mechanism in tilapia and make preliminary conjectures concerning three other fish species. Currently the knowledge of egg activation is little in biology of fish, and existing research only concerns laboratory models like zebrafish and medaka. We do know, however, that Ca^{2+} appears to play a vital role in the activation of fish eggs. Calcium waves have been described at egg activation in medaka eggs (Gilkey *et al.*, 1978; Fluck *et al.*, 1991) and zebrafish eggs (Lee *et al.*, 1999; Gilland *et al.*, 1999). Injections of IP_3 , Ca^{2+} and cADPR, caused regenerative Ca^{2+} release in medaka eggs (Lee *et al.*, 1996; Fluck *et al.*, 1999). Injection of cADPR caused Ca^{2+} release in egg homogenates prepared from gilthead sea bream (*Sparus auratus*) (Polzonetti *et al.*, 2002). This investigation provided the partial characterization of potential sperm factor activity in four important aquaculture species. Further research might help to alleviate some of the problems observed in egg activation and fertilization in other species of cultured fish.

6.4.2 Sea urchin egg homogenate bioassay

The sea urchin egg homogenate bioassay is a well established bioassay which has been used to demonstrate Ca^{2+} release and PLC activity in several different organisms (Galione *et al.*, 1997; Jones *et al.*, 1998a; Parrington *et al.*, 1999; Jones *et al.*, 2000; Rice *et al.*, 2000; Saunders *et al.*, 2002). This bioassay operates at low Ca^{2+} conditions (160 nM), and provides conditions suitable for the sperm factor, but avoids the activation of other PLC subtypes (Rice *et al.*, 2000). A similar bioassay was used by Howell *et al.* (2003) to

demonstrate Ca^{2+} release produced by boar and the marine worm (*Chaetopterus pergamentacetus*) sperm extracts when injected into homogenates prepared from *C. pergamentacetus* eggs. These authors further demonstrated that a sperm PLC might be an important mediator for egg activation at fertilization in *C. pergamentacetus*.

Galione *et al.* (1997) suggested that Ca^{2+} release is activated in the sea urchin egg homogenate via two mechanisms. The first involves the IP_3 receptor mechanism and the second utilizes a mechanism involving ryanodine receptors. Galione's investigation further suggested that Ca^{2+} release is independent of the second messenger IP_3 or cyclic adenosine diphosphate ribose (cADPR) and that this might suggest that sperm extracts caused Ca^{2+} release by an enzymatic step rather than second messengers (IP_3 and cADPR). However, Rice *et al.* (2000) demonstrated that sperm extracts exhibit PLC activity, which is an important component for egg activation, and that this PLC activity regulates the production of IP_3 within the sea urchin egg homogenate.

6.4.3 Identification of PLC activity in sperm extracts from the Nile tilapia

PLC activity is a key element in egg activation. However, PLCs' involved could be present in either the egg or sperm cells (Dupont *et al.*, 1996; Carroll *et al.*, 1999; Jones *et al.*, 1998a; Parrington *et al.*, 1999; Shearer *et al.*, 1999; Ciapa and Chiri, 2000; Jones *et al.*, 2000; Rice *et al.*, 2000; Saunders *et al.*, 2002; Cox *et al.*, 2002; Howell *et al.*, 2003; Kurokawa *et al.*, 2004).

The present results demonstrate that Nile tilapia spermatozoa do indeed exhibit PLC activity, by the sperm extracts' capacity to hydrolyse PIP_2 to IP_3 and thus cause Ca^{2+} release in the sea urchin egg homogenate. The same tilapia extract was able to induce Ca^{2+} release in living mouse oocytes.

Sea urchin eggs also exhibit PLC activity (Rice *et al.*, 2000). Consequently, Ca^{2+} levels within the assay were controlled by Ca^{2+} /EGTA buffers, as previously used by Jones *et al.* (1998b). Thus, the amount of Ca^{2+} was constant in the bioassay at all times and that the concomitant increment in Ca^{2+} was caused by PLC activity within the sperm extract rather than egg PLC activity (Jones *et al.*, 1998a, 2000; Coward *et al.*, 2003.) PLC activity was confirmed to be present in tilapia sperm extract; similar results were previously reported for both mammalian and invertebrate sperm extracts (Jones *et al.*, 1998a; Howell *et al.*, 2003). Similar levels of PLC activity have been observed in sperm extracts of the marine worm *C. pergamentacetus*; this was the first demonstration of PLC activity in invertebrate sperm (Howell *et al.*, 2003).

Mammalian studies show that the ability of sperm extracts to generate Ca^{2+} release in the sea urchin homogenate is sperm-specific. This was clearly demonstrated after injection of different extracts prepared from several other tissues such as brain, liver or kidney; these extracts consistently fail to generate any Ca^{2+} release in the bioassay (Jones *et al.*, 2000).

The most recent studies of PLC subtypes demonstrate that the sperm from some mammals contains a novel PLC isoform, PLC ζ . This novel PLC ζ has been cloned in mouse, human and monkey (Saunders *et al.*, 2002; Cox *et al.*, 2002) thus far. PLC ζ was identified as the physiological trigger of egg activation and further embryonic development in mammals. However, further investigations are required in order to isolate a non-mammalian homologue of PLC ζ . Efforts are already well underway, particularly with regard to the isolation of homologue of PLC ζ , or similar molecule in fish sperm, as this will make a significant contribution to our understanding of egg activation in teleost fish, which at present, is scant at best.

6.4.4 Tilapia sperm extracts cause oscillation in intracellular Ca^{2+} when injected into mouse oocytes

Sperm extracts prepared from tilapia triggered a series of Ca^{2+} oscillations in cultured mouse oocytes. This is the first evidence to show that Ca^{2+} oscillations can be triggered in mouse eggs by a species of fish (Coward *et al.*, 2003). The pattern of these Ca^{2+} oscillations (i.e. amplitude and frequency) were similar to those previously reported when mouse eggs were injected with a variety of sperm extracts generated from boar, mouse, hamster, bovine, monkey, chicken, pig, frogs, ascidians, marine worms and even plants. Mouse eggs can be activated by sperm from several different species including hamster, rabbit, pig human and fish (Kimura *et al.*, 1998). Similar Ca^{2+} transients were observed when somatic cells (i.e. rat hepatocytes) were injected with hamster sperm extracts (Berrie *et al.*, 1996), or when porcine sperm extracts were injected into nemertean or bovine oocytes (Stricker *et al.*, 2000; Knott *et al.*, 2002). Similar results have also been observed in ascidians (Kyoizuka *et al.*, 1998; Runft and Jaffe, 2000). Jones *et al.* (2000) further demonstrated that boar sperm extract, when injected into intact mouse eggs, caused a series of six Ca^{2+} transients; these transients were observed from 600 seconds post-injection.

When injected into mouse oocytes, tilapia sperm extracts exhibited initial Ca^{2+} transients which lasted for ~ 150 - 200 seconds; these were similar to those initial Ca^{2+} transients produced by extracts of bovine, boar, chicken, frog and plant origin (Dong *et al.*, 2000; Li *et al.*, 2001). A larger initial Ca^{2+} transient was reported when hamster and porcine extracts were injected into mouse oocytes; the duration of the initial transients lasted 5-6 minutes for the hamster extract, and more than 10 minutes for the porcine extract respectively (Oda *et al.* 1999; Lee *et al.*, 2001). Nevertheless, the subsequent transients were similar in amplitude but smaller in frequency. Similar Ca^{2+} transients were observed when mouse oocytes were injected with cRNA isolated from spermatogonia cells of

mouse, hamster, human and monkey (Parrington *et al.*, 2000; Saunders *et al.*, 2002; Cox *et al.*, 2002). The injection of mRNA from hamster liver, skeletal muscle, brain and liver failed to produce Ca^{2+} transients when injected into mouse oocytes, suggesting that the ability to cause Ca^{2+} release, or Ca^{2+} transients, is found only in spermatogenic cell or testis tissue (Parrington *et al.*, 2000). Our present findings were in total accordance to those Ca^{2+} traces already reported in other studies (Oda *et al.*, 1999; Dong *et al.*, 2000; Li *et al.*, 2001) using sperm extracts prepared from other organisms.

6.4.5 Induced Ca^{2+} release in the sea urchin egg homogenate bioassay in response to the injection of sperm extracts prepared from a variety of commercially important fish

In this study, four different teleost species were tested for their ability to cause Ca^{2+} release in the sea urchin egg homogenate. Rainbow trout, halibut, and catfish also have the ability to release Ca^{2+} in the cell free bioassay, thus concurring with the tilapia results discussed earlier (Coward *et al.*, 2003). The four sperm extracts caused a rapid increment in Ca^{2+} when injected into the sea urchin egg homogenate. The pattern of Ca^{2+} release was similar to those traces previously reported for hamster, human, boar, mouse, monkey, ascidians (Swann, 1990; Parrington *et al.*, 1996; Galione *et al.*, 1997; Jones *et al.*, 1998a; Parrington *et al.*, 1998, 1999; Swann and Parrington, 1999; Jones *et al.*, 2000; Rice *et al.*, 2000; Cox *et al.*, 2002; Saunders *et al.*, 2002).

A similar mechanism of egg activation is likely to exist amongst the four teleost species investigated. The capacity to generate Ca^{2+} release in the sea urchin bioassay was similar when the four species were compared, despite the fact that these species exhibit different reproductive strategies, and have different modes of life. All four of these species share a similar pattern of egg activation, in which egg activation is likely to be triggered by a sperm-derived PLC in a manner similar to that seen in mammalian sperm. However,

more investigations are required to totally understand the precise mechanism involved in these species. PLC activity must be assayed in these species to confirm that a PLC molecule is involved. Further studies might include the use of pharmacological inhibitors to investigate whether the calcium release seen in the homogenate in response to our fish sperm extracts is due to signalling mechanisms including IP₃, cADPR, or nicotinic acid adenine dinucleotide phosphate (NAADP), or indeed whether we are dealing with an entirely new Ca²⁺ mobilising molecule.

6.4.6 PCR approach for tilapia PLC ζ isolation and tilapia BAC library screening

Intense investigation involving degenerate PCR and tilapia BAC library screening failed to isolate a homologue of PLC ζ from tilapia. Several attempts were made to isolate this novel molecule in tilapia. This investigation is still on-going and has been carried out as an on-going collaborative project with researchers at the Department of Pharmacology, University of Oxford. Present strategies include cDNA library screening, screens of zebrafish and fugu genomes and the use of zebrafish EST database.

In conclusion, tilapia sperm contains a molecule that exhibits PLC activity, is able to release Ca²⁺ in the sea urchin egg homogenate bioassay, and induces Ca²⁺ oscillations when injected into mouse oocytes. Sperm extracts from Atlantic halibut, African catfish and rainbow trout also had the ability to release Ca²⁺ in the bioassay. Collectively, these data suggest that these fish species, particularly, the tilapia, possess a molecule similar to the mammalian PLC ζ , although further pharmacological test are required to show whether the signalling mechanism involved in fish used IP₃ receptor or not. Additional attempts to elucidate a PLC ζ homologue from tilapia have failed, thus far, but research continues to address this. Isolation of a homologue of PLC ζ in fish is likely to make a significant

contribution to both general reproductive biology, and our knowledge on egg activation in fish.

7 Chapter Seven General Conclusions

This Thesis describes the effects of the photoperiod on the reproductive biology of the Nile tilapia *Oreochromis niloticus*, the melatonin profiles in these fish reared under controlled conditions under different light regimes. To fully understand the relationship between photoperiod, melatonin levels and tilapia reproduction, the molecular characterization of the melatonin receptor was described and its tissue distribution analysed. This investigation has opened up an interesting research area in which tilapia has become an important species as a research model that obviously responded to light. This is the first information on plasma melatonin levels, melatonin receptors identification and distribution and its relationship with the environmental conditions (i.e. photoperiod and light intensity) in this species. The characterization of melatonin receptors has given us an insight into the link between light perception and reproductive physiology in a tropical fish species. This study is also of practical significance for the aquaculture industry. The photoperiod results could be directly transferred to the hatchery operators and should help to increase the efficiency of seed production of this species.

Furthermore, this multidisciplinary doctoral study also contributed to understand the process of egg activation in several species in collaboration with the Department of Pharmacology, University of Oxford. Results have shown for the first time the presence of sperm factor and PLC activity in tilapias and other important species for aquaculture, such as the rainbow trout, the Atlantic halibut and the African catfish. These results support further our understanding of the egg activation mechanism in fish species and will help to improve the reproduction and performance of such species. Moreover, this investigation provides new tools that can be used in ploidy manipulation.

7.1 The effect of photoperiod on tilapia reproduction

In this study, four different light regimes were investigated, these included short (6L:18D), normal (12L:12D), and long daylength (18L:6D) and continuous illumination (24L:0D). The reproductive performance of tilapia was enhanced by light manipulations. Long daylength (18L:6D) significantly improved egg production (58%), increased fecundity and relative fecundity in fish. However, egg size was smaller than those produced by fish exposed to normal daylength (12L:12D). The egg production reported as a number of eggs per month or accumulated over the time showed that long daylength was producing a constant supply of eggs. Spawning activity was also studied, using the Inter-Spawning-Interval (ISI) as an indicator of spawning synchrony. The shortest ISI was found in fish held under long daylength, these fish spawned every two weeks, and this resulted in a constant spawning production of 15 spawns per month in this treatment (i.e. 2 spawns per fish per month). The longest ISI was obtained in fish held under normal daylength (20 days). In this study, the environmental conditions and especially temperature was constant for all treatments, thus the only variable that could have an effect on tilapia reproduction was the daylength. This study reported that fish reared under normal daylength produced significantly bigger eggs and were characterised by the longest ISI. This was suggested to be attributed to the fact that these eggs have spent more time in the gonad, increasing the duration of the vitellogenesis and also the accumulation of vitellogenin from the liver. On the other hand, eggs produced by fish held under long photoperiod remained in the gonad for a shorter period of time, which could explain why they accumulated less vitellogenin and were smaller. Thus, this is the first study to suggest that egg size is directly influenced by the ISI.

Photoperiod manipulations have been successfully applied in many important fish species for aquaculture. For example: in pink salmon, initial spawning and spermiation

activity was advanced by six months, when fish were exposed to long days (18L:6D) during March (1990) and January (1991) and short days (6L:18D) during September (1990) and April (1991) respectively (Beacham and Murray, 1993), in Masu salmon, the use of long daylength (18L:6D) or continuous illumination at the beginning of the reproductive cycle followed by a change to short daylength (6L:18D) advanced maturation and additionally one month of photoperiod manipulation was necessary to trigger maturation in this species (Takashima and Yamada, 1984), in Atlantic salmon, long daylength or continuous illumination are commonly used to arrest oocyte development and thus avoid gonadal maturation (Taranger *et al.*, 1998, 1999), in Atlantic halibut and cod the use of constant illumination was used to advance, delay or inhibit maturation depending of the time of exposure (Björnsson *et al.*, 1998). Use of constant long daylength in sea bass resulted in 2 -3 months delay of spawning activity (Carrillo *et al.*, 1989), and carp matured within 3 -4 months of exposure to long daylength (16L:8D). However maturation was delayed when fish were exposed to normal daylength (12L:12D). Additionally, the use of long daylength increased fish maturation at any time of the year (Davies, 1986), and finally, the use of extended photoperiod during the first winter in turbot resulted in a significant increment of fish growth and maturation (Imsland *et al.*, 1997)

In tilapia, Ridha *et al.*, (1999) and Ridha and Cruz (2000) reported that long daylength with strong light intensity is required to enhance tilapia seed production. However, no reliable information was reported regarding total fecundity and ISI. In Ridha's investigation, fish were reared in groups, making the assessment of individual female performance difficult. In the study, tilapia broodstock were individually tagged and stocked allowing an accurate record of all individual spawning activity and egg production. Our results are in total accordance with

Ridha and Cruz (2000), in which a greater number of eggs were obtained under long daylength.

The information generated in this study could be easily transferred to hatcheries, allowing a more reliable seed production. The use of photoperiod as a tool to enhance spawning activity may help to overcome the problems reported in some places, in which due to changes in temperature and photoperiod, the spawning activity is characterised by a marked seasonality. In tilapias, to avoid problems associated with early maturation, several methods have been described, such as hand sexing, the use of predator fish, and hormone sex reversal. However, the most successful method currently used to generate all-male populations in tilapia culture is the hormone sex reversal; this method has some limitations, such as the necessity of high numbers of same age first-feeding fry suitable to hormonal treatment. To overcome this problem, hatchery operators rely on large numbers of broodstock in order to guarantee a large supply of the same age first-feeding fry. Thus the use of photoperiod could be an effective tool to reduce the broodstock requirement by increasing individual performance to produce a desired number of fry suitable for hormonal treatment.

7.2 Plasma melatonin levels in tilapia

Plasma melatonin levels were also studied in tilapia in order to investigate how fish perceived photoperiod variation. There was variation in the shape of the melatonin profiles reported, especially when fish were reared under different light regimes. The variation encountered in these profiles was attributed to low numbers of fish were sampled at each sampling point (2 or 3 fish). However, the long daylength regime (18L:6D) was characterised by low plasma melatonin night time concentrations in comparison to the other treatments.

Although, some variation in melatonin levels had been observed, most of the profiles generated under different light regimes displayed maximum night time levels of 50-80 pg/ml with a quick increase within 1 or 2 hours after the onset of the dark period.

Overall, results have shown that melatonin levels in tilapia are low compared with other fish species and especially, salmonid species. The melatonin production in tilapia is clearly regulated by the diurnal variation of light. The patterns of melatonin production in tilapia are similar to those already reported, in which a high melatonin biosynthesis is reached just after the onset of the night period, and then levels remains constant throughout the night and finally decrease to basal levels soon after of the start of the light phase. Fish perceived light and that melatonin levels responded to that light perception. However, the involvement of melatonin in the control of tilapia reproduction is still uncertain and needs to be further investigated. Interestingly, the fish that exhibited the highest melatonin levels during the night, produced the lower fecundities, and spawned less frequently than fish that exhibited low melatonin levels (18L:6D). This may suggest that other internal factors are linked to this process. This research allowed us to partially understand the melatonin rhythmicity in tilapia, however, this study suggested that further research is needed, using a higher number of fish and also a more reliable assay.

The possible explanation of this variation of melatonin levels and tilapia reproduction could be attributed to the natural distribution of this fish. In tropical species, the onset of the reproductive season is basically triggered by changes in water temperature, water quality or may be influenced in some cases by the rainy season. In this study, we used tilapia as a research model; this is a typical tropical species. It is possible that this fish has no answer to

photoperiod variations under natural conditions because photoperiod variations in the tropics are low.

However, in this study, fish reared under short daylength (6L:18L) exhibited low reproductive performance and high melatonin levels. This may suggest that fish have experienced a very strong change in photoperiod and made them secrete more melatonin and this could be an issue in the internal regulation of reproduction. However, on the other hand, fish exposed to long daylength (18L:6D) have not shown the same effect. In this case melatonin levels were lower and their reproductive performance was far better. This may indicate that tilapias are more sensitive to short days than long days. Another possible cause was the history of this fish. Before the beginning of this experiment, fish were kept under controlled conditions of temperature and photoperiod, however, this original photoperiod was (12L:12D). This may explain why fish exposed to short daylength were more affected by photoperiod than fish exposed to long daylength (18L:6D). High melatonin levels were reported in fish exposed to short days and low melatonin were exhibited by fish reared in long daylength treatments. Thus, this high level of melatonin may have some effects on tilapia reproduction in the sense that fish have interpreted this short photoperiod as inappropriate time for reproduction.

A similar pattern in egg size was observed in which fish reared under short daylength produced bigger eggs than fish reared under long daylength. Bigger eggs will result in higher levels of fry survival. Variation in photoperiod is accompanied by variation in water productivity (phytoplankton and zooplankton). In this way under short days the productivity is lower and the opposite will occur under long days. This may suggest that tilapia exposed to

short photoperiod may have produced low numbers of fry, but these fry will have better chances of survival. However, those fish exposed to long photoperiod produced more eggs but of smaller sizes which result in small fry, however these fry are produced under long days and high quantities of food are more likely to be available.

In this Thesis the comparison was made with salmonids, because little information on photoperiod, melatonin and reproduction in tropical species is available. However, the salmonid species show a totally different reproductive strategy and habitat preferences. Salmonid species are temperate species, which present just one reproductive cycle in a year. These species are naturally distributed in temperate regions in which photoperiod changes are very strong. Thus these (salmonid) use the light information to synchronise the spawning activity (Bromage *et al.*, 2001), and the melatonin levels exhibited by this species are higher than tropical species.

A reduction in melatonin levels during the night-time, is highly efficient in reducing grilising in Atlantic salmon (Porter *et al.*, 1999), although the link between melatonin and reproductive physiology had not yet been discovered. Salmon held under night imposed illumination exhibited just 6% grilising, however, those fish reared under ambient condition presented 61.5% grilising. The use of continuous light increased the growth in these fish. This suggests that melatonin levels have to be above a certain threshold in order to initiate fish reproduction. Low melatonin levels reported by Porter *et al.* (1999) in Atlantic salmon, could be part of the signalling pathway by which reproduction had been suppressed in most of the fish. This study, using a totally different species as a research model, suggests that melatonin may not play a crucial role in the control of reproduction. Fish held under continuous illumination, with low melatonin levels (10 – 20 pg/ml) throughout the experiment and which

have exhibited a lack of rhythmic variation in melatonin levels between day and night, produced the second highest fecundity, spawn numbers and short ISI. Overall this light regime was successful enhancing reproductive activity, compared with short and normal daylength trials. These results may suggest that melatonin production in this fish was not necessary to maintain spawning activity. The melatonin levels reported for those fish held under continuous illumination were different from Porter *et al.*, (1999). There is a large variation in melatonin production between species and this variation may be related to the environmental background of each species and its evolution.

Tilapia may possess a similar mechanism controlling reproduction than that reported for the stickleback fish, which like tilapia shows a better reproductive performance on longer days. However, when melatonin was used to see whether it has any effect on its reproduction, no positive results were found. This may indicate that in the three-spined stickleback, melatonin is not playing any direct role upon fish reproduction, although, this species exhibited a great response to light stimuli (Wootton, 1973 ab, Wootton and Evans, 1976; Mayer *et al.*, 1997b, Bornestaf *et al.*, 2001).

Thus, further investigations are needed in order to elucidate the importance of melatonin in tilapia reproduction. Studies investigating the effect of constant darkness for example on spawning activity are needed in order to confirm the present results and further understand the relationship between melatonin and tilapia reproduction. This study suggests that some other molecules and genes at the pineal, retina and brain are probably involved in the transduction of light stimuli, which control or regulate reproduction levels in tilapia and other species. Also further investigations are needed in order to elucidate the relationship between melatonin, sex steroids and tilapia reproduction.

7.3 Melatonin receptor

A partial sequence of the Mella receptor was isolated in tilapia; this allowed us to further investigate the specific tissue distribution in tilapias. Mella was shown to be highly expressed in the brain, however, it was difficult to determine in which precise region of the brain this receptor was more abundant as the brain was studied as a whole. However, using the *in situ* hybridization technique in fish, several melatonin binding sites have been demonstrated. These binding sites are widely distributed in the brain, however, the highest concentration was found in the visual region located in the optic tectum, nucleus rotundus, pretectum and dorsal thalamus. Some binding sites were also found in the gustatory regions in the hypothalamus, preoptic area and cerebellum. (Davies *et al.*, 1994; Iigo *et al.*, 1994, 1997; Ekstrom and Meissl, 1997; Gaildrat *et al.*, 1998; Mazurais *et al.*, 1999, 2000; Amano *et al.*, 2003a, 2003b)

Our study also revealed that the gonad did not show any expression of Mella, similar results were found for most of the peripheral tissues. This investigation supported the results on plasma melatonin levels and together suggested that in tilapia, there is no real effect of melatonin in reproduction. There was a lack of Mella expression in gonads. The high expression of Mella in the brain may suggest that the possible link between melatonin and fish reproduction is a complex process, which may involve some other biochemical pathway in the fish brain. Additionally, Mazurais *et al.* (2000a) also reported that melatonin has no direct effect on the liver estrogen receptor (ER) and vitellogenin in rainbow trout. These authors also reported that the Mella and Mel1b receptors are not expressed in the liver.

The only study in which a melatonin receptor has been found in fish pituitary came from Gaildrat and Falcon, (2000), they reported the expression of melatonin clone 2.6 in pike pituitary gland. However, no expression was detected in the ovaries, liver and intestine.

More investigations are needed in order to physically localise expression of the melatonin receptor and some others hormones receptors such as the GnRH. The *in situ* hybridization approach may help to elucidate the exact location of these receptors and their relationship to other signalling pathways that might influence fish reproduction. Thus, a series of investigations involving both reproductive physiology experiments and molecular biology assays may help to understand in a better way the relationship between melatonin, the brain and reproduction. It may be helpful to try to demonstrate if there is any increase in melatonin receptors in the day or during the night and then extrapolate this to GnRH receptors and see whether these is a direct relationship between receptors oscillation and their abundance in the fish brain.

Recent genomic advances mean it is now possible to identify and characterize patterns of gene expression among the site of gene that control circadian rhythms in relation to photo stimulation and regulate the physiological response such as reproduction.

The relationship between melatonin – melatonin receptors and reproduction is not simple, and complex pathways are involved in this regulation. Another possible factor playing part in this is the clock gene, which is responsible for several physiological functions, such as the regulation of body temperature, sleep-awake mechanisms and reproduction. It is also believed that the clock gene is also involved in the regulation of biological rhythms in the organism, including melatonin activity (Delaunay *et al.*, 2000).

7.4 Mechanisms of egg activation in commercially important species of teleost fish

A continual growth in aquaculture activity has been observed over the last two decades, yet the mechanism of egg activation and fertilization is still unclear. In aquaculture, many species suffer problems associated with low fertilization, hatching and early embryonic development. However, the most common problem is low fertility, for example in Atlantic halibut it was reported that only 1% of the fertilized eggs hatched (Norberg *et al.*, 1991). Similar levels of fertility have been reported for sole and turbot reared under captive conditions, in which fertilization rates can be reduced to 50% of the annual egg production (Houghton *et al.*, 1985; Bromley *et al.*, 1986). In this study we try to describe the mechanisms of egg activation in tilapia. After this was achieved this investigation was extended to three other important species for aquaculture. This investigation will increase the knowledge of the mechanism of fertilization and egg activation in fish and could help to overcome some of these problems.

The use of the sea urchin egg homogenate bioassay, allowed us to partially elucidate the mechanisms of egg activation in tilapia. Results demonstrated that tilapia sperm contain a molecule which triggers the release of calcium in the bioassay. This was confirmed by the PLC activity found in tilapia sperm extract, this extract has the ability to hydrolyze PIP_2 into PI_3 , and when injected into the bioassay has triggered calcium release. The tilapia sperm extract also has the ability to trigger calcium oscillation when injected into live mouse oocytes. These patterns of calcium oscillation were similar to those reported for different organisms such as marine worms, ascidians, sea urchin, chickens and amphibians.

In order to expand this investigation, additional extracts were made from the Atlantic halibut, African catfish and rainbow trout. Sperm extract of these species also had the ability to trigger calcium release, when injected into the bioassay. Although a lack of information is

observed regarding PLC activity in these species. A similar mechanism of egg activation is thought to be present in these three species.

The molecule responsible for egg activation has been isolated in mice, humans and monkeys. This novel molecule was named PLC ζ . In this investigation, several unsuccessful attempts were made to isolate a PLC ζ homologue in tilapia. This was the first attempt to isolate a non-mammal homologue of PLC ζ .

The isolation of non-mammal PLC ζ in particular a teleost one, may make a large contribution to the development of aquaculture, this novel molecule can be used to trigger egg activation without the physical sperm. This will be of great interest in the further development of aquaculture biotechnology, especially in the ploidy manipulation such as the production of gynogenetics organisms. In this case, the UV irradiation of the sperm will be eradicated from this protocol and just a single injection of a novel molecule will trigger the egg activation without the paternal genetic information or the inconvenience of any male nuclear organelle.

Another possible application could be the optimization and enhancement of fish sperm and thus an increment in fertilization rates which could be possible once the total structure and function of this novel molecule has been elucidated and described.

More investigations are needed to further isolate this novel molecule in teleost, and its possible implication in reproductive physiology, not just in fish. This research has many possible applications and could be applied in order to solve infertility problems in humans, in which there is more information and technology and commitment is greater.

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9 Appendix I

9.1 Research publications

Work presented in this Thesis has been published in the following papers:

Refereed papers

- Coward, K., Campos-Mendoza, A., Larman, M., Hibbitt, O., McAndrew, B., Bromage, N., and Parrington, J. (2003) Teleost fish spermatozoa contain a cytosolic protein factor that induces calcium release in a sea urchin egg homogenates and triggers calcium oscillations when injected into mouse oocytes. *Biochemical and Biophysical Research Communications* **305**, 299-304.
- Campos-Mendoza, A., Bromage, N., and McAndrew, B.J. (2004) The effect of photoperiod on the reproductive performance on the Nile tilapia, *Oreochromis niloticus*. *Fish Physiology and Biochemistry* **28**, 503-504.
- Campos-Mendoza, A., McAndrew, B.J., Coward, K. and Bromage, N. (2004) Reproductive response on Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effect on spawning periodicity, fecundity and egg size. *Aquaculture* **231**, 299-314.

Conference articles / Published in meeting abstract

- Campos-Mendoza, A., Bromage, N., and McAndrew, B.J. (2003) The effect of photoperiod on the reproductive performance on the Nile tilapia, *Oreochromis niloticus*. In K. Okazuwa (Ed), *7th International Symposium on Reproductive Physiology of Fish*. 18th – 24th May, 2003. Mie, Japan. [Poster presentation]
- Campos-Mendoza, A., McAndrew, B.J., Coward, K. and Bromage, N. (2004) Photoperiodic manipulation and its effect upon the reproductive performance of the Nile tilapia *Oreochromis niloticus*. *The Society For Experimental Biology, Annual Meeting*. 29th March 2004. Edinburgh, Scotland. [Oral presentation] (This talk was selected to be presented in the Young Scientist Award Session)

Sequences published on Molecular Databases

- Campos-Mendoza, A., McAndrew, B.J., Coward, K., and Bromage, N. (2004) Identification of melatonin receptor Mella in *Oreochromis niloticus*. Partial cds 492 bp. [Accession number AY569971]

Teleost fish spermatozoa contain a cytosolic protein factor that induces calcium release in sea urchin egg homogenates and triggers calcium oscillations when injected into mouse oocytes

Kevin Coward,^{a,*} Antonio Campos-Mendoza,^b Mark Larman,^c Olivia Hibbitt,^a
Brendan McAndrew,^b Niall Bromage,^b and John Parrington^a

^a Department of Pharmacology, University of Oxford, Oxford OX1 3QT, UK

^b Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

^c Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

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Abstract

Established studies in a variety of organisms including amphibians, fish, ascidians, nemerteans, echinoderms, mammals, and even a species of flowering plant, clearly demonstrate that an increase in intracellular egg calcium is crucial to the process of egg activation at fertilization. In echinoderms, egg activation appears to involve an egg phospholipase C gamma (PLC γ). However, numerous studies in mammalian species suggest that calcium is released from internal egg stores at fertilization by a sperm-derived cytosolic protein factor. Recent studies in the mouse have identified this sperm-derived factor as being a novel sperm-specific PLC isoform with distinctive properties (PLC ζ). Homologues of PLC ζ have since been isolated from human and cynomolgus monkey sperm. In addition, sperm factor activity has been detected in non-mammalian species such as chicken, *Xenopus*, and a flowering plant. Here we report evidence for the existence of a similar sperm-derived factor in a commercially important species of teleost fish, the Nile tilapia *Oreochromis niloticus* (L). Using an established bioassay for calcium release, the sea urchin egg homogenate, we demonstrate that protein extracts obtained from tilapia spermatozoa exhibit PLC activity similar to that seen in mammalian sperm extracts, and also induce calcium release when added directly to the homogenate. Further, tilapia sperm extracts induced calcium oscillations when injected into mouse oocytes.

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Keywords: Fertilization; Calcium; Phospholipase C; PLC ζ ; Sperm; Egg activation

Activation of the egg at fertilization, in practically all species studied thus far, is initiated by a sperm induced increase in free cytosolic calcium (Ca²⁺) concentration in the egg [1]. This Ca²⁺ rise is essential for successful activation of the egg and for early embryonic development [2]. In sea urchins, frogs, and fish (developmental model species such as medaka and zebrafish), the union of a sperm and an egg leads to a single explosive wave of Ca²⁺ traversing the egg from one pole to the other [1,3,4]. In contrast, in mammals, nemertean worms, and ascidians, a series of periodic increases in Ca²⁺ known as oscillations are observed [1,5,6].

The primary mechanism of Ca²⁺ release in the egg appears to involve increased levels of inositol 1,4,5-triphosphate (IP₃) subsequently activating IP₃ receptor-mediated Ca²⁺ release from intracellular egg stores [5,7]. Although the IP₃ pathway appears to play the primary role in egg activation, it is important not to dismiss the possible involvement of other naturally occurring calcium release agents such as cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [8–10]. Along with cADPR, nitric oxide (NO) and cyclic GMP (cGMP) pathways are also thought to be involved at fertilization in sea urchin eggs [11]. The mechanism by which sperm initiate the signalling cascade resulting in calcium release at fertilization remains a hotly debated issue [12]. In mammals, there is now

* Corresponding author. Fax: +44-1865-271853.

E-mail address: kevin.coward@pharm.ox.ac.uk (K. Coward).

good evidence to suggest that a soluble 'sperm factor' is introduced into the egg at sperm–egg fusion. This factor hydrolyses stores of phosphatidylinositol-4,5-bisphosphate (PIP₂) within the egg creating IP₃. Calcium is then released from internal egg stores such as the endoplasmic reticulum via the IP₃ receptor [2,4,6,12–16]. The cytosolic factor responsible was recently identified at the molecular level in the mouse, human, and cynomolgus monkey as a sperm-specific novel isoform of phospholipase C (PLC), which has been named PLC ζ [16,17]. Injection of cRNA encoding for PLC ζ triggered Ca²⁺ oscillations in mouse eggs that were indistinguishable from those at fertilization. Moreover, removal of endogenous PLC ζ from sperm extracts totally abolished Ca²⁺ release in eggs [16].

In mammals, injection of soluble sperm extracts from boars, hamsters, or humans, can trigger Ca²⁺ oscillations similar to those seen at fertilization in mouse, hamster, human, and cow eggs [14,18–23]. Soluble sperm extracts have also been shown to induce Ca²⁺ oscillations in non-mammalian species including marine worms [24], ascidians [25], and sea urchins [26,27]. Sperm extracts from non-mammalian species such as chicken and *Xenopus* [28], and even a flowering plant [29], have also been shown to contain a cytosolic soluble sperm factor that can trigger calcium oscillations when injected into mouse eggs. Dong et al. [28] suggested that the discovery of sperm factor activity in non-mammalian species implied that the sperm factor's ability to induce calcium oscillations in mouse eggs may not be species specific in vertebrates. The discovery of a cytosolic sperm factor in a species of plant is particularly interesting since it shows that although plants and mammals are evolutionary divergent, the activity of the putative sperm factor in triggering calcium release in mammalian eggs may not be specific to the animal kingdom [29]. However, it has yet to be established whether a sperm factor does play a universal role during fertilization in animals and plants [29].

The precise physiological mechanisms involved in fertilization and egg activation in the bony fish (teleosts) remain to be determined. Uncovering the mechanism of egg activation in teleost fish would be of particular interest given that the commercial culture of many important freshwater, but especially marine, teleosts remains constrained due to productivity problems associated with fertilization, hatching, and early embryonic development [30]. There are major concerns over the "capacity to fertilize" certain captive species. In captivity, fertilization rates and hatching success are often much lower than those witnessed in the wild. Our present knowledge about the pattern of egg activation in fish is currently limited to small laboratory species, such as zebrafish *Brachydanio rerio* (Hamilton) and medaka *Oryzias latipes* (Temminck and Schlegel), that, while being valuable experimental tools, have no real

commercial importance [31]. Even in these species, however, the mechanism whereby egg activation is triggered at fertilization remains far from clear.

Existing data from studies of teleosts do, however, demonstrate clear changes in Ca²⁺ at fertilization. Gilkey et al. [32] were the first to describe a free Ca²⁺ wave traversing the activating teleost egg (medaka); aequorin-injected eggs exhibited an explosive rise in free Ca²⁺ during fertilization, followed by a slow return to the resting level. This wave began at the animal pole, close to the site of germinal vesicle breakdown, and vanished at the antipode some minutes later. Similar results were later obtained in zebrafish eggs [33]. Injection of IP₃ and Ca²⁺ into medaka eggs resulted in Ca²⁺ release in a cytoplasmic region close to the egg surface [34]; results suggested that cytoplasmic Ca²⁺ induced Ca²⁺ release in the medaka egg from cytoplasmic stores indirectly, probably via a membrane factor such as IP₃. Lee et al. [35] further reported a single wave of Ca²⁺ traversing the zebrafish egg when activated by ionomycin. Microinjection of the established Ca²⁺-agonist, cADPR, triggered a regenerative wave of Ca²⁺ release in medaka eggs [36]. Interestingly, this mechanism was associated with the exocytosis of cortical alveoli [36]. Polzonetti et al. [37] further showed that cADPR can induce Ca²⁺ release in a homogenate made from Gilthead sea bream *Sparus aurata* (L.) eggs, indicating a potential role at fertilization. One study has suggested that zebrafish eggs may be activated by contact with the spawning medium [33]; there then follows a short time window (5–30 s) within which fertilization can occur. This activation mechanism, however, is very different from that described in medaka [32,38] in which sperm appear to activate the egg and not the external media.

In this paper we introduce some novel preliminary findings that suggest that the sperm of a commercially important species of teleost fish, the Nile tilapia (*Oreochromis niloticus* L.), may contain a sperm factor similar to that found in mammalian species. These findings are of significance for two reasons. First, a non-mammalian version of PLC ζ has yet to be isolated and characterized and our findings provide the first indication that a sperm PLC may play a role in egg activation in fish. Second, given the current concern over fertilization rates of some teleost species, the present findings are likely to make an important contribution to our understanding of egg activation in fish and, hence, their commercial culture.

Materials and methods

Choice of fish species. The Nile tilapia (*O. niloticus* L.) was selected for use in this study. While representing one of the most commercially important groups of cultured freshwater fish in global aquaculture, the

Nile tilapia, by virtue of its short reproductive cycle, amenability to handling, and ease of maintenance, remains a highly useful teleost model for research on reproductive physiology [31].

Preparation of cytosolic fish sperm extracts. Two separately prepared batches of concentrated tilapia sperm extract [Extract #1 and Extract #2] were used in this study. For each extract, fresh milt (sperm) was collected from 30 male Nile tilapia held at the tropical aquarium suite, Institute of Aquaculture, University of Stirling. Fish were anaesthetized with 10% (w/v) ethyl 4-aminobenzoate (benzocaine) dissolved in ethanol and sperm were released by gentle abdominal pressure. Sperm samples were collected in glass micro-capillary tubes. Samples were checked under a light microscope to confirm that they were not contaminated with urine. Clean (non-contaminated) sperm were pooled in 1.5 ml microcentrifuge tubes and kept on ice to await further processing. Samples were then washed in PBS (phosphate-buffered saline, pH 7.4) and centrifuged at 800g for 10 min. The resultant pellet was then resuspended and washed three times in PBS; during the final wash, 0.2 mM PMSF (phenylmethylsulphonyl fluoride) was added to the PBS. The washed pellet was finally resuspended in a solution containing 120 mM KCl (potassium chloride), 20 mM HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*[2-ethanesulphonic acid]), and an excess of chelex 100. Resuspended pellets were transferred to a 1 ml cryovial, carefully frozen in liquid nitrogen, and then thawed to room temperature. The freeze-thaw process was repeated 3–4 times in order to aid lysis of sperm cells. Sperm lysate was subsequently transferred to an ultracentrifuge tube and centrifuged at 100,000g for 1 h at 4°C. Supernatant was concentrated by using a combination of Centricon and Microcon centrifugal concentrator units (Millipore Corporation) in accordance with the manufacturer's instructions. Final concentrated sperm extracts (50–100 µg/µl protein ideally) were stored in aliquots of 5 µl at –80°C to await further analysis.

The sea urchin egg homogenate bioassay. Concentrated Nile tilapia sperm extracts were first tested for sperm factor activity (the ability to cause Ca²⁺ release) in an established cell free Ca²⁺ bioassay, the sea urchin egg homogenate. This assay preserves the structure of the sea urchin egg's membrane bound Ca²⁺ stores and utilizes a fluorescent dye (Fluo-3), which in association with a fluorimeter, allows us to detect Ca²⁺ release from internal egg stores. The sea urchin homogenate system is well characterized and has been used extensively to study Ca²⁺ release patterns in eggs. Unfertilized sea urchin (*Lytechinus pictus*) egg homogenates (2.5%) were prepared as previously described using Fluo-3 (3 mM) fluorescence as an indicator of Ca²⁺ release [26]. The homogenate system was first tested by injecting 2 µM IP₃. A further system test involved the injection of 3 µl (~50 µg/µl) of boar sperm extract, previously shown to be extremely effective in inducing Ca²⁺ release in the sea urchin homogenate [39]. Boar sperm extract was prepared as described previously [39]. Having established that the homogenate was responding to positive controls (IP₃ and boar sperm extract) in an appropriate manner, a series of experiments were undertaken using each of the two tilapia sperm extracts (Extract #1 and Extract #2). In each experiment, 3 µl (40–70 µg/µl) of tilapia sperm extract was injected directly into the sea urchin egg homogenate and monitored for a total time period of 500–800 s.

Concentrated tilapia sperm extracts were then tested for PLC activity. Concentrated Nile tilapia sperm extract (2 µl, 40–70 µg/µl) was incubated with 2 µl of 1 mM PIP₂ at room temperature for 1 min, prior to being injected into the sea urchin egg homogenate bioassay system [26,39]. Ca²⁺ levels were controlled in this assay by use of Ca²⁺/EGTA buffers [26] such that levels remained at 160 mM. IP₃ generated via the hydrolysis of PIP₂ by a potential cytosolic factor in the fish sperm extract would thus induce the release of Ca²⁺ from internal egg stores, which could be readily assayed using the Ca²⁺ sensitive Fluo-3 dye.

Microinjection of tilapia sperm extract into mouse oocytes and imaging of intracellular egg Ca²⁺ levels. The ability of concentrated Nile tilapia sperm extract to cause Ca²⁺ release in a living mammalian oocyte was then investigated by micro-injecting small volumes of extract directly into isolated mouse oocytes and visualizing resultant

changes in intracellular egg Ca²⁺ using laser confocal microscopy. In brief, female MF1 mice were superovulated by an injection of five international units (IU) of pregnant mare's serum gonadotropin (PMSG; Intervet). This was followed by an injection of human chorionic gonadotropin (HCG; Intervet) 48 h later. Eggs were collected after a further 13.5–14.5 h, as previously described [40], and maintained in 100 µl droplets of HEPES-buffered KSOM under mineral oil at 37°C. Intracellular Ca²⁺ changes were measured with Fura red-AM (Molecular Probes) and oocytes were injected with tilapia sperm extract (Extract #1, 40 µg/µl) as previously described [16,18]. The volume injected (1–3% of egg volume) was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. Control experiments involved the injection of a HEPES-buffered sperm-extract vehicle buffer (120 mM KCl, 20 mM HEPES).

Results and discussion

Our present knowledge of the key intracellular and molecular events that accompany egg activation in teleost fish is limited to only small laboratory species such as zebrafish and medaka. We know little about these key mechanisms in larger, commercially important teleosts. Previous experiments have described a wave of Ca²⁺ traversing the activating medaka egg [32] and zebrafish [33], and have shown that injections of IP₃, Ca²⁺, and cADPR can cause regenerative Ca²⁺ release in medaka eggs [34,36]. In a more recent study, cADPR was shown to cause Ca²⁺ release in an egg homogenate prepared from Gilthead sea bream *Sparus auratus* (L.), a commercially viable teleost [37]. Nevertheless, the mechanism of egg activation in fish remains far from clear. The present study attempts to address this shortfall, using a commercially important species of teleost fish, the Nile tilapia.

Concentrated sperm extracts derived from Nile tilapia induce Ca²⁺ release in the sea urchin egg homogenate bioassay and exhibit activity characteristic of a PLC

An established bioassay for Ca²⁺ release, the sea urchin egg homogenate, was first used to test tilapia sperm extracts for sperm factor activity (the ability to cause Ca²⁺ release in eggs). The ability of the sea urchin homogenate system to respond to IP₃ and boar sperm extract was tested, prior to testing the tilapia sperm extracts. Injections of either 2 µM IP₃ (data not shown), or 3 µl (~50 µg/µl) of boar sperm extract (Fig. 1A), resulted in significant Ca²⁺ release within the homogenate, demonstrating that the system was working correctly. Injections (*n* = 5) of 3 µl (40–70 µg/µl) of each tilapia sperm extract (Extract #1 and Extract #2) also induced Ca²⁺ release (Figs. 1B and C). Levels of Ca²⁺ within the homogenate more than doubled within seconds of injecting the tilapia sperm extracts, reaching a peak approximately 50–60 s after addition.

Experiments using the sea urchin egg homogenate bioassay of Ca²⁺ release further demonstrated that

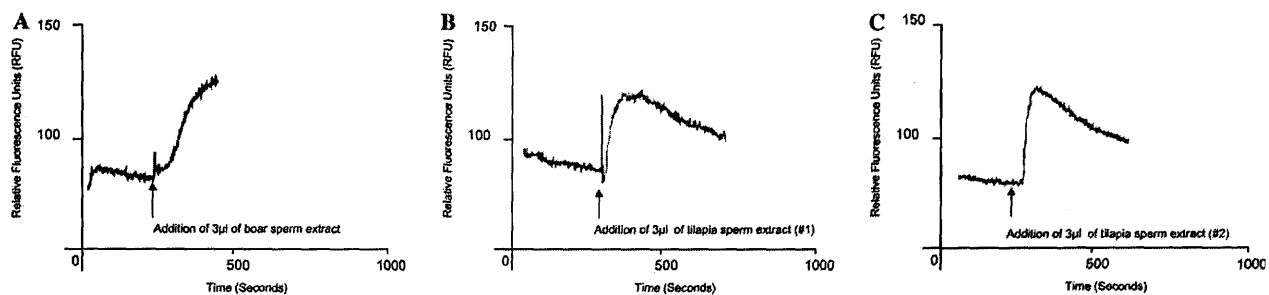


Fig. 1. Calcium release in the sea urchin egg homogenate induced by: (A) boar sperm extract and (B,C) Nile tilapia sperm extract. Arrows indicate when the injections were made.

sperm extracts made from Nile tilapia exhibited characteristic PLC activity, in that they were able to hydrolyse PIP₂ within the egg homogenate to create IP₃ and hence cause Ca²⁺ release from internal egg Ca²⁺ stores when added to the sea urchin egg homogenate (Fig. 2). This assay has previously been used to demonstrate PLC activity in mammalian sperm [26,39] and operates at low Ca²⁺ conditions (160 nM) under which the sperm factor PLC is active, but not other PLCs [39]. Our homogenate experiments, in which the sperm extract was incubated with PIP₂ at low Ca²⁺ concentration (160 nM), showed significant Ca²⁺ release as soon as the tilapia sperm extract/PIP₂ cocktail was injected into the sea urchin egg homogenate system; within 25 s, Ca²⁺ levels within the egg homogenate had more than doubled. These observations were readily reproducible and clearly indicate that Nile tilapia spermatozoa do indeed contain protein(s) exhibiting PLC activity. Given that spermatozoa from Nile tilapia have the ability to hydrolyse PIP₂ into IP₃ and cause the release of Ca²⁺ in an established bioassay system, it follows that the same

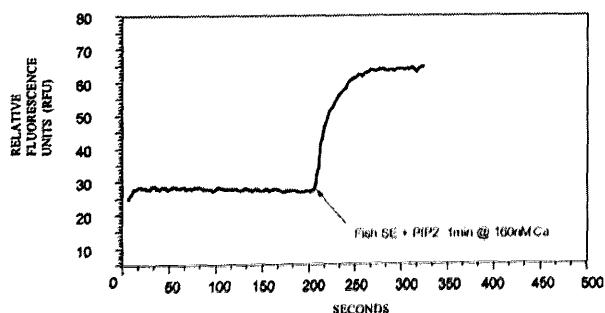


Fig. 2. Demonstration of PLC activity in Nile tilapia sperm extract. Concentrated tilapia sperm extract was incubated with PIP₂ (phosphatidylinositol-4,5-bisphosphate) for 1 min (Ca²⁺ in the reaction was buffered at 160 nM) and the resultant IP₃ (inositol triphosphate) generated was assayed using the cell-free sea urchin egg homogenate assay. Arrow shows the point at which the fish sperm extract (fish SE)/PIP₂ cocktail (previously incubated for 1 min) was injected into the homogenate.

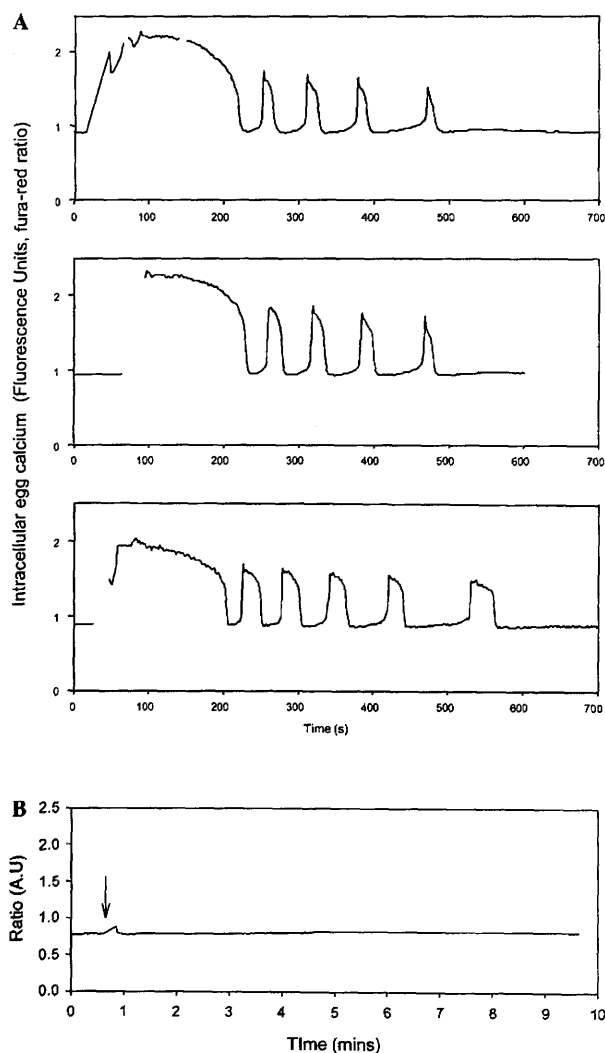


Fig. 3. (A) Oscillations of intracellular calcium in mouse oocytes following microinjection of concentrated tilapia sperm extract. Each trace represents a different mouse oocyte ($n = 3$) and (B) control injection of the injection of a HEPES-buffered sperm-extract vehicle buffer (120 mM KCl, 20 mM HEPES) (arrow represents the point of injection). Intracellular calcium changes were measured with Fura-Red AM as previously described [12,25] and represented here as fluorescence units.

sperm extracts may induce Ca^{2+} release within a living oocyte.

Injection of Nile tilapia sperm extract causes oscillations in intracellular egg Ca^{2+} when injected into mouse oocytes

Concentrated protein extracts prepared from tilapia spermatozoa were then injected into mouse oocytes. Micro-injection and whole cell fluorescence experiments, using a total of three mouse oocytes, showed that injection of concentrated Nile tilapia sperm extract induced an initial large single Ca^{2+} transient followed by a series of 4–5 subsequent oscillations (Fig. 3A). The initial transient, during which egg Ca^{2+} approximately doubled, began 10–20 s after injection and lasted for ~150 s; this was then followed by 4–5 further oscillations over a total subsequent period of ~400 s. Control injections failed to induce significant Ca^{2+} release in mouse oocytes (Fig. 3B). In the present study, patterns of Ca^{2+} oscillations (e.g., amplitude and frequency) induced by Nile tilapia sperm extracts were similar to those seen previously when mouse and sea urchin oocytes were injected with a variety of sperm extracts [2,15–17,27,28,41].

Our present data indicate that tilapia sperm extracts contain a sperm factor activity similar to that found in mammalian sperm extracts. Sperm factor activity has been reported previously in several other non-mammals such as the chicken, *Xenopus*, and even a species of flowering plant [28,29]. The present data, however, represent the first demonstration of sperm factor activity in fish and provide evidence to suggest that fish egg activation may be triggered by a sperm derived PLC. Furthermore, whilst providing further support to previous descriptions of sperm factor activity in non-mammalian organisms [28,29], our data provide the first demonstration of a sperm PLC-like sperm factor activity in a non-mammal. Current research is investigating whether sperm extracts derived from Nile tilapia can also cause Ca^{2+} release upon injection into teleost oocytes. Isolation and the molecular characterization of a sperm factor in commercially important teleosts may prove highly valuable in furthering our understanding of fertilization and egg activation in this important animal group, and may help improve fertilization and hatching rates in future commercial scenarios.

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The effect of photoperiod on the reproductive performance of the Nile tilapia, *Oreochromis niloticus*

A. Campos-Mendoza, N. Bromage and B.J. McAndrew

Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK (E-mail: ac7@stir.ac.uk)

Key words: aquaculture, broodstock management, reproduction, seed production

Abstract

The reproductive performance of *O. niloticus* was evaluated in four different photoperiods. Results suggested that long days were the most suitable for egg production and reduction of inter-spawning interval.

Introduction

Low fecundity and asynchronous spawning behaviour are the most important constraints on tilapia culture (Coward and Bromage 2000). Photoperiodic manipulation is applied in several fish species in order to control their reproduction (Bromage et al. 2001). In tilapias the effect of photoperiod on reproduction is poorly understood, however, it has been reported that photoperiod could play an important role on tilapia reproduction (Ridha and Cruz 2000). The aim of the present work was to understand and develop photoperiodic manipulation of *O. niloticus* broodstock in order to improve egg production and inter-spawning interval.

Materials and methods

32 sibling female *O. niloticus* were exposed to four trials (6L:18D, 12L:12D, 18L:6D and 24LL) for 180 days, eight fish per trial. Fish were fed twice daily. The reproductive performance was evaluated in terms of number of spawns, total fecundity and inter-spawning interval (ISI). Fish biometry was recorded and specific growth rate (SGR) evaluated. This experiment was carried out in a closed recirculation system with

mean light intensity of 530 lux and water temperature of 27 ± 1 °C.

Results

No significant differences were found between the group mean weight and length at the beginning or end of the experiment. However, significant differences were found in the SGR ($P < 0.05$) between groups and the reproductive performance shown in Table 1.

Discussion

Our results showed that 18L:6D was the most cost effective way of enhancing egg production. This trial gave the highest fecundity and number of spawns, suggesting that these fish increased oogonial proliferation as a reproductive strategy, as well as a reduction in ISI. The SGR demonstrated that those fish reared under shorted photoperiods (6L:18D and 12L:12D) had the highest increase in weight but the lowest fecundity and number of spawns as well as the longer ISI. Results were similar to those reported by Ridha and Cruz (2000) who observed that seed production was enhanced more by extended photoperiods rather than

Table 1. Reproductive performance on Nile tilapia *Oreochromis niloticus* reared in four different photoperiods

Parameter	18L:6D	12L:12D	18L:6D	24LL
Total spawns*	65	61	90	75
Total eggs*	129,269	124,675	216,701	177,331
Fecundity	2019 ± 82 ^b	2043 ± 68.8 ^b	2407.8 ± 65.9 ^a	2396.4 ± 77.5 ^a
ISI d ⁻¹	18.9 ± 1.5 ^{ab}	19.8 ± 1.3 ^b	14.7 ± 0.5 ^c	16.3 ± 1.0 ^{ac}
SGR (%d ⁻¹)	0.47 ± 0.03 ^{bc}	0.51 ± 0.05 ^c	0.31 ± 0.02 ^a	0.38 ± 0.04 ^{ab}

The values are the mean ± S.E.M., different superscript indicate statistically significant differences from each other (ANOVA, Fisher's comparison test, P < 0.05).

*Indicates the total value.

light intensity. Photoperiod manipulation could be a reliable and powerful tool in tilapia broodstock management; basically it allowed the reduction of the ISI, resulting in more spawns per fish in less time.

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Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effects on spawning periodicity, fecundity and egg size[☆]

A. Campos-Mendoza^{a,*}, B.J. McAndrew^a,
K. Coward^b, N. Bromage^a

^a *Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK*

^b *Department of Pharmacology, University of Oxford, Oxford, OX1 3QT, UK*

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Abstract

Nile tilapia (*Oreochromis niloticus*) has rapidly become an important species for aquaculture, although their intensive culture remains constrained by poor spawning synchrony and low fecundity, adding significant cost to hatchery production. Previous research has indicated that spawning synchrony may be improved in some fish species by photoperiod manipulation. There is limited information on the effects of photoperiod manipulation on tilapia. In this paper, the reproductive performance of 32 individually housed Nile tilapia was evaluated under four different photoperiods: short day (6L:18D), normal day (12L:12D), long day (18L:6D), and continuous illumination (24L:0D). Significantly larger eggs ($P < 0.05$) were produced under normal daylength (12L:12D) compared to other treatment groups. Fish reared under long daylength (18L:6D) exhibited significantly higher ($P < 0.05$) total fecundity (2408 ± 70 eggs spawn⁻¹) and relative fecundity (7.2 ± 0.2 eggs g⁻¹ body weight) concomitant with a significant reduction in inter-spawn-interval (ISI, 15 ± 1 days) in comparison with the rest of the trials. This investigation shows that long daylength (18L:6D) helps improve some important reproductive traits in Nile tilapia, and suggests that such methodology may be used to alleviate the production problems caused by low fecundity and poor spawning synchrony, and thus play a valuable future role in tilapia culture.

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Keywords: Reproduction; Photoperiod; Broodstock management; Nile tilapia

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* Corresponding author. Tel.: +44-1786-46-78-84; fax: +44-1786-47-21-33.

E-mail address: ac7@stir.ac.uk (A. Campos-Mendoza).

1. Introduction

Tilapias are now one of the most important groups of aquaculture species; production increased significantly over the last decade and according to FAO statistics had reached almost 1.5 million tonnes by 2001 (FAO, 2002). These are versatile species now found in almost any tropical aquatic system, from subsistence culture in small ponds and paddy fields to highly intensive production systems, e.g. hapas, cages and tanks. Furthermore, tilapia perform well in freshwater, brackish water and seawater environments (Chervinski, 1982; Philippart and Ruwet, 1982; Beveridge and McAndrew, 2000).

Female tilapia have individual patterns of ovarian development so that in breeding populations they tend to spawn asynchronously every 3 to 4 weeks, depending upon environmental conditions (Rana, 1988; Macintosh and Little, 1995; Coward and Bromage, 2000). Low fecundity and asynchronous spawning behaviour are major constraints on mass tilapia seed production (Mires, 1982; Rana, 1988; Baroiller and Jalabert, 1989; Macintosh and Little, 1995; Coward and Bromage, 1998; Bhujel, 2000; Little and Hulata, 2000). In order to optimise tilapia seed production and obtain a homogeneous stock of first-feeding fry suitable for sex reversal, hatchery operators have tended to increase the number of broodfish (Macintosh and Little, 1995; Little et al., 1997; Coward and Bromage, 2000; Bhujel, et al., 1998, 2001a; Bhujel, 2000). In Thailand, for example, a commercial hatchery maintains over 60,000 broodfish in order to guarantee production of 10 million fry per month and thus satisfy market demand (Bhujel and Suresh, 2000). Although the use of high numbers of broodfish helps overcome these problems, this method is far from ideal because of the increased costs needed to house and maintain these fish.

Photoperiodic manipulation has been applied successfully in several fish species to alter their reproductive cycle (Bromage et al., 2001). Using this technique, hatchery operators of rainbow trout (*Oncorhynchus mykiss*) produce sufficient numbers of eggs and fry at desired times, and thereby ensure all-year round production.

In the case of tilapias and other tropical species, the effect of photoperiod on reproduction is poorly understood, although several authors have reported that photoperiod and light intensity might play an important role in controlling reproduction (Cridland, 1962; Hyder, 1970; Rothbard and Pruginin, 1975; Balarin and Haller, 1982; Jalabert and Zohar, 1982; Bhujel, 2000). Ridha et al. (1998), showed that photoperiodic manipulation improved seed production in *Oreochromis spilurus* using 14L:10D light regimes. However, Ridha and Cruz (2000) reported that longer and brighter days (18L:6D with 2500 lx) produced more fry and improved spawning synchrony in Nile tilapia compared with short days (12L:12D; 15L:9D) and low light intensity (500 lx). Considering the constraints currently imposed upon tilapia culture by poor spawning synchrony, any method that helps to improve hatchery efficiency should be investigated.

The aim of the present work therefore was to investigate the effects of photoperiodic manipulation on the reproductive performance of *Oreochromis niloticus* broodstock to see whether this identifies possible broodstock management strategies that may be adopted by hatcheries to improve egg production.

2. Materials and methods

2.1. Experimental fish

Thirty two siblings of red strain *O. niloticus* (McAndrew et al., 1988) were taken from The Tilapia Reference Collection (McAndrew and Majumdar, 1983) held at the Tropical Aquarium, Institute of Aquaculture, University of Stirling. Fish were 18 months old at the beginning of the experiment.

2.2. Culture system

Fish were maintained in a gravity-fed recirculation system incorporating eight glass aquaria (114 × 45 × 42 cm; 200-l capacity, Fig. 1). Each aquarium was subdivided into four individual holding spaces by perspex sheets (Coward and Bromage, 1999a). Aquaria out-flows were connected to four settling tanks and a gravel filter unit. Settling tanks contained large numbers of bio-rings (Dryden Aquaculture, UK) to aid particulate and biological 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 system was covered with a special frame made with tubular steel covered in white blown PVC sheets to exclude all external source of light. Light in each tank was provided by a lamp (Lampways, Tripleplus, 60 W, EC) attached to the ceiling of the frame. All lights were controlled with digital timers (Smiths Industries, UK) in order to achieve the desired photoperiod. The system was subdivided into four individual chambers (one chamber per experimental group) with two aquaria in each chamber.

The water temperature was maintained at 27 ± 0.5 °C (using a 3-kW thermostatically controlled water heater). Water was oxygenated via airstones in the header tank and each

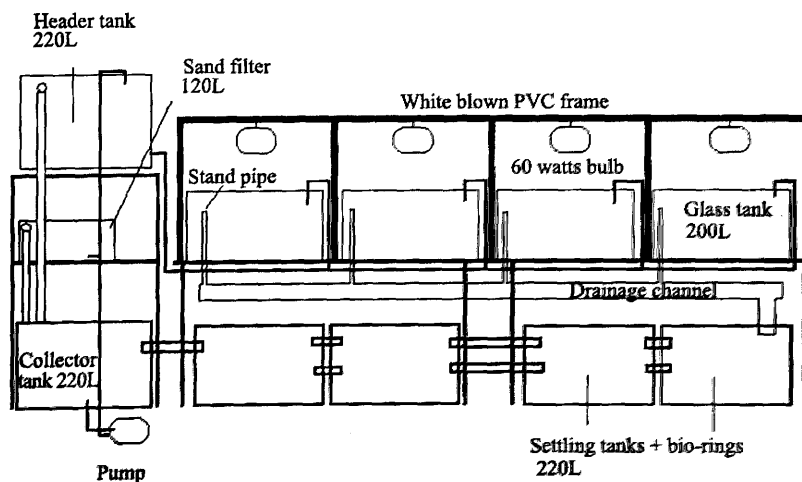


Fig. 1. Lateral view of the closed recirculation system used to hold experimental fish.

aquarium by a low-pressure blower. The water inflow was constant at $252 \text{ l h}^{-1} \text{ tank}^{-1}$. Fish were fed *ad libitum* twice daily with a commercial pelleted trout feed (Trouw Aquaculture, UK). 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, fish were 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 before being returned to their respective tank. At the end of the experiment, fish were re-weighed in order to estimate daily weight gain (DWG) as described by Bhujel et al. (2001b).

Fish were checked at two hourly intervals during the light phase for the evidence of spawning. Signs of spawning in this species include dilation of the genital papillae, and/or the presence of eggs inside the buccal cavity. Fish were allowed to spawn naturally or were manually stripped if observed close to natural spawning. Females with broods were captured in a fine mesh net and transferred to a bucket containing fresh water; eggs were then removed and washed. Fish were then anaesthetised, measured and weighed and then returned to the experimental tank and all data recorded.

Fecundity in tilapia has been defined in several ways (Coward and Bromage, 1999a, 2000) and remains a source of debate. In the present study, we used Rana's (1988) definition which describes total fecundity as the number of eggs in a freshly spawned clutch of eggs. After counting the whole clutch, a sub-sample of 50 eggs was taken and each egg individually measured. Since tilapia eggs are ovoid, it was important to measure both axes (long and short axis). These values were used to estimate egg diameter and volume. Inter-spawning-interval (ISI) was also determined (time elapsed between one spawn and the next). For further details of how egg diameter, egg volume and ISI were determined, consult Coward and Bromage (1999a).

2.3. Photoperiod and light intensity

Four discrete light regimes were studied (Table 1): short days (6L:18D), normal days (12L:12D), long days (18L:6D) and continuous illumination (24D:0L). Only the 12L:12D and 18L:6D photoperiods are likely to be experienced by *O. niloticus* in its natural geographical distribution (Fishelson, 1966; Philippart and Ruwet, 1982).

Table 1
Experimental design

General description	Photoperiod (L = hours light, D = hours dark)	Number of tanks (replicates)	Number of fish per tank replicate
Short day	6L:18D	2	4
Normal	12L:12D	2	4
Long day	18L:6D	2	4
Continuous light	24L:0D	2	4

Light intensity in each aquarium was evaluated with a lux meter (Photometric sensor SKL310, Skye Instruments, Llandrindod Wells). Measurements were taken centrally at the surface of the water column. Light intensity was constant at 530 lx in each chamber over the entire experimental duration.

2.4. Statistical analysis

Statistical analyses were performed using MINITAB (version 13.1). Statistical significance between treatments was evaluated at the 5% probability level. General linear model (ANOVA) and regression analyses (linear and quadratic) were used to further analyse data. Data were \log_{10} or Arcsine transformed as appropriate. Values are expressed as the mean \pm S.E.M.

3. Results

3.1. Fecundity and egg size

A total of 291 spawns were recorded over the 6-month experiment, 65, 61, 90 and 75 spawns occurring in the 6L:18D, 12L:12D, 18L:6D and 24L:0D treatments, respectively (Table 2). Fig. 2 shows the distribution of these spawns over time (months); it is clearly

Table 2
Reproductive and growth performance of Nile tilapia (*O. niloticus*) reared at four different light regimes

	Photoperiod treatment			
	6L:18D	12L:12D	18L:6D	24L:0D
<i>Reproductive parameter</i>				
Total spawns*	65	61	90	75
Spawns month ⁻¹	10.8 \pm 1.3 ^a	10.2 \pm 1.3 ^a	15 \pm 1 ^a	12.5 \pm 1.5 ^a
Spawns fish ⁻¹ month ⁻¹	1.6 \pm 0.1 ^{bc}	1.5 \pm 0.1 ^c	2 \pm 0.1 ^a	1.9 \pm 0.1 ^{ab}
Total eggs*	129,269	124,675	216,701	177,331
Eggs month ⁻¹	21,545 \pm 3234 ^b	20,347 \pm 3188 ^b	36,988 \pm 1667 ^a	29,555 \pm 3903 ^{ab}
Fecundity	2020 \pm 80 ^b	2043 \pm 70 ^b	2408 \pm 70 ^a	2396 \pm 80 ^a
Relative fecundity (egg g ⁻¹)	6 \pm 0.3 ^b	6 \pm 0.2 ^b	7.2 \pm 0.2 ^a	6.4 \pm 0.2 ^b
Egg diameter (mm)	2.41 \pm 0.03 ^{ab}	2.47 \pm 0.02 ^a	2.37 \pm 0.01 ^b	2.36 \pm 0.02 ^b
Egg volume (mm ³)	6.6 \pm 0.3 ^{ab}	7.0 \pm 0.2 ^b	6.2 \pm 0.1 ^{ac}	6.1 \pm 0.1 ^c
Total egg volume (mm ³)	13,173 \pm 620 ^a	14,058 \pm 476 ^a	14,804 \pm 376 ^a	14,348 \pm 496 ^a
ISI (days)	19 \pm 2 ^{ab}	20 \pm 1 ^b	15 \pm 1 ^c	16 \pm 1 ^{ac}
<i>Growth parameter</i>				
Initial weight (g)	255 \pm 20 ^a	256 \pm 20 ^a	276 \pm 30 ^a	305 \pm 40 ^a
Final weight (g)	560 \pm 50 ^a	537 \pm 40 ^a	477 \pm 40 ^a	577 \pm 40 ^a
Initial length (cm)	23 \pm 1 ^a	23 \pm 1 ^a	24 \pm 1 ^a	24 \pm 1 ^a
Final length (cm)	30 \pm 1 ^a	30 \pm 1 ^a	29 \pm 1 ^a	30 \pm 1 ^a
DWG (g fish ⁻¹ day ⁻¹)	1.8 \pm 0.2 ^b	1.9 \pm 0.2 ^b	1.1 \pm 0.1 ^a	1.5 \pm 0.1 ^{ab}

Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

* Indicates the total value for the entire experimental period.

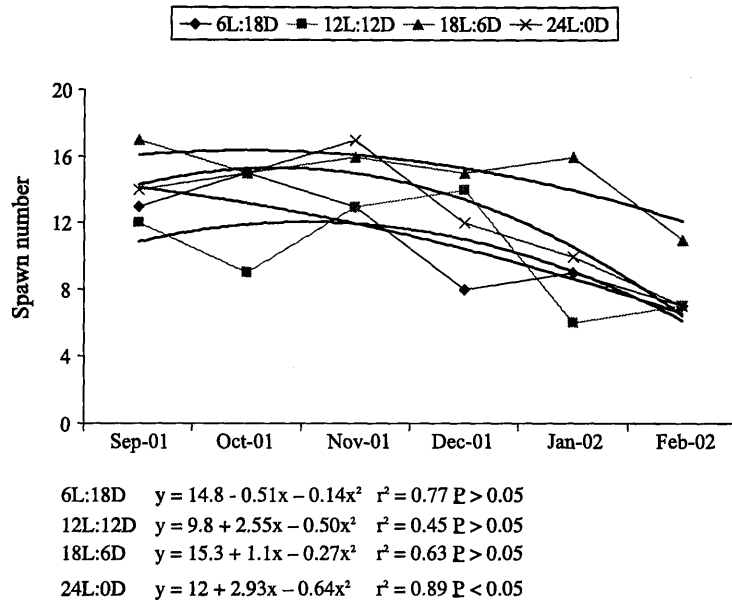


Fig. 2. Number of spawns per month produced by Nile tilapia reared under four different light regimes.

evident that the 18L:6D photoperiod produced the highest and most consistent number of spawns. There were significant differences between the mean number of spawns fish^{-1} month^{-1} between the photoperiod treatments (Fig. 3A, Table 2), which varied between 1.5 ± 0.1 for the 12L:12D trial and 2 ± 0.1 for the 18L:6D trial.

A total of 647,976 eggs were collected during the experiment. The least number of eggs were produced by the 12L:12D group with 124,675 eggs (19% of total egg production), followed by the 6L:18D group with 129,269 eggs (20%), the 24L:0D group with a total of 177,331 eggs (27.5%), and finally the 18L:6D group which exhibited the highest production of eggs with a total of 216,701 eggs (33.5%). Total egg production per month for each treatment for the whole 180-day experiment is presented in Fig. 4. This graph shows that the long day (18L:6D) group consistently produced the highest number of eggs per month over the experiment, with the exception of the October total. Mean egg production was significantly reduced under the 12L:12D treatment with a mean of $20,347 \pm 3188$ eggs month^{-1} , while the highest was in the 18L:6D group ($36,988 \pm 1667$ eggs month^{-1}).

There were significant differences in total fecundity between photoperiods. The 6L:18D treatment had the lowest mean total fecundity with 2020 ± 80 eggs clutch^{-1} whilst the highest mean total fecundity was produced under the 18L:6D trial with a total of 2408 ± 70 eggs clutch^{-1} (Fig. 3B, Table 2). Consequently, there were also significant differences between photoperiods in relation to relative fecundity (number of eggs produced per gram of body weight) (Fig. 3C, Table 2); relative fecundity varied between 6 ± 0.2 and 7.2 ± 0.2 eggs g^{-1} , the 18L:6D treatment group being significantly higher than the others.

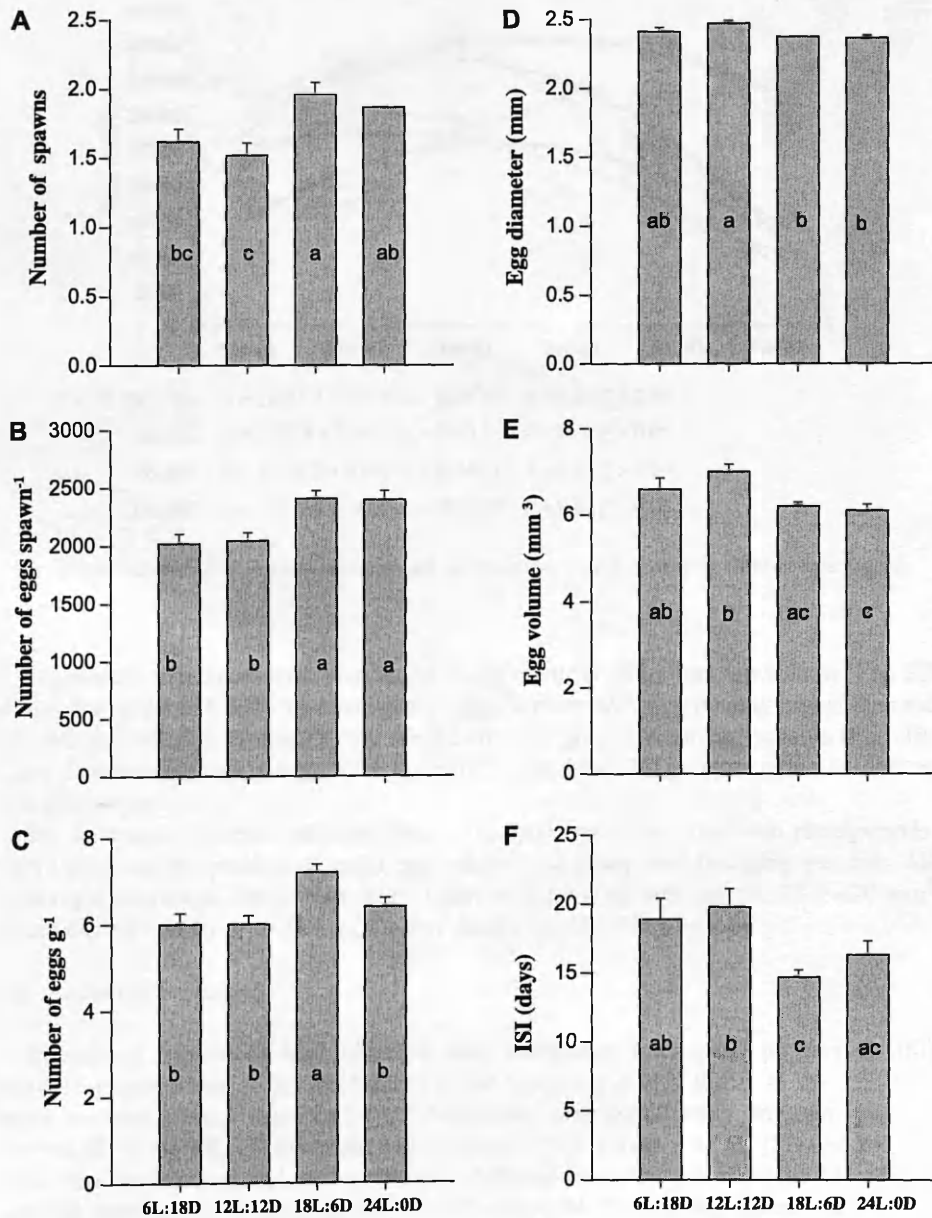


Fig. 3. Reproductive performance of Nile tilapia cultured under four different light regimes: (A) number of spawns fish⁻¹ month⁻¹, (B) total fecundity, (C) relative fecundity, (D) egg diameter, (E) egg volume, and (F) inter spawning interval (ISI). Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

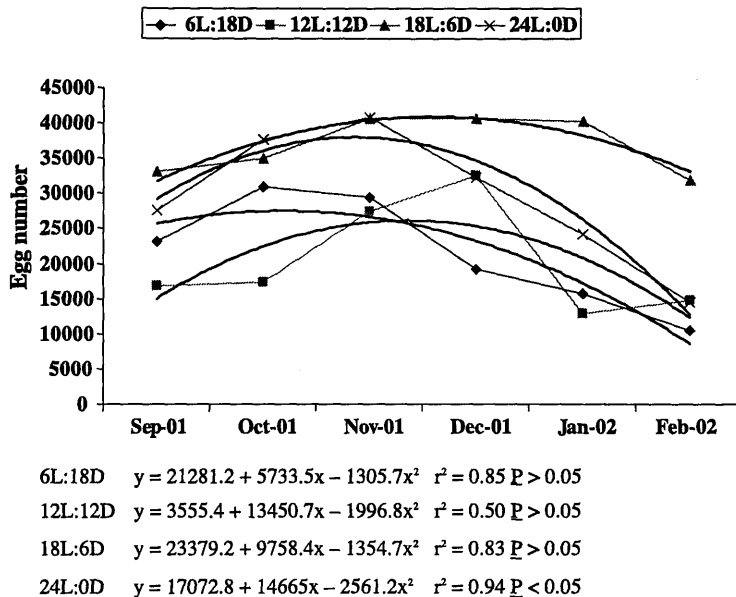


Fig. 4. Total number of eggs per month produced by Nile tilapia reared under four different light regimes.

Significant differences were also found in egg diameter and mean egg volume. Fig. 3D shows that in the 12L:12D treatment group, egg diameter was significantly higher than the 18L:6D and 24L:0D treatments, but not the 6L:18D group. Mean egg volume (Fig. 3E, Table 2) was significantly larger ($7 \pm 0.2 \text{ mm}^3$) in the 12L:12D group than the 18L:6D or 24L:0D groups.

No differences were found in relation to the total egg volume between photoperiods. This value was the product of mean egg volume and mean total fecundity per fish. All light regimes showed similar total egg volume, which varied between $13,173 \pm 620 \text{ mm}^3$ in the 6L:18D group to $14,804 \pm 376 \text{ mm}^3$ under the 18L:6D treatment.

3.2. Spawning periodicity

Significant differences were detected when comparing inter-spawning-interval (ISI) between photoperiods (Fig. 3F, Table 2). The longest ISI was found in the 12L:12D treatment with a mean value of 20 ± 1 days which was significantly different from the shortest ISI in the 18L:6D treatment with a mean of 15 ± 1 days. The 6L:18D and 24L:0D trials were intermediate and not significantly different from each other but the 24L:0D ISI was still significantly shorter than the 12L:12D treatment. Positive significant relationships were found between ISI and egg diameter and between ISI and egg volume in the total data set as well as within treatments. However, there was a strong negative correlation between ISI and fecundity and relative fecundity. There was no significant relationship between total egg volume and ISI in the data. There were also statistically significant

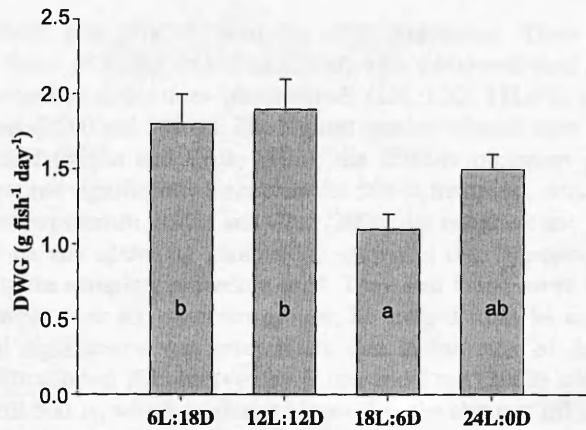


Fig. 5. Daily weight gain (DWG) of tilapia cultured under four different photoperiods. Values are mean \pm S.E.M. Annotation with different superscripts indicate statistically significant differences (ANOVA, Fisher's comparison test, $P < 0.05$).

relationships between fish size (both weight and length) and ISI in the total data set; this relationship was positive in all the treatment groups, but was only significant in the 18L:6D treatment.

3.3. Growth performance

No significant differences were found between the treatment groups in terms of fish size (weight and total length) at the beginning of the experiment (Table 2). Group mean weight ranged from 255 ± 20 to 305 ± 40 g and total length ranged from 23 ± 1 to 24 ± 1 cm. At the end of the experiment, mean weight ranged from 477 ± 40 to 577 ± 40 g, whilst total length from 29 ± 1 to 30 ± 1 cm. No significant differences were observed between groups in terms of weight or length at the end of the experiment.

Nevertheless, significant differences were detected in daily weight gain (DWG) between the groups. A significantly higher DWG (1.9 ± 0.2 g fish⁻¹ day⁻¹) was observed under 12L:12D compared to 18L:6D treatment (1.1 ± 0.1 g fish⁻¹ day⁻¹) over the 180 days (Fig. 5, Table 2).

4. Discussion

4.1. Egg production and spawning periodicity

Fig. 4 shows that fish under the 18L:6D photoperiod consistently produced more eggs per month than all other treatments, some 58% more eggs than the 12L:12D photoperiod. Fish under the 18L:6D treatment exhibited significantly increased total and relative fecundity concomitant with significantly reduced ISI resulting in more frequent spawning

and a greater clutch size (Fig. 2) than the other treatments. These findings are in accordance with those of Ridha and Cruz (2000) who compared seed production (egg, sac fry and swim-up fry) under three photoperiods (12L:12D, 15L:9D, and 18L:6D) and two light intensities (2500 and 500 lx). The highest number of seed were observed under a 18L:6D photoperiod (Ridha and Cruz, 2000); the 2500-lx treatment gave the highest number of seed but not significantly more than the 500-lx treatment, which is close to that used in the present experiment. Ridha and Cruz (2000) did not show any significant effect of light intensity on any spawning parameters; spawning data appeared to be periodic, probably related to the sampling procedure used. They also found no statistical difference for mean ISI between their six treatment groups; ISI ranged from 14 to 55 days and the lack of statistical significance was most likely due to the lack of data pertaining to individual fish. The shortest ISI observed by Ridha and Cruz (2000) was 14 days in fish under 18L:6D with 500 lx, which is almost identical to the shortest ISI of 15 days in our experiment (also in the 18L:6D group).

The present experiment effectively 'robbed' eggs (removed eggs from the buccal cavities of mouth-brooding females) within a few hours of spawning or resulted in fish being stripped. Both of these techniques have been shown to reduce ISI in tilapia (Fishelson, 1966; Dadzie, 1970; Siraj et al., 1983; Rana, 1988; Little, et al., 1993; Tacon et al., 1996; Coward and Bromage, 2000). The similarity in ISI between the results of Ridha and Cruz (2000) and this present study would suggest that we are getting close to an optimum ISI using these techniques. It is known that ISI is normally shorter in smaller tilapia (Siraj et al., 1983). The fish used in this present experiment were two to three times bigger than those females used by Ridha and Cruz (2000) and it would also suggest that the rapid removal of eggs from actively brooding females might have helped to reduce the ISI. Egg robbing is time consuming and labour intensive and may therefore only be a useful contribution to broodstock management at a very intensive commercial scale.

It is interesting to note that the fish under continuous illumination (24L:0D) were not significantly different to the 18L:6D trial for most of the reproductive parameters measured. They produced fewer eggs than the 18L:6D trial, mainly because of the drop in monthly egg production after the third month that characterised the other treatments (Fig. 4). This suggests that the use of a long day photoperiod (18L:6D) helps to extended spawning intensity.

4.2. Egg size and fecundity

Photoperiod manipulation had an effect on egg size. Significant differences in egg size were observed between photoperiods. The largest eggs (diameter and volume) in this experiment were produced by fish under a 12L:12D photoperiod (Table 2). This group had the lowest number of spawns and total fecundity and the longest ISI. Significant relationships were detected in this group, between ISI and egg size (Fig. 6A). This might simply imply that because ISI was longer, eggs had more time to sequester vitellogenin from the bloodstream, hence resulting in a larger final egg size. In contrast, the fish in the 18L:6D treatment had the shortest ISI and produced the smallest eggs (Fig. 6B, Table 2). This is the first clear evidence that ISI directly effects egg size in this species. In previous

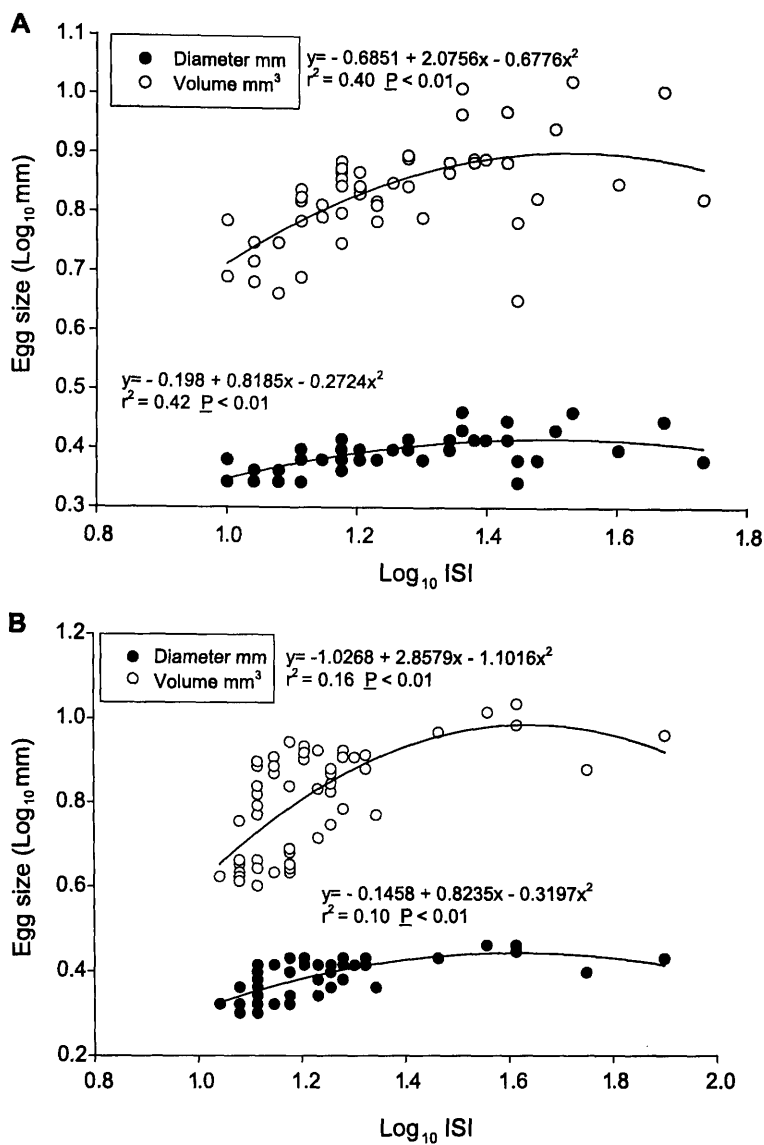


Fig. 6. Relationship between ISI and egg size (volume and diameter): (A) normal day 12L:12D, and (B) long days 18L:6D. (Data has been log₁₀ transformed, and analysed by polynomial quadratic regression.)

studies, egg size in tilapia has been related to genotype, nutritional condition, age and size of the broodstock (Rana, 1985).

In this study, it appears that those fish reared under long daylength showed advancement in gonadal recrudescence, producing discrete batches of smaller eggs very quickly compared to those fish reared under shorter days. This effect was clearly observed in salmonids and is discussed by Bromage and Cumaranatunga (1988), but the

impact on ISI is unlikely to be as profound as in a species that can spawn as frequently as tilapia. The advantage of producing high numbers of smaller eggs has to be balanced against the fact that bigger eggs produce larger and stronger larvae at hatching, with consequent improved chances of survival under some farming regimes (Rana and Macintosh, 1988; Rana, 1985, 1988). It is unclear whether this advantage would outweigh the loss of production associated with having to use larger females or extending the ISI to get larger eggs.

Ridha et al. (1998) compared the seed production of *O. spilurus* under ambient light and temperature conditions and controlled light intensity with 13L:11D and 14L:10D photoperiods with a water temperature of 29 ± 2 °C. They found that reproduction was reduced during winter due to a reduction of water temperature and daylength under ambient conditions. The longer day 14L:10D treatment gave significantly more seed than either ambient or 13L:11D day treatments.

Highest mean total fecundity was recorded in fish experiencing long daylength and continuous illumination in the present study. It is well documented that total fecundity is more related to tilapia size rather than age (Rana, 1988; Coward and Bromage, 1999a). In the case of the substrate spawning *Tilapia zillii*, Coward and Bromage (1999a) reported a strong relationship between fish size (weight and length) and fecundity but did not find any significant relationship between egg size and maternal weight and length. In the present experiment, all fish were of the same age and were of similar size. The 18L:6D trial produced the highest relative fecundity (7 ± 0.2 eggs g^{-1}), which is very similar to that reported by Bhujel et al. (2001a), who reported relative fecundity of 7331 ± 618 eggs kg^{-1} in a shaded hapa system suspended in outdoors tanks under tropical conditions of light and temperature. It is still unclear why long daylength and continual illumination resulted in increased total fecundity in our experiment. Further studies are required to confirm and explain these observations.

4.3. Growth

There were significant differences in DWG between the four photoperiods which changed the relative ranking of the mean weights and length of the 6L:18D, 12L:12D and 18L:6D treatment groups over the 180 days of the experiment. The fish received excess food and the change appears to be related to energy given over to reproduction, as there is a negative correlation between DWG and the total number of eggs produced over the experiment.

At the beginning of this study, the fish were 18 months of age and had therefore been sexually mature for about 10 to 12 months. Sexually active tilapia partition more energy into reproduction rather than somatic growth, (Mair and Little, 1991; Macintosh and Little, 1995). Although there were no significant differences between the mean weights of the fish in the treatment groups at the beginning of the experiment, the means could be ranked (Table 1). Fish in the 24L:0D trial remained the largest mean weight but the relative ranking in size of the 6L:18D, 12L:12D and 18L:6D groups has reversed by the end of the experiment suggesting that the fish at the shorter and normal day (6L:18D, 12L:12D) were growing more quickly. The 18L:6D group was growing at the slowest rate, containing the smaller broodstock but the highest reproduction performance. The results suggest that the

diet was adequate to maintain both the growth and reproductive capacity of these fish for the duration of the experiment.

4.4. Effect of photoperiod on fish reproduction

It is, of course, highly likely that photoperiod directly influences the dynamics of ovarian development in tilapia. The precise mechanism involved has yet to be elucidated but is likely to be endocrine in origin. Tilapia have a similar melatonin secretion pattern to other vertebrates; high circulating levels by midnight which are totally abolished by the beginning of the light phase (Campos-Mendoza, unpublished). Photoperiod exerts influence on reproduction in fish by affecting the brain–pituitary–gonadal axis (for review, see Bromage et al., 2001). In brief, photoperiod manipulation brings about changes in gonadotropin releasing hormone (GnRH), and pituitary and plasma FSH (GtH I) and LH (GtH II) (Amano et al., 1994; Davies et al., 1995, 1999). Moreover, it is well established that light intensity and duration influences circulating levels of melatonin. Bromage et al. (1995, 2001) reported that melatonin levels are strongly correlated with photoperiod manipulation in salmonids resulting in the advance or delay of spawning time, suggesting that melatonin works as a regulator in reproductive behaviour. Furthermore, Amano et al. (2000) reported that melatonin is one of the factors that mediated the photoperiodic signals in the control of gonadal development in Masu salmon (*Oncorhynchus masou*), and that these changes in photoperiod are transduced by the melatonin rhythms which transfer this information to the brain–pituitary–gonadal axis.

A further explanation as to why fecundity varied between photoperiods might be simply due to basic reproductive strategy. The population of *O. niloticus* (Manzala, Egypt) is the most northerly occurring natural population of this species and as such would experienced seasonal changes in daylength. Light may act as an additional cue along with increasing water temperature to help the species to optimise and synchronise spawning early in the season. It would be interesting to see whether species with a much more restricted equatorial distribution respond in the same way to longer days. This study however, also demonstrates that fecundity and egg size may be determined, at least in part, by the ISI, confirming the earlier findings of Coward and Bromage (1999a). The basic mechanisms underlying the dynamics of ovarian development in a substrate-spawning species of tilapia were described in a highly quantitative manner by Coward and Bromage (1998, 1999b). Further research, however, is needed to assess how these basic dynamics might be influenced by exogenous (e.g. temperature and photoperiod) and endogenous (e.g. melatonin, gonadotropin, and sex steroid hormones) factors.

In conclusion, this study has shown that photoperiodic manipulation appears to be a reliable and powerful tool for tilapia broodstock management. Further research is needed to fully understand how photoperiod imparts such a powerful effect upon tilapia reproduction. Particular areas of concern include the effects of ‘biological’ and ‘reproductive’ age, the precise effects of light intensity, the possible interactions of photoperiod and temperature, and how photoperiod/light intensity might influence the reproductive endocrinology of tilapia, and thus affect the dynamics of ovarian development.

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class of proteins, called PLC ζ . We have been investigating how PLC ζ is generated in the testes and sperm using a novel transgenic technique, in vivo gene transfer. In vivo gene transfer is a new technique whereby a trans-gene is micro-injected directly into the seminiferous tubules of the testis. An electrical current is applied, opening pores in the spermatogenic cell membranes, introducing the transgenic DNA into the cell, to be expressed using the cells transcriptional machinery. We are using mammalian expression vectors to link PLC ζ to a fluorescent marker, namely green and yellow fluorescent protein, allowing us to visualise and track the expression pattern of PLC ζ within a living tissue. The ability to assess the function of PLC ζ in a living system is vital to our understanding of its activity during development, how it is regulated and how it behaves at fertilisation. Without the luxury of a working in vitro spermatogenic cell culture system the use of in vivo gene transfer should allow us to assess the function of PLC ζ while bypassing traditional time-consuming and complicated transgenic techniques.

A5.9

Interactions between Eph and FGF signalling revealed by somitogenesis in zebrafish

O. Ozkaya and C. Brennan, (School of Biological Sciences, Queen Mary University of London, London, UK)

Somites are the segmental units of the vertebrate mesoderm and give rise to all the skeletal muscles and the vertebral column. They are formed periodically during vertebrate segmentation by the process of somitogenesis. It has been suggested that somitogenesis involves a molecular segmentation clock: gene expression oscillates on and off in the cells of the presomitic mesoderm (PSM). A wavefront of maturation then sweeps back through this tissue, arresting oscillation and initiating somite differentiation. Cells arrested in different phases of their cycle express different genes and give rise to the anterior or posterior half of a somite (1).

Eph receptors are cell surface receptor tyrosine kinases and, together with their membrane bound ephrin ligands, they have been shown to have important roles in somite boundary formation (2). Disruption of Eph signalling by injection of a dominant negative ephrin leads to disruption of somite boundary formation (2). Timelapse analysis showed that later in development irregular shaped and bigger somites are formed on the injected side. According to the clock and wavefront model (1), larger somites can be formed by either a change in the period of oscillation of the clock, or a delay in the operation of the wavefront. We investigated these possibilities using in situ hybridisation analysis of dominant negative ephrin injected embryos.

(1) Cooke and Zeeman. *J Theor Biol.* 1976 May 21;58(2):455-76.

(2) Durbin et al. *Genes Dev.* 1998 Oct.1;12(19):3096-109.

A5.10

Photoperiodic manipulation and its effect upon the reproductive performance of the Nile tilapia, (*Oreochromis niloticus*)

A. Campos-Mendoza^a, B.J. McAndrew^a, K. Coward^b, N. Bromage^a, (^aInstitute of Aquaculture, University of Stirling Stirling, UK; ^bDepartment of Pharmacology, University of Oxford, Oxford, UK)

Tilapias are now a major aquaculture species with production levels of over a million tonnes annually. The hatchery production of fry is still very inefficient due to the relatively low fecundity and lack of spawning synchrony. Any methodology that enables farmers to synchronise the reproductive cycles of their broodstock would have immense practical advantages. Light is already known to play an important role in the initiation of gonad maturation in other fish species. In this investigation, the reproductive performance of 32 siblings of Nile tilapia was evaluated under four different photoperiods: short day (6L:18D), normal day (12L:12D), long day (18L:6D), and continuous illumination (24L:0D). Significantly larger eggs ($P < 0.05$) were produced under normal daylength (12L:12D) compared to other treatment groups. Fish reared under long daylength (18L:6D) exhibited significantly higher ($P < 0.05$) total fecundity (2408 ± 70 eggs spawn⁻¹) and relative fecundity (7.2 ± 0.2 eggs g⁻¹ body weight) concomitant with a significant reduction in inter-spawn-interval (ISI, 15 ± 1 days) in comparison with the rest of the trials. This investigation shows that long daylength (18L:6D) helps improve some important reproductive traits in Nile tilapia, and suggests that such methodology may be used to alleviate the production problems caused by low fecundity and poor spawning synchrony, and thus play a valuable future role in tilapia culture.

A5.11

Studies on the biosynthesis and reproductive functions of novel eicosanoids in barnacles

A.F. Rowley, B.H. Maskrey, (School of Biological Sciences, University of Wales Swansea, UK); G.W. Taylor, (Imperial College School of Medicine, London, UK)

Barnacles are key hard fouling organisms that encase man-made structures submerged in the marine environ-