Photoperiod effects on circadian rhythms and puberty onset in African catfish *Clarias gariepinus*

A thesis submitted to the University of Stirling for the degree of Doctor of Philosophy

By

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DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It is neither accepted, nor has been submitted for any other degrees. All the sources of information have been fully acknowledged.

Signature of Candidate ……

Signature of Supervisor ……

Signature of Supervisor ……

Date ……

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ABSTRACT

Photoperiod manipulation is routinely used in the aquaculture industry with the aim to enhance growth by manipulating the timing of reproduction in several commercially important temperate fish species. However, there are clear gaps in our understanding of how photoperiod is perceived by the circadian axis and transmitted to the brain to alter reproduction. Furthermore, due to the wide range of environments inhabited by fish, it is unlikely that one single organization exists. It is therefore believed that comparative studies of temperate species "models" with tropical species such as the African catfish (*Clarias gariepinus*) that adapted to different environments characterized by weaker light signals can help in such an aim. A number of studies were therefore performed in this PhD project to expand our knowledge on circadian biology and environmental physiological effects in African catfish.

The first aim was to characterize the circadian melatonin system in this species (chapter 3). Results clearly showed that the control of melatonin production by the pineal gland was very different in the African catfish as compared to temperate species such as salmon and trout. Indeed, melatonin production appeared to mainly depend on light stimuli perceived by the eyes as opposed to salmonids where light directly perceived by the pineal gland regulates its own melatonin production within photoreceptors. The main evidence was obtained in ophthalmectomised fish that were unable to synthesize and release melatonin into the blood circulation during the dark period. This was the first time that such a decentralized organisation, similar in a way to the mammalian system, was found in any teleost species. In vitro results also supported such findings as African catfish pineal glands in isolation were not able to normally produce melatonin at night as usually seen in all other fish species studied so far. This indirectly suggested that pineal gland photo-sensitivity might be different in this tropical species. Further studies were performed to better determine the amount of light that

can be perceived by the African catfish pineal gland depending on light transmittance though the skull (where the pineal gland is located). Surprisingly, it appeared that catfish cranium act as a stronger light filter than in other species resulting in lower light irradiance of the pineal gland. This could explain, although it still needs to be further confirmed, why African catfish photic control of melatonin produced by the pineal would have evolved differently than in temperate species.

The work then focused on better characterizing diel melatonin production and endogenous entrainment through exposure to continuous photic regimes (continuous light, LL or darkness, DD) (chapter 4). Daily melatonin profiles of fish exposed to 12L:12D photoperiod (routinely used in indoor systems) confirmed low melatonin production at day (<10 pg/ml) and increase at night (50 pg/ml) as reported in most vertebrate species studied to date. Interestingly, results also showed that melatonin production or suppression can anticipate the change from night to day with basal melatonin levels observed 45 mins prior to the switch on of the light. These observations clearly suggest the involvement of a clock-controlled system of melatonin secretion that is capable of anticipating the next photophase period. Furthermore, when constant light (LL) was applied, day/night melatonin rhythms were abolished as expected due to the constant photic inhibition of AANAT activity (e.g. one of the enzyme responsible for the conversion of serotonin into melatonin). However when fish were exposed to constant darkness (DD), a strong endogenous melatonin rhythm (maintained for at least 4 days and 18 days in catfish and Nile tilapia respectively) was found, demonstrating once again the presence of robust circadian oscillators in this species.

The next aim of the doctoral project was then to investigate circadian behaviour of catfish through locomotor activity studies (Chapter 5). African catfish is again a very interesting “model” due to its reported nocturnal activity rhythmicity as compared to most other teleosts species. Locomotor activity is considered as a very useful tool to elucidate the

mechanisms of circadian organization in both invertebrates and vertebrates circadian. Results first confirmed the nocturnal activity rhythms in the species. Furthermore, clear circadian endogenous rhythms were observed under constant light (LL) or darkness (DD) during several days before losing rhythmicity. Interestingly, the activity levels varied depending on the stocking density.

Finally, the last aim of this project was to test the effects of a range of photoperiodic manipulations on growth performances, sexual development and reproductive performances in African catfish reared from eggs to puberty. Results did not show any differences at the early sages (up to 90 days post hatching) in growth performances nor mortality (high) between control 12L:12D and LL treatments. In contrast, during the juvenile-adult period (from 120 to 360 DPH), significant growth effects were observed, as previously reported in other catfish species, with fish under LL displaying lower growth rate, food consumption and feed conversion efficiency in comparison to most other treatments (12:12, LL, 6:6, 6:18, 12-LL and LL-12) especially 12l:12D. However, no major effects of the photoperiodic treatments were observed with all fish recruited into puberty and developing gonads although differences in the timing of gametogenesis could be observed, especially a delay (circa 2 months) in females exposed to short daylength (6L:18D and 6L:6D). As for egg quality, egg diameter was the only parameter to differ between treatments (slightly larger in egg batch from LL treated females). Overall, none of the photoperiodic regime suppressed maturation in African catfish as opposed to some temperate species.

The work carried out during this PhD project clearly advanced our understanding of circadian rhythmicity, light perception and effects of photoperiod on physiology in a tropical species. Future studies are now required to further characterise the circadian system and link it to evolutionary trends within vertebrates.

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GLOSSARY OF COMMON AND SCIENTIF NAMES USED WITHIN THIS THESIS

Common name	***Latin name***
African clawed frog	*Xenopus laevis*
Asian sea bass (barramundi)	*Lates calcarifer*
Atlantic cod	*Gadus morhua*
Atlantic croaker	*Micropogonias undulatus*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Ayu	*Plecoglossus altivelis*
black porgy	*Mylio macrocephalus*
Brazilian cave catfish	*genus Taunayia*
brook trout	*Salvelinus fontinalis*
brown bullhead catfish	*Ictalurus nebulosus*
brown trout	*Salmo trutta*
burbot	*Lota lota*
channel catfish	*Ictalurus punctatus*
Chinese longsnout catfish	*leiocassis longirostris*
common carp	*Cyprinus carpio*
common mummichog	*Fundulus heteroclitus*
Eurasian perch	*Perca fluviatilis*
European catfish	*Silurus glanis*
European eel	*Anguilla anguilla*
European sea bass	*Dicentrarchus labrax*
gilthead sea bream	*Sparus aurata*
goldfish	*Carassius auratus*

haddock	*Melanogrammus aeglefinus*
Iberian wall lizard	*Podarcis hispanica*
Indian catfish	*Heteropneustes fossillis*
Italian green frog	*Rana peruzi*
Japanese amberjack	*Seriola quinqueradiata*
Japanese catfish	*Silurus asotus*
Japanese eel	*Anguilla japonicus*
Japanese sea catfish	*Plotosus lineatus*
Japanese sea perch	*Lateolabrax japonica*
Kusa fugu	*Takifugu niphobles*
lamprey	*Petromyzon marinus*
masu salmon	*Oncorhynchus masou*
Mozambique tilapia	*Oreochromis mossambicus*
North African catfish	*Clarias lazera*
Philippine catfish	*Clarias batrachus*
pike	*Esox lucius*
pink salmon	*Oncorhynchus gorbuscha*
Rabbitfish	*Siganus canaliculatus*
rainbow trout	*Oncorhynchus mykiss*
red sea bream	*Pagrus major*
Ruin Lizard	*Podarsis sicula*
sailfin molly	*Poecilia velifera*
sea catfish	*Arius felis*
Silver catfish	*Rhamdia quelen*
spotted sea trout	*Cynoscion nebulosus*

striped bass	*Morone saxatilis*
tench	*Tinca tinca*
Tengara catfish	*Mystus tengara*

CHAPTER 1

GENERAL INTRODUCTION

1.1 BIOLOGY OF AFRICAN CATFISH *C. GARIEPINUS*

1.1.1 Taxonomy of catfish

Catfish are highly diverse fish with a probable benthic ancestral habitat. They evolved during the Cretaceous/Ecocene about 70 million years ago and are considered as tertiary fish like most euteleosts. Ancestral catfish originated before the break-up of Gondwanaland and then evolved in both the New and Old worlds (Bruton, 1996).

Catfish belong to the superorder Ostariophysi which consists of two main series; the *Anolophysi* and the *Otophysi*. The *Anolophysi* has one single order; *Gonorhynchiformes*, represented by the milkfish. The *Otophysi* series has two orders; the *Cypriniformes* represented by the carp and the *Siluriformes* represented by the catfish (Figure 1.1). Catfish consist of 38 families containing approximate 416 genera and 2,584 species (Figure 1.2; Hecht and Britz, 1988). Two of these families correspond to marine catfishes; the *Ariidae* containing about 149 species and the coral catfishes or *Plotodisae*, about 32 species (Teugels, 1996). The African catfish belongs to the genus *Clarias* which has six subgenera; *Clarias (dinotopteroides), C. (Brevicephaloides), C. (Platycephaloides), C.* (*Clariodes), C. Anguilloclarias)* and *C. (Clarias)* (Teugels, 1982a). The species that are most often referred to in fish aquaculture in Africa belong to the subgenus *Clarias* Gronovius 1781 which, in the new classification (Teugels, 1982a), contains two species:

- *Clarias anguillaris* (Linnaeus 1758) synonym: *Clarias senegalensis* (Cuvier & Valenciennes 1840).
- *Clarias gariepinus* (Burchll 1822) synonyms: *Clarias lazera* (Cuvier & Valenciennes 1840) *Clarias mossambicus* (Peters 1852).

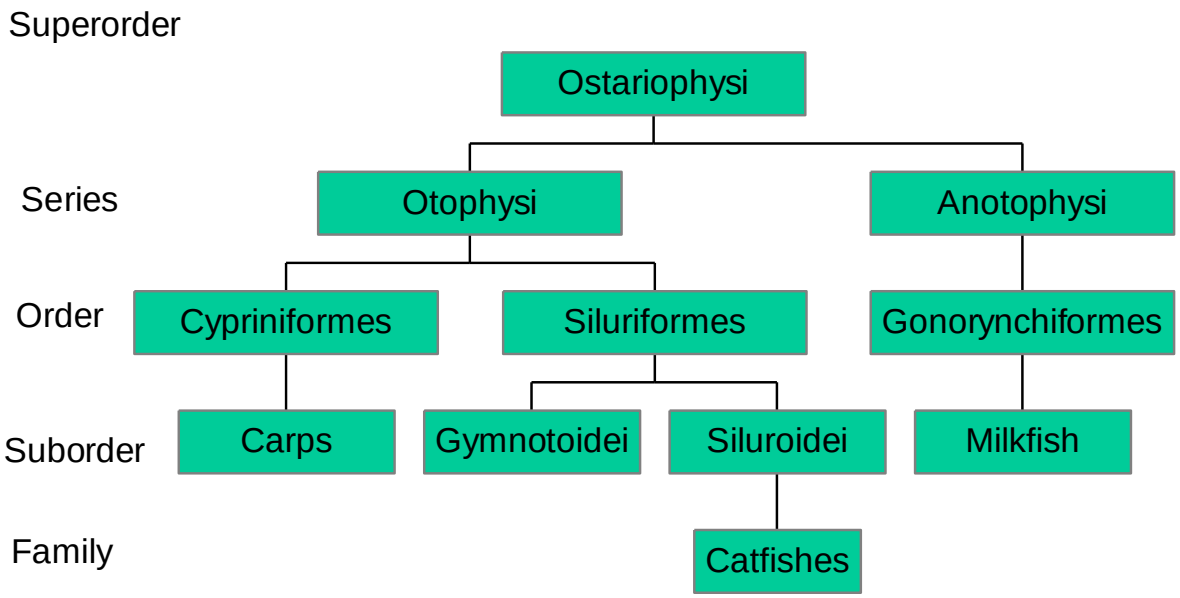

Figure 1.1 Taxonomy of Catfish (Teugels, 1996)

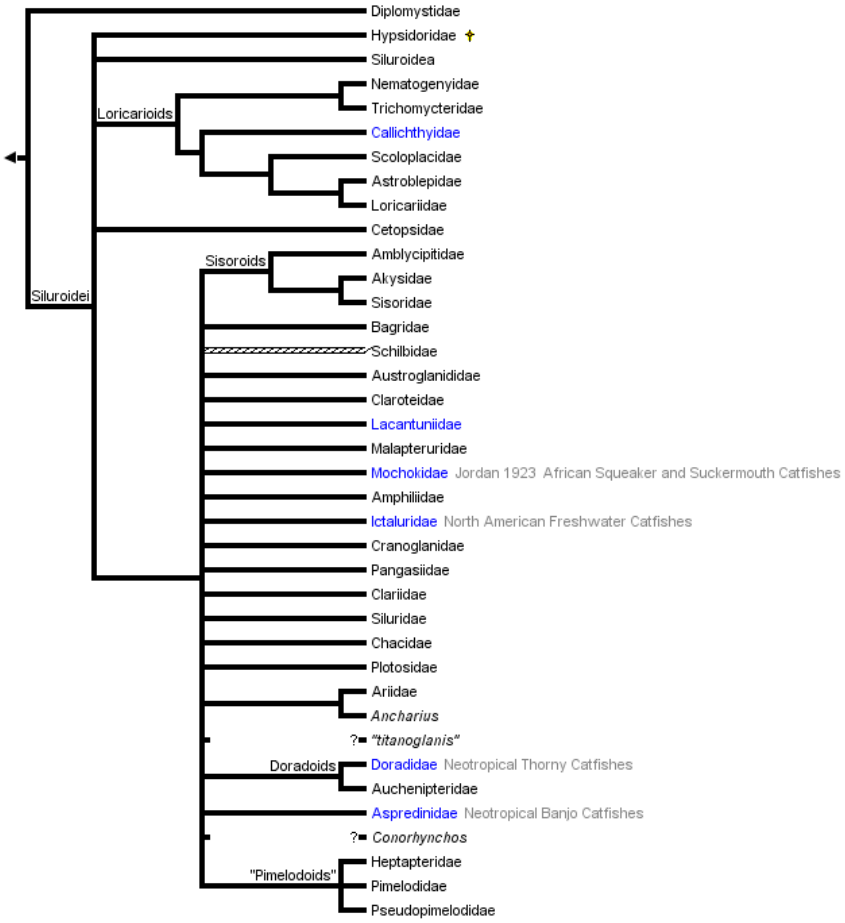

Figure 1.2 Taxonomy of Siluriforms (Lundberg and Friel, 2003).

1.1.2 Distribution and production of *C. gariepinus*

Catfish are commonly found in fresh and brackish water on all continents, and are especially abundant in the tropics of South America, Africa and Asia (Bruton, 1996). Naturally, 64% of all species known are confined to Central and South America, 19% of catfish species can be found in Africa, 15% in Eurasia and south-East Asia while about 2% of species are found in North America (Teugels 1996; Bruton, 1996). Generally, the most economically important species from the family *Clariidae* are *Clarias lazera* from the North and Central part of Africa, *C. mossambicus* from the Western part, *C. gariepinus* from the Southern part, *C. anguillaris* and *C. senegalensis* from the Eastern part of Africa.

At present, African catfish, especially *C. gariepinus*, are cultured in at least twelve African countries, the most important producers being Mali, Nigeria, Ethiopia and Ghana (FAO, 2008; Figure 1.3). In Asia *Clarias* spp. are farmed mainly in Thailand, Philippines, China, Israel, Malaysia and Indonesia. In Bangladesh *C. gariepinus* was introduced from Thailand in December 1989 and has been successfully bred since 1990 and was transferred from Bangladesh to Indian fish markets from 1993 (Singh *et al*., 2003). In Europe, *Clarias* spp have been cultured in the Netherlands, Germany and Belgium (Verreth *et al*. 1993). The Czech Republic (Adamek and Sukop, 1995) has begun to farm this species on both an extensive and intensive basis. In Latin America, catfish is mainly produced in Brazil (Hecht *et al*., 1996). The total capture (FAO, 2008) of *C. gariepinus* remained steady over the last decade (40-45,000 tones), but aquaculture production has shown a 6 fold increase from 2001 to 2007 (from 7,500 to 45,000 tonnes) (Figure 1.3).

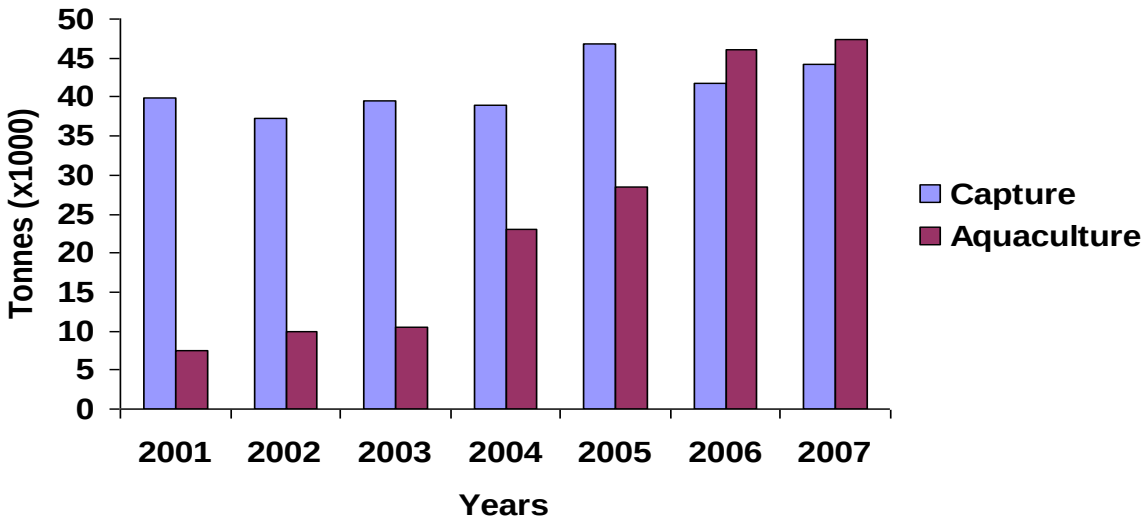

Figure 1.3 Global *C. gariepinus* production (FAO, 2008).

1.1.3 Life cycle of *C. gariepinus*

In nature, the size of *Clarias* individuals at first maturation varies from region to region and specific environmental conditions (Schulz and Goos, 1999). Generally, *C. gariepinus* reaches first sexual maturity at 6-9 months of age and at lengths of 200 - 300mm (Table 1.1). Spawning takes place at night usually after heavy rain in flooded marginal areas. During this time and before spawning, males compete and become very aggressive towards each other (Hecht and Britz, 1988).

Catfishes are found in three major breeding categories: non guarders, guarders and bearers (Hecht *et al*., 1988). *Clarias gariepinus* is a non guarder type with females spawning when environmental conditions are suitable (Haylor, 1992). There is no parental protection of the progeny; however, the spawning site is carefully selected. Absolute fecundity is high with 30,000 – 80,000 eggs per fish (mean weight of 540g) and fecundity of a ripe female represents 15-20% of the fish body weight (De Graaf *et al*., 1995).

Under laboratory and fish farming conditions, the African catfish reproductive cycle can be delayed or even suppressed due to confinement and stress as natural environmental conditions are difficult to mimic in such enclosed environments (Haylor, 1992). Rearing conditions are usually characterized by constant high temperature and relatively short daylength, which suppress the strong seasonality of *C. gariepinus* reproduction (annual rhythm of ovarian recrudescence and regression), observed in its natural habitats. Once puberty was reached, broodstock showed constant ovarian activity (Richter *et al*., 1987a). Fish can therefore be stripped all year long (monthly) for more than one year and fertilized eggs develop into normal fry. In the event that fish are not stripped, they then remain at a post-vitellogenic stage with intense atresia and recycling of oocytes. In captivity, *C. gariepinus* does not spawn naturally and must be induced by hormonal therapies. *C. gariepinus* seed has been produced artificially using a range of hormones such as

desoxycorticosterone acetate (DOCA), human chorionic gonadotropin (HCG), carp pituitary gland suspension and GnRH analogues (Richter and Van der Hurk, 1982). The catfish farming community in Africa has mainly been using a homoplastic pituitary gland suspension to induce spawning (De Graaf and Janssen, 1996). This method has been found to be highly reliable and, in comparison to synthetic hormone analogues, the technique is cheap and practical (Richter *et al*., 1995). This is of particular importance in African countries where sophisticated chemicals are often expensive and difficult to obtain.

For successful seed production, the timing of stripping after the hormonal treatment is important to obtain good quality eggs. It can be achieved by monitoring oocyte final maturation and ovulation time. Usually, at temperatures of 26-28ºC, stripping can be performed 8-10 hrs after hormonal injection (Table 1.2). Furthermore, the ovulation time and the development of newly fertilized eggs up to hatching depend on water temperature (Baidya and Senoo, 2002; 2004, Table 1.2). Hatching of African catfish embryos is quicker than in most other *Clariid* species. In commercial catfish hatcheries in southern Africa, a rearing temperature of 28^{o}C is used at which the larvae hatch after 16-18 hours. After swim-up, larval rearing is restricted to a 10-15 day period during which the fish are usually kept indoors under optimal conditions. For the first 3-5 days they receive a supplement of Artemia nauplii three times a day although several studies have shown that *Clariid* larvae can be reared successfully without *Artemia* or other zooplankton supplementation (De Graaf and Janssen, 1996).

African catfish is a group synchronous species. Ovogenesis can be classified into 6 chronological stages (Owiti and Dadzie, 1989): immature virgin (stage I), developing virgin (stage II), oocyte ripening (stage III), (oocyte maturing) (stage IV), spawning (stage V) and spent (stage VI, Table 1.3). Spent oocytes that are artificially released have a green appearance and can reach 1.2 mm diameter. Spermatogenesis in *C. gariepinus* is separated

into the following five phases (Schulz and Goos, 1999): spermatogonia (stage I), primary spermatocytes (stage II), secondary spermatocytes (stage III), spermatids (stage IV) and spermatozoa (stage V, Table 1.4).

Table 1.1 Median sizes at first maturity for *C. gariepinus* in various African countries (Haylor, 1993).

Total length (mm)		Location
Males	**Females**	
-	200	Lake Chad
380	380	Rhodesia
430-450	450-480	Vaal River
350-400	350-400	Transvaal
260	260	Malawi
650-750	650-700	South West Africa
350	350	South Africa

Table 1.2 The time interval between injection and stripping/ fertilization and hatching in relation to water temperature (Hogendoorn and Vismans, 1980).

Water Temperature (°C)	Time between injection and stripping (hours)	Time between fertilization and hatching (hours)
20	21	57
21	18	46
22	15.5	38
23	13.5	33
24	12	29
25	11	27
26	10	25
27	9	23
28	8	22
29	7.5	21
30	7	20

Table 1.3 Phases of oogenesis in *C. gariepinus* (Owiti and Dadzie, 1989).

Stage	Appearance
immature (ST1)	The ovary is colourless to translucent brown, lanceolate and lobular in appearance, occupying the posterior quarter of the body cavity. In fish larger than 10 cm the ovary can be distinguished from the testis due to its smoothness in contrast to the serrated edges of the testis. Histological description: nests of oogonia, numerous early, darker staining and few advanced lighter-staining, per-vitellogeneic oocytes visible.
developing virgin (ST2)	The ovary is translucent to brown in colour and occupies about one third of the length of the peritoneal cavity. Individual oocytes are visible with the naked eye as tiny specks. Histological description: the oocytes do not yet contain yolk. The size and number of primary oocytes increases.
oocyte ripening (ST3)	The ovary is opaque, brownish-green in colour, occupying about one half the length of the ventral cavity. Eggs are visible as yellowish-green or brownish-yellow granules. Blood capillaries are visible around the ovary. Histological description: oocytes in early vitellogenetic phase can be observed. The oocyte yolk is formed.
oocyte maturing (ST4)	Large ovary, opaque, and brown-green in colour. Eggs yolk clearly visible to the naked eye. Ovary occupies more than three quarters of the peritoneal cavity. Histological description: the oocyte increases to its final size of 1000-1200µm. A large nucleus (200µm) is clearly visible with signs of migration towards the periphery of the oocyte. The oocytes remain at this stage until environmental factors (rainfall or a hormonal injection) stimulate ovulation.
spawning (ST5)	Eggs are translucent and easily extruded on slight pressure or just by handling the fish. Histological description: large eggs contain coarse granules throughout the cytoplasm. Germinal vesicles start to migrate to the animal pole, where they remain.
Spent (ST6)	Ovary flaccid, flabby with thick whitish tough walls. Genital aperture of female looks inflamed. Some translucent and opaque (residual) eggs visible to the naked eye. Histological description: large numbers of ruptured follicles visible in ovary. Some yolky eggs undergoing atresia as well as presence of early yolky oocytes, non-yolky oocytes and oogonia.

Table 1.4 Phases of spermatogenesis in *C. gariepinus* (Owiti and Dadzie, 1989).

Stage	Appearance
Spermatogonia (ST1)	Testes are organised as a pair of small, thread-like elongated colourless organs with slightly serrated edges. Histological description: primary germ cells, spermatogonia and cysts containing primary and secondary spermatocytes can be found.
Primary Spermatocytes (ST2)	A translucent white colour, occupying about one-third of the length of the body cavity. Serrations at edges become sharp and more clear. Histological description: similar to the stage 1 germ cells, few cysts containing spermatids are visible.
Secondary Spermatocytes (ST3)	Testes are more enlarged, occupying half the length of the body cavity, opaque and grey-white in colour. Histological description: increase in number of germ cells especially cysts containing secondary spermatocytes and spermatids. Cysts containing spermatozoa become evident towards the end of this stage.
Spermatids (ST4)	Testes are turgid, greasy- white in colour, greatly enlarged, occupying almost two-thirds of the ventral cavity. Serrated edges become roundish and blunt. Milt is not extruded by pressure as in other fishes due to interference from a finger-like structure separating the testes from the genital opening. Histological description: lobules packed with spermatozoa evident as well as cysts containing developing germ cells.
Spermatozoa (ST5)	Testes shrunken and flaccid. Serrated edges revert back to original sharp appearance. Histological description: numerous collapsed lobules and some residual sperm evident.

1.2. ENVIRONMENTAL CONTROL OF PHYSIOLOGY IN TELEOSTS

In teleost fishes, photoperiod, temperature and seasonal rainfall, are important in regulating reproductive cycles among other environmental factors. These exhibit large seasonal fluctuations in temperate regions, whereas in the tropics, dry and wet seasons alternate leading to seasonal differences in water quality and food availability with small variations in temperature and photoperiod (Haylor, 1992).

1.2.1 Importance of biological rhythms

Most living organisms have adapted to the daily and seasonal changes in daylength which are driven by the rotation of the earth and moon, or temperature, salinity and food availability that entrains their diel activity rhythms. These rhythms are crucial for the synchronisation of most physiological functions such as sleeping patterns, feeding, locomotor activity, migration, reproduction as well as daily rhythms at the molecular and endocrine levels (Gerkema, 1992; Boujard and Leatherland, 1992; Foster and Kreitzman, 2005). The daily light/dark cycle is generally considered to be the most important driver of circadian clocks as well as temperature cycles, feeding schedules, and social interactions that can also affect the timing of clock mechanisms (Bromage *et al*., 2001). These rhythms provide a selective advantage to animals by allowing them to anticipate changes in their environment to adjust their period of activity and optimize their survival as a result of millions of years of evolution. They also confer many other advantages such as energy saving and avoidance of UV-mediated DNA damage as well as crucial synchronization of metabolic requirements (Langmesser and Albrecht, 2006).

1.2.2 Photic cues

Light can be described in terms of its properties of periodicity (seasonal daylength changes), quality (e.g. spectral profile) and quantity (e.g. light intensity) (Downing and Litvak, 2002).

1.2.2.1 Photoperiod

Photoperiod (length of day *vs* night) is the most predictable "noise free" environmental signal which remains precisely the same from year to year and reflects seasonality. It is therefore the main cue used by most seasonal animals to entrain/synchronise their physiology and especially reproduction. Studies have shown that photoperiod is the principal factor regulating reproduction in salmonids, gadoids, bass, flatfish, breams, mullet, sciaenids, cyprinids and serriolids (Bromage and Cumaranatunga, 1988; Poncin, 1989; Bromage *et al*., 1993, 2001) although temperature can also play a pivotal role especially towards the end of the reproductive cycle (timing of spawning). All these species inhabit temperate latitudes where seasonality is at its strongest. Photoperiodic animals have been classified into long-day and short-day breeders. In long-day temperate animals such as Atlantic salmon (Taranger *et al*., 1999), rainbow trout (Randall *et al.*, 1998b) Atlantic halibut (Norberg *et al*., 2001) and turbot (Imsland *et al.*, 2003), it is the increasing of daylength (from winter to summer) that triggers the onset of maturation (decision window) and short days are inhibitory. In short-day animals such as Atlantic cod (Davie *et al*., 2007b), haddock (Davie *et al*., 2007a), Eurasian perch (Fontaine *et al*., 2006) and European sea bass (Felip *et al*., 2008), it is the decreasing daylength that entrains reproductive cycles with long-days being inhibitory (Bromage *et al*., 2001). In aquaculture, the strong seasonality of spawning is one of the major constraints that must be addressed due to the restriction of eggs and fry supplies at specific times of the year. Photoperiod

manipulation can therefore be a very effective, non invasive and cheap tool to manipulate reproduction and spawning in aquaculture systems (Bromage *et al*., 2001) (see section 4.2). In tropical species, although seasonal changes in daylength can be weak, photoperiod is still a key signal that entrains the animal physiology and behaviour.

1.2.2.2 Light intensity and spectrum

One major question regarding light manipulation in fish farming relates to the duration and quality of the photic signal (Bromage *et al.,* 2001; Boeuf and Falcon 2001). If photoperiod varies along the annual cycle in a regular and predictable manner, light quality in terms of intensity and spectrum is less predictable and will depend on habitats characterized by depth, clarity and structure. Thus, in order to optimize the rearing conditions and eventually being able to manipulate fish physiology one has to consider the respective durations of day and night and, in both situations, light intensity, spectral composition and orientation (Migaud *et al*., 2006). It is important to emphasize that fish perceive light both from above (*via* the pineal organ), the sides (*via* the eyes) and possibly through deep brain photoreceptors. All these light centres may have different sensitivities.

Fish are able to visually discriminate colours although species specific sensitivities exist probably as a result to adaptation of the visual and non visual systems to specific natural habitats (Cheng and Flamarique, 2004). Light of different spectral composition can therefore affect fish growth and survival as shown in different species (Head and Malison, 2000; Downing and Litvak, 2001; Ruchin, 2004), body pigmentation (Van der Salm *et al*., 2004), stress response (Head and Malison, 2000; Volpato and Barreto, 2001), behaviour (Marchesan *et al*., 2005) and reproduction (Naor *et al*., 2003; Boulcott *et al*., 2005).

In African catfish, Britz and Pienaar (1992) and Almazan-Rueda *et al*. (2005) have reported that different photoperiod and light intensity regimes can affect African catfish

behaviour and growth rate, and they suggested that light can act as a stressor. Light conditions can probably partly explain differences in fish performance and behaviour observed in catfish species. Of interest, Han *et al.* (2005) showed reduced growth in Chinese longsnout catfish exposed to low (5 lux) or high (443 lux) light intensities, with specific growth rate (SGR) and feed conversion efficiency (FCE) higher in fish exposed to medium light intensities (74 lux). Similar results were found in *C. gariepinus* with lower growth rate and higher activity under 150 lux as compared to 15 lux, this was suggested to be related to stress (Almazan *et al*., 2004). Britz and Pienaar (1992) showed that stress, aggression and cannibalism were reduced and growth enhanced in *C. gariepinus* exposed to lighting conditions mimicking natural conditions (continuous darkness or low light intensities).

However, some species can grow better and develop quicker at low light intensities, such as striped bass larvae at 1 lx (Chesney, 1989), juvenile halibut at 1–10 lx (Hole and Pittman, 1995) and juvenile haddock at 30 lx (Trippel and Neil, 2003). On the other hand, some species were reported to show improved growth at very intense light levels, European sea bass larvae at 1400–3500 lx (Barahona–Fernandes, 1979), Atlantic cod larvae at 2400 lx (Puvanendran and Brown, 2002), and black porgy juveniles at 3000 lx (Kiyono and Hirano, 1981). It seems that the effect of light intensity on growth and survival are species-specific (Puvanendran and Brown, 2002).

1.2.3 Temperature

Reproduction of many carps and other cyprinids, catfish and other tropical and sub-tropical species is affected by temperature. Thermal manipulations can therefore be used to control the timing of reproduction and especially spawning time (Bromage *et al*., 2001). This is especially true in Cyprinids in which it has been reported that low temperature

(<3°C) in common carp can affect the final stages of oocytes maturation and inhibit ovulation (Davies, 1986; Bromage *et al*., 2001). Temperatures ranging from 8 to 11°C appear to have no effect on timing of the maturation and reproduction cycle. A study performed in the North African catfish tested the effects of two temperatures (25°C and 30°C) on gonadogenesis and found significantly higher hatching rates in eggs obtained from females kept at 25°C (Richter *et al.*, 1982). Similar results have been reported for Chinese catfish *Clarias fuscus*. Young (1990) reported that females developed mature ovaries and were induced to spawn successfully under constant or long photoperiod at 25°C but at 30°C, regardless of photoperiod, all female ovaries matured. In another experiment, Richter *et al*., (1987a) suggested that seasonality of the ovarian cycle of *C. gariepinus* can be suppressed by constant high water temperature. In the Indian catfish, photoperiod and temperature can affect gonadal activity, but apparently, temperature is the predominant (proximate) cue with photoperiod playing a more permissive (ultimate) role (Vasal and Sundararaj, 1976; Sundararaj and Vasal, 1976). Long day could stimulate ovarian recrudescence in Indian catfish when exposed during the gametogenesis period (February-April) for 6 weeks at a temperature of 25°C. Interestingly at temperatures over 30°C, vitellogenesis was stimulated regardless of the photoperiod (e.g. 12L:12D, 14L:10D, continuous light, or darkness). Temperature is clearly a key signal in tropical and subtropical regions where the changes in daylength are not strongly marked, but rainfall and water quality are also important environmental factors regulating reproduction in catfish (Haylor, 1993).

1.2.4 Rainfall and salinity

Catfish reproduction is definitely influenced by rainfall. In natural habitats, African catfish reproduction is synchronized to the rainy season with postvitellogenic oocytes

observed during the period of maximum rainfall (Haylor, 1992). This phenomenon is probably due to increasing water levels associated with changes in temperature (relatively cooler) as well as water quality changes (hormones, chemical, waste) (Bruton 1979a).

Adult North African catfish (the common name of *Clarias gariepinus*) can live in brackish water between 12-16ppt, although the maximum salinity tolerance of fingerlings is 9–9.5 ppt (Chervinski, 1984). In laboratory conditions, restrictions in water and decrease in salinity (<5‰) during spawning time give best results. Salinities above this threshold can result in very significant egg mortalities. Gbulubo and Erondu (1998) studied the effects of salinities on the hatching and survival of African catfish eggs and hatched fry. They showed that optimal salinity for the hatching of the eggs ranged from 0–5‰. Above 5‰, hatching was significantly lowered and no hatching occurred at 8‰. African catfish body fluid osmolarity is equivalent to a salinity of approximately 8–10 ‰ (Giese, 1973). Survival and growth of African catfish fry have been found not to be significantly different in salinities between 0 and 5‰ (Britz and Hecht, 1989).

Other environmental factors (biotic and abiotic) that do not act as cues *per se* but will inevitably affect the reproductive and growth performance of fish are: water quality, stocking densities and stress level (Ross 2000; Pankhurst and Porter 2003). These factors are normally inevitably interlinked in the wild and if appropriate conditions occur, will create opportunistic windows for enhanced reproductive activity and/or performance. In seasonal species these factors will also contribute to the timing and synchronizing of the final stages of reproductive development and spawning time (Pankhurst and Porter 2003). Stocking densities also play an important role in African catfish performance. Maximal stocking densities were reported to be 250 larvae/L during early rearing stages, 600 fish/m3 for juveniles and up to 150 fish/m3 for adults (Haylor, 1992). Importantly, when

reared at low densities, African catfish cannibalistic behaviour is enhanced, performance reduced and overall survival can be poor.

1.3 LIGHT PERCEPTION: THE CIRCADIAN AXIS AND PHOTOPERIODIC SENSING

1.3.1 Melatonin: the light perception hormone

Melatonin is a common output signal of the vertebrate circadian clock which is produced primarily by the pineal organ and released into the blood stream (Falcon *et al*., 2007). Melatonin is also synthesized in the retina and has been detected in the gastrointestinal tissues (Besseau *et al.*, 2006; Bubenik *et al*., 1997). There is evidence indicating that pineal melatonin contributes largely to the circulating plasma and cerebrospinal fluid (CSF) levels (Tricoire *et al*., 2002), whereas ocular melatonin serves local functions in an autocrine/paracrine mode. The role of melatonin is still not elucidated in fish, it can act on reproduction in seasonal breeders, be involved in the regulation of circadian rhythms (including locomotor activity, body temperature and feeding) amongst other suggested roles (Arendt, 1995; Goldman, 2001; Pevet *et al.*, 2002; Zawilska and Nowak, 1999). However, crucially, direct evidences of melatonin actions are still lacking in fish (Mayer *et al.,* 1997; Falcon *et al.,* 2007) as opposed to higher vertebrates (mainly mammals) where clear links between reproduction and melatonin have been reported (Ardent, 1998). Retinal melatonin acts primarily within the eye, where it is involved in the control of rhythmic processes, such as retinomotor movements, dopamine synthesis, release and metabolism, rod outer segment discs shedding and phagocytosis (Cahill and Besharse, 1995; Zawilska and Nowak, 1992 and Zawilska and Nowak, 1999). In mammals, however, the pineal gland is the only organ capable of synthesizing melatonin, and hence its removal completely abolishes the melatonin rhythm. In fish, the retina can also synthesize melatonin, which could explain why pinealectomy diminishes, but does not abolish, the daily rhythm of melatonin in the blood in some species, but these melatonin levels are not significant and will not effect blood stream concentration (Bromage *et al*.,

2001). The retinal melatonin production profile of 32 teleosts has been studies by Ligo *et al*. (2007) and classified into three types; (1) normal profiles, which is parallel with the pineal releasing the melatonin hormone during the dark phase (24 out of 32 species); (2) reversed profile with higher levels of melatonin during the light phase (4 species; common mummichog, European sea bass, Nile tilapia and torafugu); (3) no significant differences in daily melatonin profile (4 species; Japanese eel, Japanese sea perch, Japanese amberjack, and Kusa fugu). These differential patterns of retinal melatonin production can be explained by differences in the molecular machinery responsible within photoreceptor cells for melatonin production and regulation (Falcon *et al*., 2007).

In almost all species studied to date, pineal melatonin levels have been shown to increase during the night and remain low during the day, thus reflecting the prevailing photoperiod throughout the seasons (Delgado and Vivien-Roels, 1989; Zachmann *et al*., 1992b; Randall *et al*., 1995a; Bromage *et al*., 1995, 2001; Falcon *et al*., 1989; Yanez and Meissl, 1996; Porter *et al*., 1996, 2001; Ekstrom and Meissl, 1997; Mayer *et al*., 1997a, 1998; Falcon, 1999; Amano *et al*., 2000; Bromage *et al.,* 2001; Falcon *et al.,* 2007). In a study on daily variation and localization of melatonin-binding sites in Japanese catfish brain, results showed that plasma melatonin levels were higher during the dark phase (40-50pg/ml) than during the light phase (5-10pg/ml) (Iigo *et al.*, 1997).

Although the pattern of production is conserved across all vertebrates (high at night/low during the day), three variants have been identified (Figure 1.4). The most common profile, C-type, is characterised by a rapid rise in melatonin immediately following the onset of the dark period (within the first hour of darkness) and is commonly found in migratory salmonids and other teleosts as well as higher vertebrates such as domestic cat, Djungarian hamster and sheep (Reiter, 1988; Randall *et al.,* 1995). A-type profiles are characterised by a delay after the start of the dark phase before melatonin rises to its peak towards the end

of the dark phase. This was observed in gadoid species such as Atlantic cod and haddock as well as mouse and Syrian hamster (Reiter, 1988; Porter *et al.*, 2001; Davie *et al.*, 2007a). Finally, B-type profiles are characterised by a discrete peak in the middle of the dark phase as observed in Mozambique tilapia and human (Reiter, 1988; Nikaido *et al.*, 2009). The meanings of these different profile types are not understood but might be linked to an ability or inability to anticipate photic signals under the control of circadian clocks (Reiter, 1988).

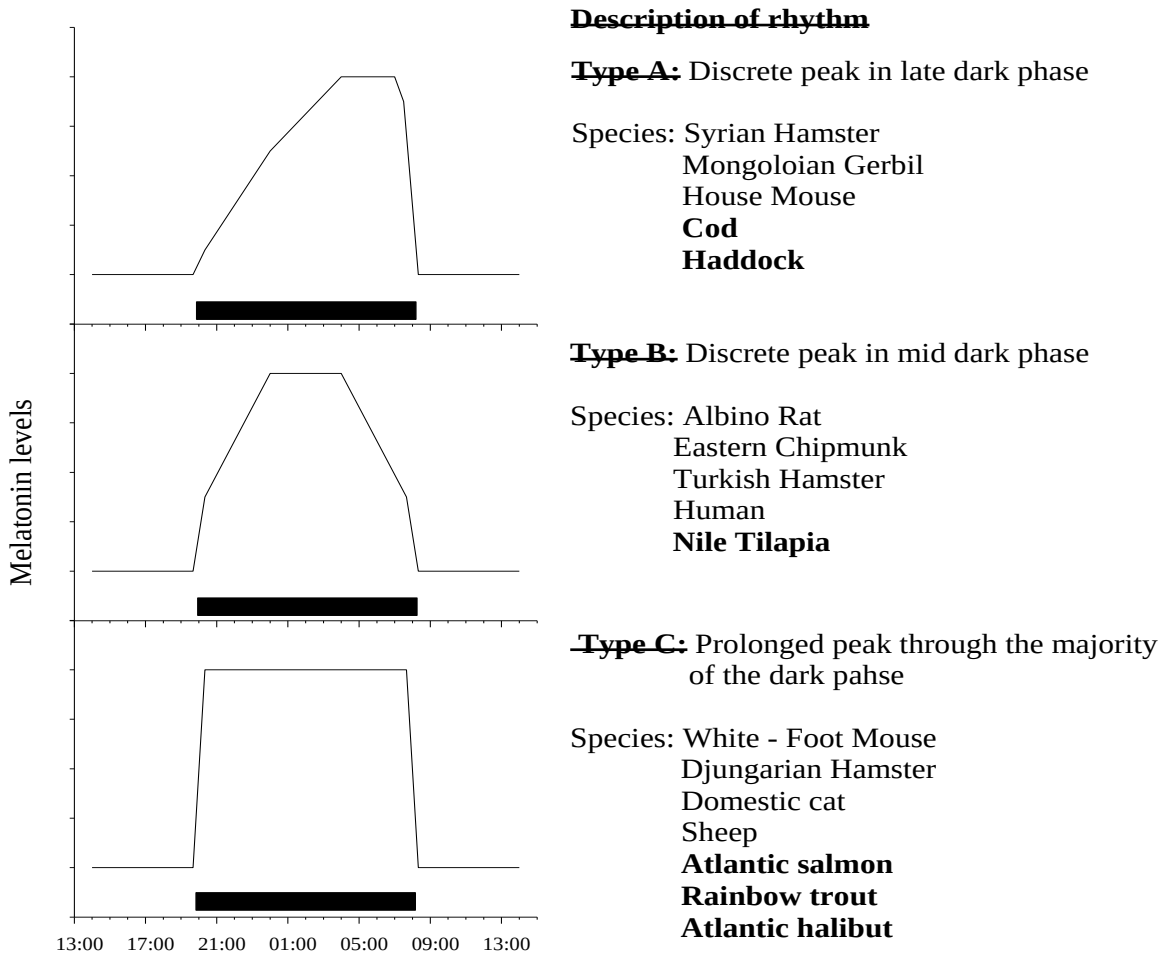

Figure 1.4 Diagrammatic representation of the different melatonin profiles recorded in vertebrates. Examples of species which express such patterns of plasma melatonin for each profile are listed. Horizontal black bar denotes subjective dark period (adapted from Reiter, 1988).

Large differences in melatonin peak levels between teleosts have also been observed ranging from 10-260 $pg.ml^{-1}$ (Table 1.5) such as in sea bass (Bayarri *et al.*,, 2004a; Migaud *et al*., 2006; Lopez-Olmeda *et al*., 2009), cod and haddock (Bromage *et al.,* 2001; Davie *et al.,* 2007a, b) to 300-800 $pg.ml^{-1}$ mainly observed in salmonid species (Randall *et al.,* 1995; Migaud *et al.,* 2006). No melatonin levels have been reported in African catfish only in Japanese catfish. However, the large variability reported between studies in the same species are probably related to a number of factors including analytical techniques (RIA, ELISA, HPLC), developmental stage (size, nutritional and reproductive status) and age of the fish, season with associated light and temperature conditions and others. Indeed, many studies highlighted the environmental regulation of daily and annual melatonin variations such as light, temperature and recently salinity as well as self-sustained endogenous rhythms (Bolliet *et al.*, 1996; Bromage *et al.,* 2001; Lopez-Olmeda *et al*., 2006). All these factors highlight the difficulties generally encountered when attempting to compare and review existing data in fish.

The production of melatonin by the pineal gland in a given fish species might also be correlated to the amount and spectral quality of the light reaching the pineal photoreceptors depending on the degree of absorbance of the skin and skull. The amount and quality of light that crosses the pineal window varies from one species to another (Gern *et al*., 1992; Migaud *et al*., 2006). Differences in light penetration through the skull range from 1 to 8% of simulated daylight in Atlantic salmon and sea bass and long wavelengths (650–700 nm) are far more effective at penetrating the skull than shorter wavelengths (400–450 nm) (Migaud *et al*., 2006). In terms of melatonin production the threshold of light intensity above which melatonin is suppressed depends on the species, experimental conditions, light quality and duration (Aoki *et al.*,, 1998; Bayarri *et al.*,, 2002; Vera *et al*., 2005; Migaud *et al*., 2006; Oliveira *et al*., 2007).

All these findings have led researchers to suggest that a threshold value of light intensity and water quality (such as salinity) must exist in order to influence physiological functions in fish (Oppedal *et al*., 1999; Porter *et al*., 1999, 2001; Migaud *et al*., 2006). Recently, Migaud *et al*. (2006) have calculated the light intensity threshold for Atlantic salmon to be around 0.016 $W.m^{-2}$, after allowing for the 2.4% of light lost during transmission through the cranium. Importantly, large species specific differences were observed with cod, for example, being up to 10,000 times more sensitive than salmon.

1.3.1.1 The Pineal gland

Simonneaux and Ribelayga (2003) reviewed the history of the vertebrate pineal gland focusing on mammals. Descartes was the one of the first to describe the pineal gland as the third eye during the 17^{th} century. At the end of 19^{th} century, Ahlborn and Rabl-Ruckhardt described the anatomy, histology, innervation, and embryology of the mammalian pineal gland but its function remained unknown. At the beginning of the 20^{th} century the physiological role of the pineal gland was studied. In 1943, Bargman suggested that the endocrine function of the pineal gland was regulated by light, via the central nervous system. From the 1970s, the number of publications on the pineal gland increased.

In most poiklothermic vertebrates, the pineal complex has two components. In fish, they are the pineal and parapineal organs, which look similar during ontogeny, but reach different levels of differentiation in the adult (Ekstrom and Veen, 1983; Borg, *et al*., 1983). The parapineal organ remains more or less rudimentary, while the pineal organ grows to form a relatively large vesicle located dorsally to the forebrain, immediately below or within the skull roof. The pineal organ is often differentiated into a proximal slender pineal stalk and a distal expanded end-vesicle. The wall of the pineal organ is formed by unistratified epithelium, which is strongly folded and may almost obliterate the central

lumen of the pineal organ (Ekstrom and Meissl, 1997). The pineal epithelium of teleosts consists of photoreceptor cells, neurons, and ependymal interstitial cells which are often called supportive cells, interstitial cells, or glial cells (Figure 1.5). Some oligodendrocytes have been found surrounding neural axons and macrophages are found in the central lumen of the pineal. Photoreceptor cells produce the indolamine melatonin. The pineal gland translates photoperiodic information into a hormonal signal which would then serve as a messenger to every organ of the body (Borg, *et al*., 1983). The size of the pineal organ varies considerably amongst fish species.

In some species, pineal glands are large and cover most of the telencephalon (e.g. pike or salmon) and in other species such as cod, sea bass and tilapia, the pineal gland is less conspicuous and lacks its characteristic vesicle form (Ekstrom and Meissl, 1997).

1.3.1.2 The Retina

Yasutake and Wales (1983) and Chinabut *et al*. (1991) have described the structure of the fish eye. It is composed of different tissues, including the cornea, the iris, the lens and the retina (Figure 1.6). The retina is the most complex and is made up of six layers: the pigmented epithelial (PE) covering the visual layer of rods and cones that determine how much light can enter the eye. The rod and cone photoreceptors of the visual layer detect light and colour, making up the outer nuclear layer (ONL), and are arranged into three configurations: twin cones, single cones, and rods. The tips of the rods and cones extend into the outer plexiform layer (OPL), which is covered by three layers characterized by the predominance of different neuron types, the inner nuclear layer (INL), the inner plexiform layer (IPL) and the ganglion cell layer (GCL). Finally, there is a ganglion cell layer consisting of nerve fibers that extend, via the optic tract, to the brain.

Table 1.5 Plasma melatonin concentrations measured during day (light phase, L) and night (dark phase, D) in different fish species at different developmental stages and reared under different culture conditions of photoperiod, salinity (freshwater, FW or sea water, SW) and temperature.

Fish common name	Melatonin levels (pg/ml)	Fish size	Culture conditions	References
Rainbow trout	20 (L) / 100 (D)	Juvenile, 150g	12L:12D, FW, 6 °C	Taylor *et al.*, 2006
Brook trout	250 (L)/800 (D)	Adult, 110g	16L:8D, FW, 12°C	Zachmann *et al.*, 1992b
Atlantic salmon	200 (L) / 800 (D)	Smolt, 590g	12L:12D, SW, 10 °C	Migaud *et al*., 2006,
European sea bass	20 (L) / 265 (D)	Adult, 1193g	16L:8D, FW, 12°C	Porter *et al*., 2000
	10 (L) / 150 (D)	Juvenile, 87g	14L:10D, SW, 19 °C	Bayarri *et al.*, 2003
	10 (L) / 100 (D)	Juvenile, 107g	18L:6D, SW	Bayarri *et al.*, 2004
	20 (L) / 200 (D)	Juvenile, 110g	12L:12D SW, 23 °C	Migaud *et al.*, 2006
	50 (L) / 110 (D)	Juvenile, 117g	12L:12D, SW, 23°C	Lopez-Olmeda *et al.*, 2009
Mozambique tilapia	80 (L) / 180 (D)	Adult, 430g	12L:12D, FW, 25°C	Nikaido *et al.*,2009
Lamprey	12(L) / 80 (D)	Adult, 300g	7.5L:16.5D,FW,4.5°C	Mayer *et al*., 1998
Japanese Catfish	5 (L)/ 40 (D)	Adult, 220g	12L:12D, FW, 22°C	Iigo *et al.*, 1997
Rabbitfish	80 (L) 250 (D)	Adult, 450g	12L:12D, SW, 24°C	Rahman *et al*., 2004

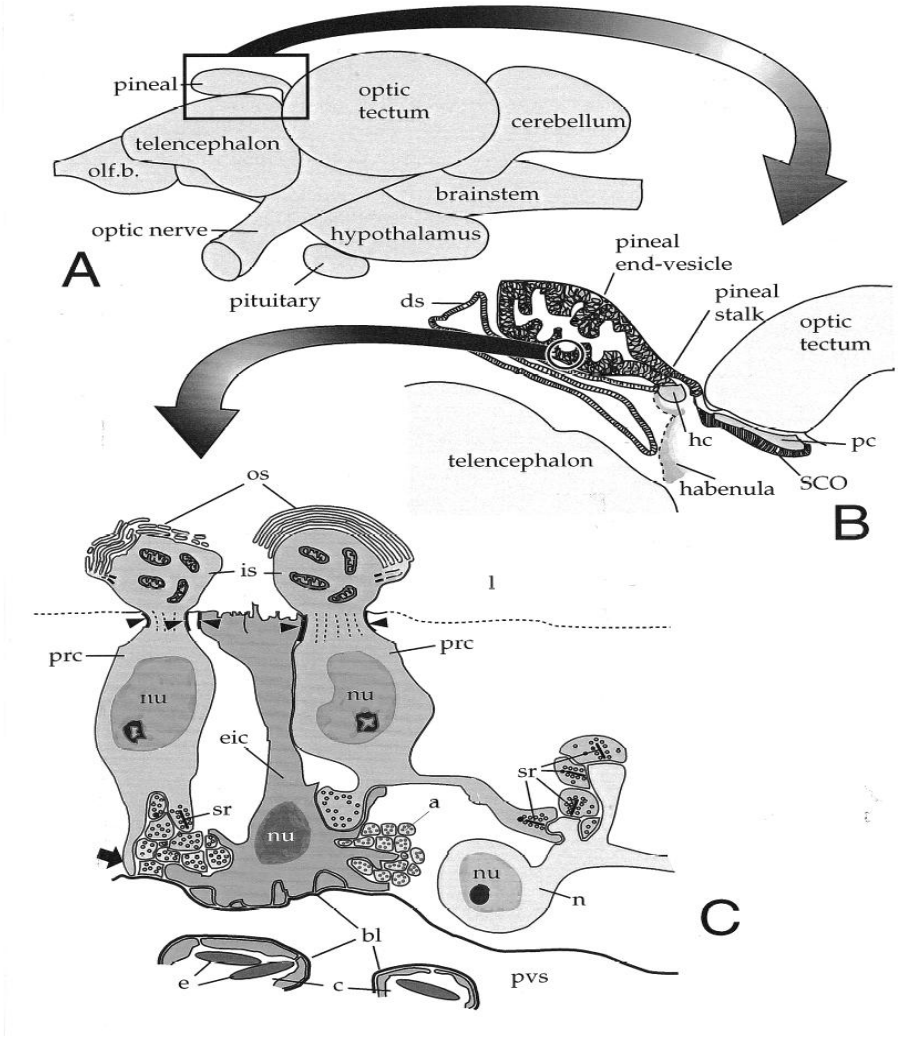

Figure 1.5 Pineal anatomy: (A) Overview of the major subdivision of the teleost (here, salmonid) brain: olf. b., olfactory bulb. (B) Schematic rendering of a midsagittal section throug the area indicated by the rectangular fame in (A), showing the topological relation of the pineal organ to the dorsal diencephalon (habenula and habenular commissure, hc), midbrain optic tectum, and telencephalon: ds, dorsal sac; pc, posterior commissure; sea, subcommissural organ. (C) Schematic rendering of cell types in the epithelium of the pineal end-vesicle, indicated by a circular frame in (B): a, axons of pineal neurons, gathered in a bundle; bl, basal lamina; c, capillaries; e, erythrocytes; eie, interstitial cell; is, photoreceptor inner segment with mitochondria; I, pineal lumen; n, centrally projecting neuron, 'ganglion cell'; nu, nucleus; os, photoreceptor outer segment; prc, photoreceptor cells; pvs, perivascular space; sr, synaptic ribbons; arrow indicates photoreceptor basal process that terminates on the basal lamina (note that such have been described in only the pike and the rockling) arrowheads indicate tight junctions that form a barrier against the cerebrospinal fluid of the pineal lumen. Drawing modified from Falcon (1979).

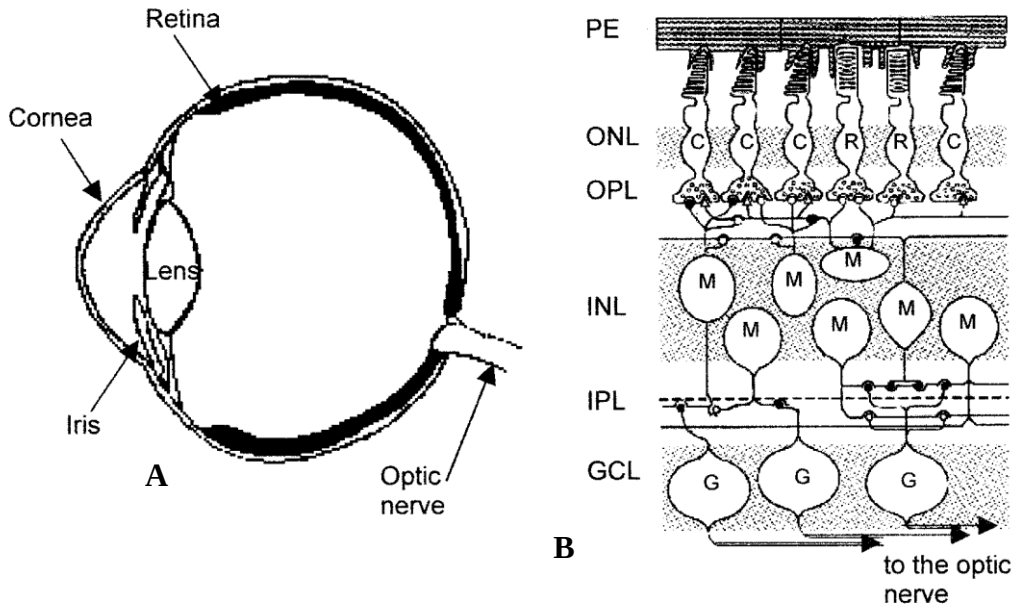

Figure 1.6 Simplified schematic representation of the structure of the vertebrate eye: A; gross morphology (adapted from Groos, 1982); B simplified organization of the retina (adapted from Morgan *et al*., 1994). Key: PE- pigment epithelium; ONL- outer nuclear layer; OPL- outer plexiform layer; INL- inner nuclear layer; IPL- inner plexiform layer; GCL- ganglion cell layer; R- rod; C- cone; M- mixed retinal cell types; G- ganglion cell.

1.3.1.3 Melatonin synthesis

Photoreceptor cells take up tryptophan from the circulation and convert it into serotonin by means of the successive action of tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (AAAD). The first step in the biosynthesis of melatonin is the conversion of tryptophan to 5-hydroxytryptophan, by means of tryptophan hydroxylase (TPOH) (Falcon 1999). 5-Hydroxytryptophan is then decarboxylated by the aromatic amino-acid decarboxylase to produce serotonin. Melatonin is then synthesized from serotonin by the action of two enzymes. The first, arylalkylamine *N*-acetyltransferase (AA-NAT), converts serotonin to *N*-acetylserotonin. The second, hydroxyindole-*O*-methyltransferase (HIOMT), methylates *N*-acetylserotonin to produce melatonin (Figure 1.7). The pineal gland transduces this neural information into hormonal information in the form of a daily rhythm of melatonin.

- Arylalkylamine *N*-acetyltransferase (AANAT): melatonin rate-limiting enzyme

In order to better understand the melatonin synthesis pathway, activities of HIOMT and AANAT enzymes were investigated in different species. An increase in melatonin production at night reflects increased AANAT activity and termination of melatonin production during the day reflects proteasomal degradation of the enzyme (Ekstrom and Meissl 1997). Therefore the AANAT is commonly known to be the melatonin rate-limiting enzyme of the melatonin biosynthetic pathway (Klein *et al*., 1997; 2002). On the other hand, HIOMT enzyme synthesis does not exhibit any significant rhythmic changes throughout the 24-hr period (Falcon *et al*., 1987; Morton and Forbes, 1988; Klein *et al*., 2002). Ribelayga *et al*. (2000) suggested that HIOMT might be implicated in seasonal, rather than daily, oscillations in melatonin production.

Different types of AANAT have been found in mammalian and non mammalian species. Only one type is present in mammals, bird and anurans, called AANAT, while there are at least three homologenous genes in teleosts localized between the retina (AANAT-1a and AANAT1b) and the pineal (AANAT2) (Coon and Klein, 2006).

Cyclic adenosine monophosphate (cAMP) is a second messenger that is important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms. cAMP is an important signal carrier that is necessary for the proper biological response of cells to hormones and other extracellular signals (Klein *et al*., 1997; 2002). It is required for cell communication in the hypothalamus/pituitary gland axis and for the feedback control of hormones. In melatonin synthesis, cAMP plays a central role in regulating through effects on AANAT activity through the pineal organs which accumulate cAMP (only in the presence of forskolin enzyme) during the dark phase (Coon and Klein, 2006).

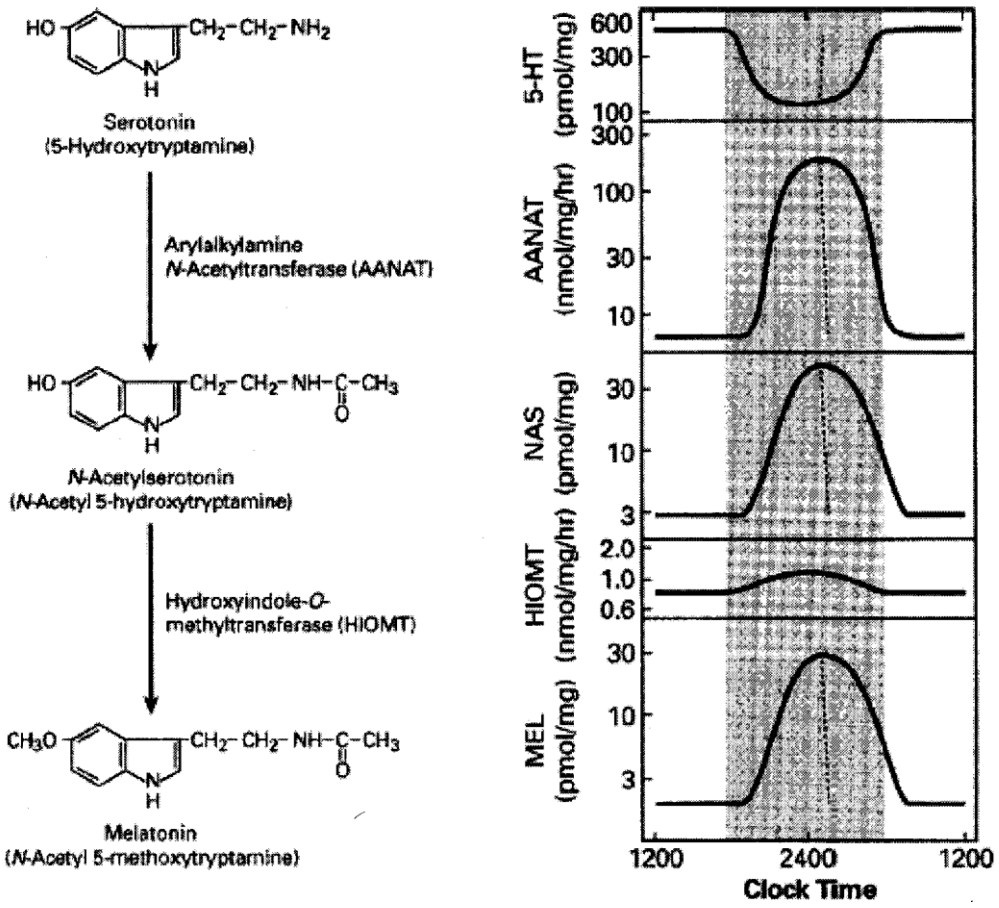

Figure 1.7 Melatonin pathway is shown on the left; changes in pineal levels of precursors and enzymes are shown on the right (Taken from Klein, 2002).

Light exposure during the scotophase suppresses AANAT activity and melatonin synthesis. However, unexpected periods of darkness during the light phase do not necessarily induce a rise in AANAT activity (Falcon *et al*., 1999). The effects of light, however, depend on the time of application at night (Falcon, 1987). Light pulses given before mid-scotophase of a 24 hr LD cycle induce a rise in AANAT activity later than expected following the scotophase in rat (Binkley, 1983; Illnerova *at el.*, 1989). The rise in AANAT activity is advanced when the light pulse is applied after the mid scotophase. In addition, melatonin synthesis responds differently to variations in light intensity and temperature (Bromage *et al*., 2001; Porter *et al*., 2001). Melatonin levels are directly correlated with light intensity (Gern *et al*., 1992, Migaud *et al*., 2006) with increased light intensity reducing levels of plasma melatonin. Plasma melatonin synthesis was shown to decrease proportionally with increasing light intensities in juvenile Atlantic salmon. Even with dark phase levels as low as 20 lux, significant differences were found between light and dark phase melatonin levels (Porter *et al*., 2001). These results were also supported *in vitro* in pineal culture of rainbow trout (Max & Menaker, 1992; Gern *et al*., 1992; Yanez & Meissl, 1996). On the other hand temperature was shown to directly affect melatonin secretion in ectothermic species (Menaker & Wisner, 1983; Underwood & Goldman, 1987; Falcon *et al*., 1994). In juvenile Atlantic salmon, melatonin levels were higher in groups maintained at 12°C compared with groups maintained at 4°C in (Porter *et al*., 2001). Similar results were found in pike and rainbow trout with greater amplitude of secretion at increased temperatures (Falcon & Collin, 1989; Max & Menaker, 1992; Randall *et al*., 1995). However, the mechanism behind temperature-dependent melatonin production is thought to occur at an early stage in its biosynthetic pathway. It was shown that the forskolin enzyme, which induced cAMP formation, then regulates N-acetyltransferase

(AANAT) production which in turn controls the synthesis of melatonin, is temperature-dependent (Falcon & Collin, 1989; Thibault *et al.*, 1993; see section 3.1.6).

1.3.1.4 Melatonin receptors

The study of melatonin receptors has been made possible with the development of commercially available 2-[125I]-Melatonin ([125I]-Mel). Such studies have allowed characterisation of the melatonin binding sites in numerous tissues (including brain, retina, and peripheral tissues) of several vertebrate species (Reppert *et al.,*, 1995, 1996). Three subtypes of melatonin receptors Mel_{1a}, Mel_{1b} and Mel_{1c} have been found; these are all of the G protein-coupled type. All these three types are present in chicken and zebrafish whereas the Mel_{1c} type is lacking in mammals. In mammals, the Mel_{1a} type was found to be present in the suprachiasmatic nucleus (SCN) of the hypothalamus and in the pituitary, and it been suggested that the Mel_{1a} type is responsible for the actions of melatonin on reproduction (Vanecek 1998). In chicken, both Mel_{1a} and Mel_{1c} type are distributed in the SCN. The Mel_{1c} type was also present in the pineal organ. Melatonin receptors have now been found in a variety of fish species including catfish, rainbow trout, salmon, pike (Ligo *et al.*, 1997; Davies *et al.*, 1994; Ekstrom and Meissl, 1997; Falcon and Gaildrat, 1997). High-affinity melatonin receptors appear therefore to have been highly conserved during evolution. Melatonin-binding sites in the catfish brain belong to the ML-1 subtype (Falcon *et al*, 2007). Several articles reported the presence of melatonin receptors in other tissues, in addition to the brain, of non-mammalian species, including the kidney, spleen and especially the gonads (Ekstrom and Meissl 1997).

1.3.1.5 Regulation of melatonin production

All teleost species have been shown to have photosensitive pineal glands which produce melatonin according to the day/night cycle (Gern *et al.,* 1992; Max and Menaker, 1992; Zachmann *et al.,* 1992b; Bolliet *et al.,* 1996) although photo-responsiveness has recently been questioned in Mozambique tilapia (Nikaido *et al.*, 2009). Furthermore, both *in vitro* (Yanez and Meissl, 1996; Migaud *et al*., 2006) and *in vivo* (Randall *et al*., 1995; Porter *et al*., 1998; Bromage *et al.*, 2001; Bayarri *et al*., 2002; Vera *et al.*, 2005; Migaud *et al*., 2006; Oliveira *et al*., 2007) studies have demonstrated that melatonin synthesis varies inversely with the irradiance of the incident light. It is thought that plasma melatonin may have threshold levels, which define the response of biological functions in fish to environmental influences (Bromage *et al.,* 2001; Migaud *et al.,* 2006). Although some electrophysiological studies have described the luminance and chromatic response of the pineal gland (Ekstrom and Meissl, 1997), better knowledge of such light requirements for melatonin regulation is needed especially regarding the quality of the light, as the aquatic environment acts as a potent filter significantly modifying spectrum and intensity.

Other factors may affect melatonin production, studies have shown that temperature acts directly on the pineal organ to modulate melatonin secretion, through the regulation of AANAT2 activity (Zachmann *et al*., 1992; Falcon *et al*., 1994, 1996; Coon *et al*., 1999; Falcon, 1999; Benyassi *et al*., 2000). Interestingly, (i) there is a good correlation between the peak of AANAT2 response and the fish optimal physiological temperature (trout: 12°C, pike: 20°C, sea bream: 27°C, zebrafish: 30°C), (ii) the response to temperature is an intrinsic property of the enzyme itself, because the same response curves were obtained when activities were measured from cultured pineal organ homogenates or recombinant AANAT2 enzymes. In the pike, temperature had no effect on the phase and period of the circadian rhythm) (Falcón *et al*., 1994). In *vitro*, Falcon *et al*. (1994) demonstrated in pike

pineal glands cultured at different temperatures, that a melatonin rhythm was expressed at 19°C up to 30°C but not at 10°C or 15°C. A similar effect was also reported in the white sucker, pineal gland by Zachmann *et al*. (1992b). Closer examination in relation to acclimation temperature prior to experimentation showed melatonin release was lower from pineals incubated at 20°C when the fish had been previously acclimated to 10°C. Thus, the concurrent action of photoperiod, that determines the duration of the melatonin signal, and of temperature, that determines its amplitude, is thought to provide accurate definitions of both the daily and annual cycles. Any changes in temperature, related to husbandry conditions or global warming, may thus have dramatic consequences on the time-keeping system of fish.

Feeding was shown to synchronize patterns of behaviour (i.e. locomotor) and physiology in different fish species (Boujard and Leatherland, 1992; Aranda *et al.*, 1999a; Aranda *et al.*, 2001; Sanchez-Vazquez *et al.*, 2001; Azzaydi *et al.*, 2007). However, importantly, in mammals, light and feed entrainable oscillators are suggested to be independent (Meijer and Rietveld, 1989). To our knowledge, in fish, no evidence of feeding entrainment affecting the melatonin synthesis rhythm system has yet been found.

Salinity is also an important environmental factor which may affect melatonin synthesis. The lifecycle of euryhaline fish involves migration between freshwater and marine environments, as is the case with salmon and eels. In gilthead sea bream and sea bass, melatonin levels varied with salinity with lower melatonin values recorded at the highest salinity (55 ‰) and higher at the lowest (5 ‰) (Kulczykowska *et al*., 2006; López-Olmeda *et al*., 2009). In addition, the influence of salinity on melatonin synthesis was revealed in the melatonin binding sites in the brain and retina. Indeed, the density of melatonin binding sites peaked in freshwater and decreased in brackish and sea waters in sea bass (López-Olmeda *et al*., 2009).

1.3.2 Circadian organization

It is known that melatonin is an important conveyer of photoperiodic information and is involved in the synchronization of many rhythmic daily physiological events and annual photoperiodic processes. There is extensive evidence demonstrating the complexity of the light perception system in fish which involves photoreceptive organs (retina, pineal), which convey information to the brain, as well as deep brain photoreceptor cells, the nature of which awaits further characterization. Different specialized structures (e.g. pineal complex, retina, parietal eye, deep brain) and pathways would have thus evolved in vertebrates, although the main basic components e.g. the non-visual photoreceptors are likely to be conserved (Menaker *et al.*, 1997; Foster and Hankins 2002; Klein 2004).

1.3.2.1 Mammals

The structures of the eye, brain (e.g. SCN) and pineal gland (known as the circadian axis) in mammals form a complex interconnected system in which photoentrainment is exclusively mediated by retinal photoreceptors. This has been demonstrated through ophthalmectomy studies (Underwood *et al*., 1990; Lockley *et al*., 1997; Foster, 1998; Yoshikawa and Oishi, 1998; Yamazaki *et al.*, 1999). The pineal photoreceptors in mammals have lost their direct light sensory abilities in comparison with lower vertebrates (Ekstrom and Meissl, 2003). Some evidence suggests that deep brain photoreceptors could act as an alternate mode of non-visual light perception (Foster and Hankins, 2002; Haldar *et al.*, 2002; Ekstrom and Meissl, 2003). This centralised model of circadian organisation is thus based on the retina in the eyes. However, light entering the eye is perceived by two different receptor types, the rod and cone photoreceptor cells. Colour and bright light are interpreted by the cones (of three types: red, blue and green), while black/white vision and dim light intensities are perceived by the rods. The photo-pigment rhodopsin (made up of

opsin and 11-*cis*-retinal) is chemically modified by the light entering the photoreceptor cells causing hyperpolarization, in turn leading to a nervous signal transmitted to the brain via the optic nerve (Moutsaki, 2003). Retina then transfer signals to the SCN in the brain (where the mammalian master clock is known to reside) through a retino-hypothalalamic tract (RHT) (Simonneaux and Ribelayga, 2003). The nature of the signal transmission into the SCN is not fully understood yet though it has been suggested that the secretion of glutamate at RHT synapses might provide impaired transmission of light signals into the SCN (Holzberg and Albrecht, 2003).

1.3.2.2 Teleosts

While teleosts have the same physical components of the circadian axis as in mammals (retina, RHT, 'SCN' and pineal), the main feature that makes them so different is the apparent absence of a central circadian master clock that connects all three components to mediate light and synchronize endogenous rhythms within the organism (Wullimann and Meyer, 1990; Tilgner *et al.*, 1990; Ekstrom and Meissl, 1997).

Unlike mammals, in all teleosts species studied so far, to our knowledge, as in birds, *in vitro* studies have shown that the pineal gland is directly photosensitive (Gern *et al*., 1992; Max and Menaker, 1992; Randall *et al*., 1995; Zachmann *et al.*, 1992b; Bolliet *et al*., 1996; Yanez and Meissl, 1996; Migaud *et al*., 2006). Photoreceptor cells in the pineal gland detect light that is transmitted through the pineal window (Figure 1.8). This signal is then converted into nervous signals that stimulate the photoreceptor plasma membrane causing hyperpolarization, which shows high sensitivity in the dark phases (Meissl and Ekstrom, 1988).

1.3.2.3 Endogenous control of melatonin production

In many species, when fish are exposed to continuous darkness (DD), they can still show free running melatonin rhythms, as well as behaviour (e.g. locomotor and feeding activity, see section 1.3.3) as if they remained under the previous LD cycle. This is known as "endogenous secretion of melatonin rhythms". This phenomenon is controlled by circadian oscillators or clocks that can be located in the pineal gland itself or in the brain or other tissues. These clocks are controlling melatonin secretion in the absence of day/night cycles (Ekstrom and Meissl 1997; Bromage *et al.,* 2001). Normally, these clocks are entrained by the LD cycle, but under constant conditions, can free run with their own endogenous periodicity.

While melatonin circadian rhythms are clearly controlled by environmental conditions, they can also be self-sustained and are under the control of circadian clocks (Holzberg and Albrecht, 2003; Falcon *et al.,* 2007). In teleosts, circadian organization specifically relating to the location and organization of clock systems are still poorly characterized with studies focusing on very few species such as zebrafish (Cahill 2002; Vallone *et al.,* 2005; Lopez-Olmeda *et al.,* 2006). Endogenous rhythms are a conserved feature observed across the animal kingdom, from photosynthetic prokaryotes to mammals (Menaker *et al.,* 1997; Ekstrom and Meissl, 2003). Studies from pineal glands in culture performed in both temperate and tropical teleosts have commonly demonstrated intrapineal oscillators, capable of self-sustaining melatonin rhythms *in vitro* in the absence of light stimuli, in species including pike (Falcon *et al.,* 1989), goldfish, *Carassius auratus* (Kezuka *et al.,* 1989; Iigo *et al.,* 1991), whitesucker (Zachmann *et al.,* 1992b), zebrafish (Cahill, 1996), sailfin molly (Okimoto and Stetson, 1999), and sea bass (Bolliet *et al.,* 1996; Bayarri *et al.,* 2004; Migaud *et al.,* 2006). However, no such endogenous rhythms

have been shown to exist in salmonids (Gern and Greenhouse, 1988; Migaud *et al.,* 2006; Iigo *et al.,* 2007) and common dentex, *Dentex dentex* (Pavlidis *et al.,* 1999).

In higher vertebrates, the molecular basis of the circadian clock has been shown to consist of a number of interrelated transcription, translation feedback loops that cycle with a period of approximately 24 hours hence the term "circadian" clock. This clock cycle is entrained by light and then maintains and synchronises self-sustained rhythms (Foulkes *et al.* 1997; Zordan *et al.,* 2001; Stehle *et al.,* 2003). Understanding these endogenous rhythms in fish is still in its infancy with the majority or work being performed in zebrafish (e.g., Whitmore *et al.*, 2000; Pando *et al*., 2001), and very limited work being published in temperate species apart from rainbow trout (Mazurais *et al.*, 2000), the golden rabbitfish (Park *et al*., 2007; Sugama *et al*., 2008) and Atlantic salmon (Davie *et al*., 2009). However, these results are already proving interesting and suggest that teleosts could provide very useful models in the field of chronobiology, not only for their plasticity but also for their diversity.

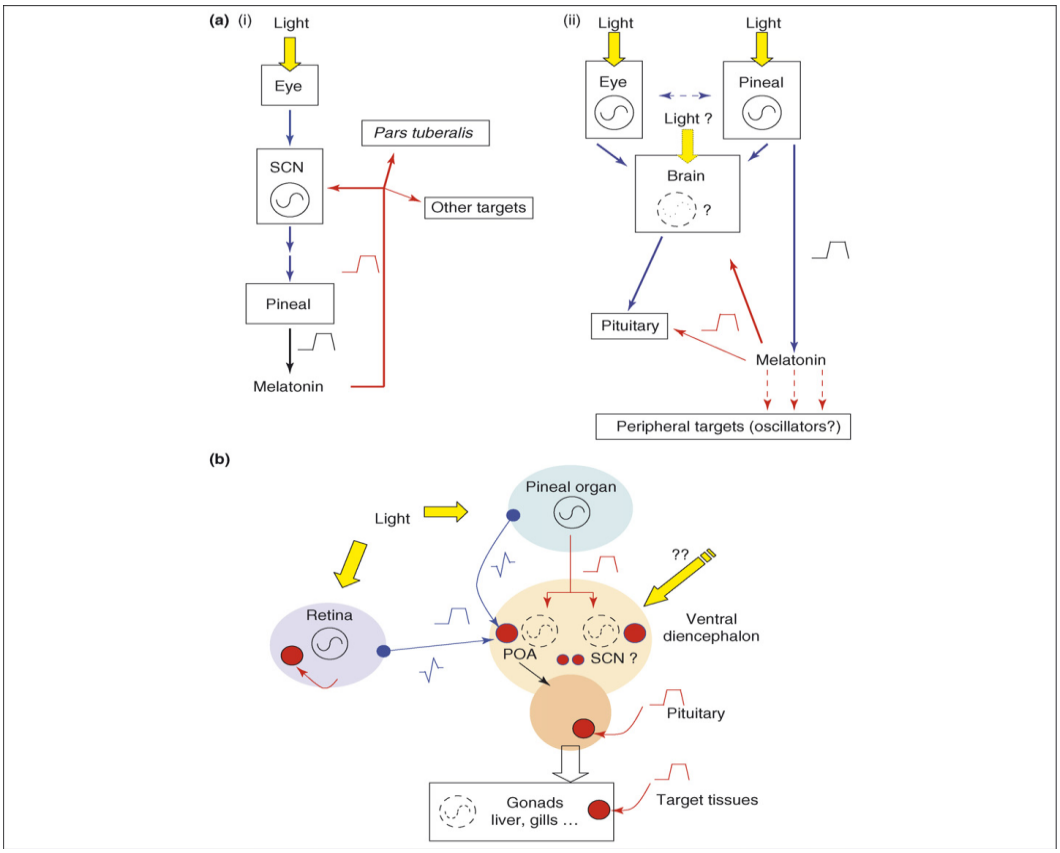

Figure 1.8 Photoperiodic and circadian control of neuroendocrine functions. (a) Fish versus mammals. In mammals (i) a linear flow leads to the rhythmic production of melatonin. Nonvisual information from the retina reaches the SCN of the hypothalamus through the retinohypothalamic tract (blue arrow). The periodic signals enable synchronizing of the circadian activity of the SCN clocks, which, in turn, impact on the pineal gland through a multisynaptic pathway (blue arrows), thus controlling cyclical melatonin secretion. Melatonin feeds back to the SCN and acts on the pars tuberalis of the pituitary and other brain areas to modulate seasonal neuroendocrine functions. The situation is more complex in fish (ii): the photoneuroendocrine system seems to be organized as a network of independent and interconnected light-sensitive oscillatory units in the retina, the pineal and, perhaps, in the brain. The dashed blue arrow indicates a hypothetical connection. '?' in the brain indicates the hypothetical presence of brain circadian oscillators. (b) Photoneuroendocrine regulation in fish. Light (yellow arrows) impacts on photoreceptor cells of the pineal organ and retina, enabling synchronization of their internal molecular clocks. Light might also impact on possible other photosensitive and circadian structures in the ventral diencephalons (POA and hypothalamic area; yellow arrow with '??') and peripheral organs. In response to the photoperiodic information, the retina and the pineal organ elaborate two types of rhythmic information. The neural information (blue arrows) from the retina and pineal organ reach the ventral diencephalon through the retinohypothalamic and the pineal tracts, respectively. This information provides an indication of day length, as well as of subtle variations in ambient illumination. The hormonal information is relayed by melatonin (red arrows), the production of which reflects day length and season. In the retina, melatonin is an autocrine and/or paracrine factor, which is metabolized locally. Pineal melatonin is released into the cerebrospinal fluid and blood, and acts on specific targets through melatonin receptors (red filled circles). In the hypothalamus, melatonin might contribute to synchronizing the activities of circadian oscillatory units [SCN and others (depicted by '?')] and modulating the production of pituitary gland releasing factors. Melatonin receptors have been identified in areas that impact on pituitary function, including the POA, which also receives nervous input from both the pineal organ and the retina. Melatonin impacts on the pituitary gland itself to modulate the production of hormones. Taken from Falcon *et al*. (2007)

1.3.3 Regulation of circadian and endogenous rhythms of locomotor rhythms

Animals have developed behavioural and physiological mechanisms to anticipate the predictable changes in the environment, thereby optimizing biological processes (Daan, 1981; Kumar, 1997; Godman, 2001; Dardente & Cermakian, 2007). Environmental changes act as synchronizers which entrain biological rhythms in their periodicity, amplitude, and phase (Aschoff, 1981; Rensing & Ruoff, 2002). In vertebrates, biological rhythms can be classified according to their periodicity: circadian (approx. 24 h), infradian (> 24 h) and ultradian (< 24 h) (Reebs, 2002).

Circadian locomotor activity rhythms of animals maintained in constant conditions have been used to screen for circadian clock mutants and to test the hypotheses about the functions of these clock genes. Locomotor activities have also been used extensively to analyze the physiological organization of mammalian, avian, and reptile circadian systems. Fish have been classified into diurnal, nocturnal or crepuscular species depending on the time of day in which their activity is displayed (Iigo and Tabata, 1996). However, in some cases the classification is not clear where some fish species are endowed with a flexible circadian system that allows them to change their phasing. A difference has been established between marine species, presumably having a strong circadian system, and freshwater fishes that would be endowed with a more plastic one (Reebs, 2002; Vera *et al*., 2006). On the other hand, some individuals of a given species can be active at different phases of the LD cycle whereas others can shift their activity patterns along their life cycle (from diurnal to nocturnal and *vice versa*) (Iigo and Tabata, 1996; Eriksson, 1978; Reebs, 2002; Favreaua, 2009). Behavioural rhythms can be resynchronized from one season to another depending on environmental changes e.g. daylength (Muiler, 1978), light intensity (Eriksson, 1978), temperature (Fraser *et al*., 1993), food availability (Sanchez-Vázquez *et*

al., 1996), intraspecific activities (e.g. migration, spawning, parental activities) (Metcalfe & Steele, 2001, Vera *et al*., 2006).

Animals exposed to such conditions lose external entrainment, and their circadian rhythms can free-run with an approximate period (*tau*) of 24 h in the case of circadian rhythms (Aschoff, 1981; Edmunds, 1988). The existence of free running rhythms can also be tested by phase-shifting the environmental light-dark cycle and investigating the appearance of transient cycles of activity. Such rhythms are self-sustained during at least some days after the animal is separated from its external *zeitgeber*, thus indicating its endogenous control (Aschoff, 1960; Iigo & Tabata, 1996; Sanchez-Vazquez *et al*., 1995). In mammals, these circadian rhythms can be sustained under constant conditions (with the absence of the environmental stimulus to synchronize the internal clock) for up to a month, whereas in teleosts fish they usually disappear after a few days (Iigo & Tabata, 1996; Naruse & Oishi, 1994; Nishi, 1990).

Many studies have investigated activity rhythms in fish however locomotor activity studies on catfish species are scarce. Results obtained to date have showed the existence of diel rhythms when fish were reared under LD cycles, with activity peaking during the dark phase as shown in Japanese sea catfish (Kasai, 2009), channel catfish (Goudie *et al*., 1983), sea catfish (Steele, 1984), Japanese catfish (Tabata *et al*., 1991), European catfish (Boujard *et al*., 1995), Brazilian cave catfish (Trajano & Menna-Barreto, 2000) and Philippine catfish (Ramteke, *et al*., 2009). This probably reflects adaptations to their natural habitat as most Siluridae teleosts are typical bottom-dwellers. However, under constant environmental conditions, different results are obtained among species. The circadian rhythms of Brazilian cave catfish for example were shown to disappear immediately after shifting the photoperiod from LD to continuous darkness (DD) and a weak free-run rhythmicity was observed (Trajano and Barreto, 2000; Trajano *et al.*, 2005). Similar results

were found for the upside down catfish (Kabasawa, 1986), and sea catfish (Steele, 1984). In contrast, a strong free-running activity was recorded under DD and continuous light (LL) in the Japanese and channel catfish (Goudie *et al*., 1983; Tabata *et al*., 1991) even in animals previously ophthalmectomised, demonstrating that the circadian oscillator regulating the endogenous rhythmic activity is not located in the eyes in this species.

Circadian activity rhythms under LD cycle have also been reported in a wide range of fish species. A clear locomotor activity patterns is observed in some fish species: e.g. zebrafish (diurnal) and tench (nocturnal) (Herrero *et al*., 2003; Hurd *et al*., 1998) whereas a large inter-individual variation of daily activity has been reported in other fish, such as tilapia (Vera *et al*., 2009) and goldfish (Iigo and Tabata, 1996). Generally activity patterns in fish show a strong plasticity, especially in freshwater species, probably due to the relative instability of their environment (Reebs, 2002).

1.4 PHOTIC PERCEPTION TO BRAIN–PITUITARY–GONAD (BPG) STIMULATION

While the presence of vertebrate photoneuroendocrine systems (PNES) has been known for many decades (Scharrer, 1964) the structure of the PNES that connects photic perception to the BPG axis remains unclear in fish.

1.4.1 Brain–pituitary–gonad (BPG) axis

The neuroendocrine system initiates and controls the process of gametogenesis and steroidogenesis primarily through the activation of the hypothalamus-pituitary-gonadal axis (Kah *et al*. 1993). The activation of this axis is controlled by environmental and endogenous cues (Munro *et al*. 1990). Brain receives these cues from the environment and activates the hypothalamus which, in turn stimulates the release of gonadotropin releasing hormone (GnRH). Then GnRH stimulates the release of gonadotropins (GTH) from the pituitary (Okubo and Nagahama, 2008; Kouril, 2009). Fish pituitaries however, similar to those of other vertebrates, have been shown to secrete two kinds of gonadotropins; GTH-I (similar to Follicle Stimulating Hormone; FSH) and GTH-II (similar to Luteinising Hormone; LH). GTH-I plays a role during initial gonadal growth and gametogenesis while GTH-II is prevalent during the final stages of maturation (Trudeau, 1997; Kouril, 2009).

In ovaries, the gonadotropins stimulate the theca and granulosa cells of the ovarian follicle to secrete steroids including androgens (e.g. testosterone), oestrogens (e.g. 17 β-oestradiol) and progestagens (e.g. 17α-20 β-dihydroprogesterone) (Cavaco *et al*., 1997). During the early oocyte growth and development phases, FSH stimulates the follicular cells of the oocyte to produce testosterone (T) which is then converted into 17β-oestradiol (E_2) in the granulosa cells via the activity of the aromatase enzyme (Redding and Patino 1993). Increased E_2 levels in the blood stimulate the hepatic system to synthetise

vitellogenin (VTG). E_2 peaks generally, during the period of most active vitellogenesis, and returns to basal levels before ovulation. Testosterone (T) starts to increase in conjunction with E_2, but peak levels are not attained until 1–2 months after E_2 (Frantzen *et al.*, 1997). VTG is a large glycophospho-lipoprotein produced by the liver and transported via the circulatory system to the ovarian follicle, processed into yolk proteins then accumulated as yolk globules in the growing oocytes. Once vitellogenesis is complete, plasma E_2 levels fall rapidly and a negative feedback on the hypothalamus and pituitary triggers the release of LH (Okubo and Nagahama, 2008; Kouril, 2009). LH stimulates the final oocyte maturation through the secretion of progestagens (17α-20βDHP or 17,20-20β, 21-P), acting as the maturation inducing hormone (MIH) (Okubo and Nagahama, 2008). This hormone then binds to the oocyte nucleus and forms the maturation promoting factors (MPF) which stimulates the germinal vesicle migration (GVM) towards the micropyle. The germinal vesicle then breaks down (GVBD) prior to the hydration stage and subsequent ovulation (Nagahama, 2008; Schulz *et al*., 1994; Kouril, 2009).

In testes, the two GTH hormones play different functional roles. FSH levels are elevated during spermatogenesis while LH levels peak during spermiation (Redding and Patino 1993). Both gonadotropins have been reported to have two types of receptors, one in the Leydig cells which is specific to LH, and the other one located in Leydig and Sertoli cells with affinities to both FSH and LH (Schultz and Goos, 1999). In fish every single primary spermatogonium is enveloped by one or two Sertoli cells which support germ cells by providing the optimal microenvironment (Okubo and Nagahama, 2008). Leydig cells are steroidogenic, e.g. responsible for the production of steroids. FSH acts by regulating Sertoli cell functions to stimulate germ cells growth during spermatogenesis (Kouril, 2009). The LH receptor is then expressed by Leydig cells, which respond to LH and release androgens (testosterone and 11-ketotestosterone) with T stimulating pituitary LH

synthesis during puberty (Okubo and Nagahama, 2008). Peak levels of 11-KT coincide with spermiation (Tveiten *et al.*, 1998). In male fish, 11-ketotestosterone (11-KT) is considered to be the main androgen hormone, stimulating the development of secondary sexual characters and spermatogenesis (Borg, 1994). Seasonal cycles of gonadal activity have been described in many teleosts species.

Sex steroids were monitored over two consecutive annual reproductive cycles in female and males of Indian catfish. In females, T and E^2 were detectable in the plasma from early ovogenesis through to the spawning period with increasing levels during the active vitellogenenic phase. Peaks levels of plasma E_2 were reached by the end of ovogenesis period and declined when the vitellogenesis was almost completed before the spawning period started (Lamba *et al.*, 1983; Garg, 1989). T levels increase up to ovogenesis and spawning periods and then return to basal levels before the postspawning started. The gonadosomatic index (GSI) coincides with the yolk accumulation in the oocytes and increases rapidly during the prespawning period to reach a peak during the spawning period (Lamba *et al.,* 1983). However, similar annual reproductive cycles were found in many other species including other catfish species (e.g. brown bullhead catfish, Rosenblum *et al*., 1987).

In male Indian (Lamba *et al.*, 1983) and brown bullhead catfishes (Rosenblum *et al*., 1987), the plasma steroid profiles in T and 11-KT are closely related to GSI changes. During spermiogenesis, T and 11-KT levels increase and reflect spermatogenesis activity, and both of GSI and steroid hormones reach a peak before spawning and then decline rapidly at the onset of the spawning period.

Although the BPG axis has been well described in fish over the last few decades, the cascade from environmental cue to brain gonadotropin stimulation resulting in the timing and regulation of gonadal development remained unknown. Recently, a new peptide,

Kisspeptin, has been identified as a key actor in the initiation of puberty and regulation of seasonal breeding in mammals (Smith and Clarke, 2007; Revel, 2007). It has been proposed that Kisspeptin actions are mediated by melatonin signalling by directly regulating KiSS-1 expression as well as changing sensitivity of KiSS-1 to sex steroid feedback (Greives *et al*. 2008). The study of kisspeptin in fish is still in its early stages, though it is becoming a very active research field.

1.4.2 Photoperiod and reproduction

The first published studies on the effect of daylength on reproduction were performed in trout in the mid-twentieth century (for reviews Bromage *et al*., 1994; 2001). Whitehead *et al*., (1978) showed that spawning in rainbow trout could be advanced by two or four months if fish were exposed to a six or nine month compressed photo-cycle respectively. The timing of spawning could therefore be altered by compressing or extending the annual photoperiodic cycle. However, these photoperiodic strategies, although very effective in enclosed indoor rearing systems, could not be used in outdoor conditions due to the ambient light signal. Therefore, studies were carried out to better understand the photic cue regulating reproduction with the aim of developing optimal photoperiodic regimes to be applied in natural environment (superimposed on ambient signal). It was found that similar effects on spawning time could be obtain in rainbow trout by exposing fish to periods of long days (LD, 16L:8D) followed by short days (SD, 8L:16D), or vice versa. The first group spawned eight weeks earlier than the control fish, while the second group (SD) was delayed by twelve to fourteen weeks (Bromage *et al*., 1984). Similar findings were reported in brook trout, brown trout, Atlantic salmon, pink salmon and Masu salmon in which exposure to constant long and short days after the summer solstice resulted in delay and advancement of spawning, respectively (Carlson and Hale, 1973; Takashima and

Yamada, 1984; Bromage *et al.*, 1990; Taranger *et al.*, 1991; Beacham and Murry, 1993; Taranger *et al.*, 1998, 1999). Long day followed by a constant short day length during winter/spring is often used to alter reproductive development in salmonids (Randall and Bromage, 1998b). Light manipulation is now a useful tool to control the spawning activity in salmonids and is also used to enhance growth performance (Endal *et al*., 2000). Importantly, any daylength can be considered long or short providing that the previous photoperiod to which the fish have been exposed to is shorter or longer respectively. Randall and Bromage (1998) showed that daylength (13.5L:10.5D or even L18:6D) can be considered as short day if fish have previously been exposed to longer daylength (i.e. 18L:6D or L22:2D , respectively). Although early studies on salmonids pioneered photoperiodic work, since then there has been extensive work carried out in a range of other temperate fish species such as the brown trout, European sea bass (Carrillo *et al*., 1989, 1991, 1993), turbot (Imsland *et al*., 2003); halibut (Norberg *et al*, 2001) and Atlantic cod (Taranger *et al*., 1998; Davie *et al*, 2008). Furthermore, the exposure to continuous light can partially or fully suppress first maturation as shown in Atlantic salmon (Taranger *et al*., 1999), Atlantic cod (Davie *et al*., 2007b), haddock (Davie *et al*., 2007a) and turbot (Imsland *et al*., 2003).

In contrast, very little information is available on the influence of photoperiod on reproduction in subtropical and tropical species. At the latitudes where these species can be found, seasonal variations in photoperiod and temperature are relatively weak as compared to temperate regions and it is therefore unclear whether these changes can play a key role in the entrainment of physiological functions. In catfish, Singh (1998) found that exposure of Philippine catfish to long photoperiod (14L:10D) during the early stages of gonadogenesis can stimulate early development of the testis by activating the brain-pituitary-gonadal axis. Sundararaj and Sehgal (1970) pointed out that both long day

(daylength of 14hrs) and gradually increasing photoperiod (from 10 to 14hr/day) are equally effective in accelerating ovarian recrudescence in the Indian catfish. Guraya *et al*. (1976) showed that long photoperiod (14L:10D) can also have effects on the latter stages of the reproductive cycle of Tengara catfish stimulating final oocyte maturation and significantly reducing the spawning window. During spawning and post-spawning stages, the same authors showed that fish were no longer receptive to the long photoperiod since it is ineffective in maintaining gravid ovaries. In the Nile tilapia, several studies have shown that long day photoperiod (18L:6D) stimulates seed production (Ridha & Cruz 2000; Campos- Mendoza *et al*., 2004). According to Biswas *et al*. (2005), a daylength of 12-14-h of artificial light with a temperature of 25-29°C gave the best results for natural spawning in the Nile tilapia. Furthermore, Galman *et al*. (1988) showed that photoperiod regime can help to produce year round red tilapia fry by extending daylength during the winter months (December-February).

However, photoperiod and light intensity regimes can also affect behaviour, growth rate and survival as shown in *C. gariepinus* (Britz and Pienaar, 1992; Appelbaum and Mcgeer, 1998; Appelbaum and Kamler, 2000; Almazan-Rueda, 2004; Adewolu, 2008). These studies suggested that different light can act as a stressor in catfish which are known to be a nocturnal fish and growth rate would be reduced as daylength increases. Previous studies performed on the effect of photoperiod on growth performance of *C. gariepinus* all concluded that growth can be affected by daylength with fish performing better under short-day photoperiods (Britz and Pienaar, 1992; Almazan-Rueda, 2004; Appelbaum and Mcgeer, 1998; Appelbaum and Kamler, 2000; Adewolu, 2008). Similar results were reported in other catfish species like Silver catfish (Piaia *et al*., 1999). However contrasting findings were also reported in channel catfish where no significant differences in growth were observed between control photoperiod (12D:12L) and a range of other photoperiodic

treatments tested (from continuous darkness to continuous light including intermediary treatments with daylength of 6, 12 or 18 hrs) (Stickney & Andrews, 1971).

Despite these environmental drivers, endogenous cycles of reproduction have become evident in a number of species exposed to constant environmental conditions, including but not only limited to rainbow trout (Duston & Bromage, 1988; Randall *et al.,* 1998b), Atlantic cod (Norberg *et al.,* 2004), European seabass (Carrillo *et al.,* 1995) and three-spined sticklebacks (Bornestaf and Borg, 2000). This clearly suggests that endogenous mechanisms underlie the timing of reproduction in fish. Evidence for the endogenous control of gonadal maturation was first provided by Duston & Bromage (1991), whereby female rainbow trout maintained on constant short days (LD 6:18) for a period of 51 months exhibited a free-running circannual rhythm for up to three cycles. During this period there was variation in the periodicity of the rhythm, both between individual fish, and also between successive spawning of individual fish. Such desynchronisation of spawning time was also reported by Bromage *et al*. (1984), typical of free-running endogenous rhythms as seen in other vertebrates. Further evidence was provided by Randall *et al*. (1998b) where a seasonal-phase response-curve in spawning time was described. It was proposed that long days, occurring earlier or later than under a natural photoperiod, were perceived as the internal clock was running slow or fast, thus initiating corrective forward adjustments (advance phase-shifts) or backwards adjustments (delay phase-shifts) respectively.

Therefore, the response to photoperiod manipulation suggests that the reproductive cycle is controlled by a circannual rhythm, and that it is the entrainment of this rhythm by seasonally light cues (*zeitgeber*) that accurately co-ordinates reproductive development with the natural pattern of daylength changes. Because they relate to annual rhythms, they have been described as circannual (Duston and Bromage 1986). If one accepts that

endogenous mechanisms underlie the timing of reproduction in fish, then it is easier to understand how the altered photoperiod regimes exert their effects, why photoperiodic history and the direction of change of daylength are important and why the same photoperiod can induce different effects if administered at different times of the year.

In a previous study performed in two catfish species (*C. gariepinus* and *C. anguillaris*), no effects of photoperiod (constant darkness/12L:12D) on percentage hatchability in both species was found. However, the illumination had a considerable influence on the development and hatching time of the eggs in the two species, where eggs exposed to continuous light hatched earlier (14-24hr) than those exposed to constant darkness (24-32hr) in both species (Inyang and Hetiarchchi, 1995). In another study on African and Asian catfish, authors have shown that a short period of light (L06:18D) can significantly increase hatching rate and exposure of eggs to continuous light can advance or speed up the hatching process (Mino *et al*., 2008). In Chinese catfish *Clarias fuscus*, Young (1988) showed that temperature had the strongest effect on ovarian maturation in terms of oocyte growth and fecundity.

1.4.3 Melatonin and reproduction

Timing of reproduction in fish can therefore be affected by photoperiod in conjunction with clear diel and seasonal patterns of melatonin. This can provide strong circumstantial evidence that melatonin is one intermediary in these processes (Bromage *et al*., 2001). Many studies have studied the role of melatonin (plasma levels or receptors) in reproduction. Such studies involved the removal of the source(s) of melatonin through ophthalmectomy (EX) and/or pinealectomy (PNX) and/or administration of exogenous melatonin (Mayer *et al.,* 1997). The pinealectomy (PNX) in male Atlantic salmon parr abolished the nocturnal rise in melatonin but failed to alter the timing of early maturation

(Mayer, 2000). Similarly, it failed to inhibit daily gonadotropin cycling in common carp (Popek *et al.,* 1994). In contrast, in the female rainbow trout, PNX performed during the summer resulted in a delay in spawning relative to controls suggesting that in this case the pineal was necessary for the entrainment of the final reproductive stages which, in part, is normally brought about by the decreasing daylengths and subsequently increased daily duration of melatonin secretion (Bromage *et al*., 2001). Following PNX and EX, Ayu still became sexually mature under SD photoperiods while under LD did not (Masuda *et al.*, 2005). Clearly these findings draw parallels with the avian model and suggest that, at least in these species, some form of deep brain photoreceptors are responsible for photic entrainment of reproduction which needs to be more thoroughly investigated.

The response to direct melatonin administration has been variable though evidence for its involvement in reproduction as part of a complex regulatory network is becoming clearer. The stronger evidence was recently found by Sebert *et al*. (2008) who showed that melatonin can stimulate the dopaminergic system of the preoptic area, which is involved in the inhibitory control of gonadotropin (LH and FSH) synthesis and release. Earlier studies have shown that injection of both male and female goldfish with melatonin can reduce gonadal development induced by long-day conditions known to cause gonadal stimulation (Fenwick, 1970). Intraperitoneal injections of melatonin in mature Atlantic croaker during the late photophase elicited significant elevations in plasma GtH II (LH) levels (Khan and Thomas, 1996). In contrast, injection of rainbow trout female with melatonin failed to impact on their reproductive seasonality (Randall *et al*., 1995) thus perhaps the effect of PNX on reproduction in this species, as described above, was via a non melatoninergic pathway. Thus it must be concluded that melatonin can influence the BPG axis, however, its functions are apparently dose dependent and can clearly operate at different levels within the cascade.

1.5 Aims of the thesis

As explained in the introduction, photoperiod is a key driver of fish physiology for circadian as well as seasonal rhythmicity. Despite the relatively weaker photoperiodic changes observed in tropical latitudes, species inhabiting such environments are likely to rely on day/night signalling to entrain their physiology. As such, they provide interesting models, different from temperate species, to better understand some of the evolutionary mechanisms leading to specific adaptations. Importantly, study of circadian rhythmicity has mainly focused over the recent decades on temperate species and knowledge on the photoperiodic mechanisms in tropical species remains scarce. This research project therefore focused on African catfish (*Clarias gariepinus*) which is becoming an increasingly important commercial species in third world countries and has a wide range of distribution across Africa. It is believed that this species can be an interesting scientific model for chronobiological studies due to its different morphology and nocturnal activity.

The overall aim of this work was to investigate how African catfish responds to different photoperiodic regimes, especially with regards to the entrainment of reproduction, locomotor activity and growth performance. Ultimately, this work will provide a better understanding of circadian/seasonal rhythmicity in this species.

The thesis is organised in chapters that first study circadian rhythms, in comparison to other commercially important species, using melatonin and locomotor activity as tools. Then the effects of photoperiod on reproductive and growth physiology are investigated. Specific objectives are outlined below:

1) Chapter 1 and 2: Investigate the organization of the circadian axis and photic regulation of melatonin production by the pineal gland of *Clarias gariepinus*

through *in vivo, in vitro,* pinealectomy, ophthalmectomy and photoperiodic studies.

2) Chapter 3: Study the locomotor activity rhythms of *Clarias gariepinus* exposed to different photoperiodic regimes and investigate potential social effects.
3) Chapter 4: Evaluate the effects of photoperiod manipulations on growth and puberty in *Clarias gariepinus*.

CHAPTER 2

MATERIALS AND METHODS

2. GENERAL MATERIALS AND METHODS

Only specific details not described in the experimental papers are given in this chapter.

2.1 Experiment facilities

2.1.1 Experimental system

Most experiments were conducted in the same system in the main tropical aquarium at the Institute of Aquaculture, Stirling University. The system consisted of sixteen self-cleaning centrally drained square tanks, each with a capacity of 65 L.

The system was covered by frame built with iron and PVC foam to keep experimental fish isolated from external luminance conditions. Each compartment had a sliding door at the front for husbandry reasons and to feed the fish. The system was split into eight cabins, with two tanks in each cabin. The light source was provided with a lamp above each tank (12 watt bulb) attached to the ceiling of the frame. All light were controlled with timers in order to set different photoperiods (Figure 2.1). Water was supplied to each tank with a flow rate of 3 L/minute from a 240 L header tank. Water drained from the tanks through central stand pipes into a series of settling and biological filter tanks. The water was then pumped back up to the 240L header tank that was aerated using airstones and heated by 3KW thermostatically controlled immersion heaters to maintain a temperature of 27 ± 1 °C throughout the experimental period. One third of the total water was replaced weekly with fresh water to maintain water quality and to avoid accumulation of wastes. A constant photoperiod of 12 hours light and 12 hours dark was maintained during acclimation periods.

2.1.2 Water quality

The dissolved oxygen content of the water in the experimental system was checked using an oxygen meter (YSI 57 Clandon, Ohio, USA), whilst the pH was monitored using a digital pH meter (Philips PW9409). Total ammonia, nitrite and nitrate of the water in the experimental system were determined using a Technicon Auto Analyser.

The water temperature of the experimental system was regulated with electric thermostatic heater controlled by a Deem 10/1193. Water quality was checked three times a week.

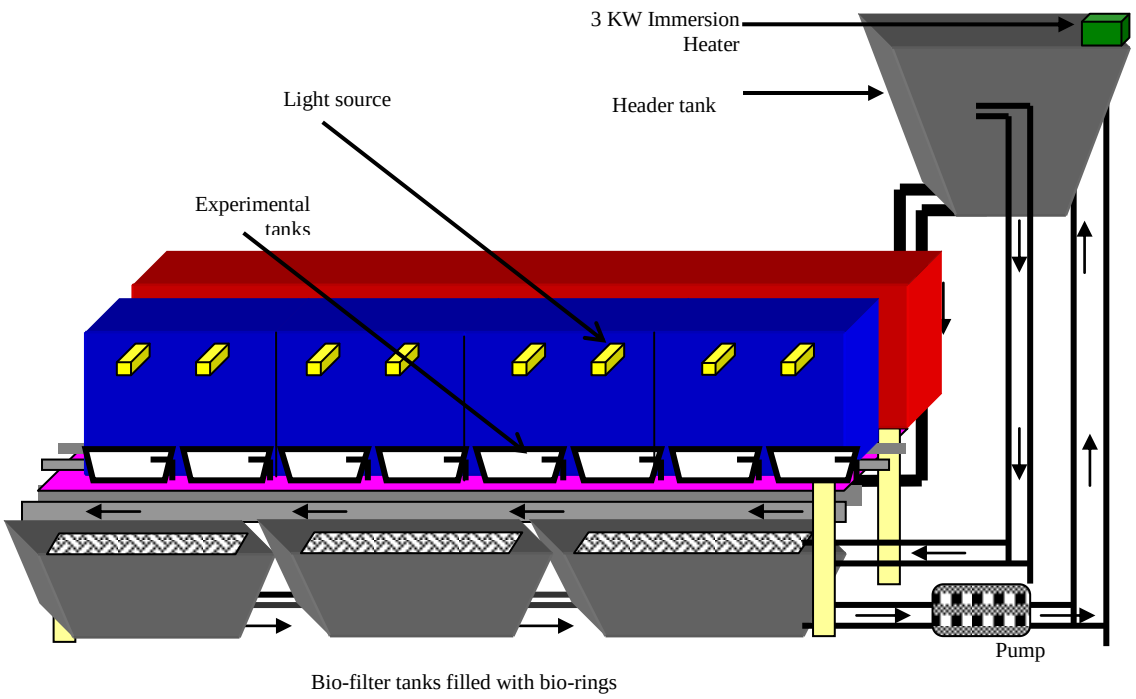

Figure 2.1 Diagram of experimental system

2.2 Experiment procedure

2.2.1 Weighing and acclimation of fish

Fish were anaesthetised using a 1% benzocaine solution (Ross and Geddes, 1979), blotted with a soft tissue paper and weighed individually on a Mettler PM 6000 balance immediately prior to the commencement of the experiments. Fish were then transferred into buckets containing fresh water with adequate aeration for recovery from the anaesthesia. Fish were always randomly allocated to the experimental tanks. Fish were left for a period of two week to recover from the stress associated with these procedures before commencing the experiment.

2.2.2 Blood sampling

Blood samples were taken from caudal dorsal aorta by using 1 ml or 2 ml syringes, dependent on the sample volume required, with 25G, 23G or 21G sterile hypodermic needles (Terumo Europe N.V., Belgium) dependent on the size of the fish. All syringes and needles were flushed with heparin ammonium salt from porcine intestinal mucosa (4mg/ml, 140 units/mg: Sigma chemicals, Ltd. UK). Blood samples were expelled into a clean Eppendorf (Fisons Scientific Equipment, UK) and placed on ice until sampling was completed. Blood samples were centrifuged (Jouan, CT422) at 2500 rpm for 15 minutes at 4 °C. The plasma was separated and stored at -70°C for later analyses.

For blood samples withdrawn during the hours of darkness, fish were removed from their housing and placed in a light proof container with anaesthetic, under complete darkness. The fish were sampled where the only light source was a dim red light (670-800 nm) with an intensity of 0.0004 W.m-2 (equivalent to 0.2 lux or 0.0021 μmoles.$m^{-2}s^{-1}$) at 0.5 m.

2.2.3 Histology

Gonads were collected from the sacrificed fish either whole or as samples if the gonad was too big. All gonad samples were fixed in 10% neutral buffered formalin and stored for later analyses. This solution was prepared as follows:

- disodium hydrogen phosphate 6.5 g
- sodium dihydrogen phosphate 3.5 g
- formalin (40% formaldehyde) 100 ml
- deionised water 900 ml

2.2.3.1 Histological analyses

Embedded and Sectioning

Fixed gonad samples were dehydrated and embedded as listed below:

1.	50%methylated spirit	30 min
2.	80%methylated spirit	90 min
3.	100%methylated spirit	90(x3)
4.	Chloroform	50 min (x2)
5.	Molten wax	105 min
6.	Molten wax	90 min (x2)

Serial of three sections were then taken from the embedded tissues at 5µm thickness using a microtone and placed on consecutive slides.

Staining

According to the procedure modified from Bancroft and Stevens (1991), the sections were stained with Mayer's haematoxylin and eosin Y as follows:

1.	Xylene	3min then 2min
2.	Absolute ethanol	2 min
3.	methylated spirit	1 min
4.	Wash in water	0.5 min
5.	Haematoxylin	5min
6.	Wash in water	0.5 min
7.	1%Acid alcohol	3 quick dips
8.	Wash in water	0.5 min
9.	Scott's tap water substitute	1 min
10.	Wash in water	0.5 min
11.	Eosin Y	5 min
12.	Wash in water	0.5 min
13.	Methylated spirit	1 min
14.	Absolute alcohol	2 min then 1 min
15.	Xylene	5 min

The slides were held in xylene until cover-slipping using Pertex mountant. Gonad development was determined using an Olympus BH-2 binocular microscope with images being captured an Olympus zoom lens linked to a computer using image capture software.

2.3 Analyses

2.3.1 Determination of plasma melatonin levels

Determination of plasma melatonin levels was carried out in the laboratory using commercially available melatonin ELISA kit (competitive enzyme immunoassay) for the quantification of plasma melatonin in the plasma (IBL, Immuno-biological Laboratories GmbH, Hamburg). Prior to the analyses, the kit was validated by confirming the

parallelism between serial dilutions of night-time pooled plasma from African catfish, *Clarias gariepinus* and Nile tilapia, *Oreochromis niloticus* to the standard curve (Fig. 2.2). All standards and samples were assayed in duplicate. The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard, was 3pg/ml. Pooled rainbow trout plasma samples were used to check the reproducibility of measurements between assays, i.e. for quality control. The intra- and inter-specific assay coefficients of variation, calculated from aliquots of pooled rainbow trout plasma used as quality controls (QCs), were 2.7 % and 5.5 % respectively.

Explanation of the test

The assay involves a competition between a biotinylated and a non- biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of anti-biotin alkaline phosphatase as a marker and p-nitrophenyl phosphate as a substrate. Quantification is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards.

Contents of the kit and preparation of reagents

This kit provided:

1. 20 pieces of extraction columns (100mg) ready to use
2. One bottle of Assay Buffer (15ml): phosphate buffer with tween and stabilizer; dilute 1:10 with bidistilled water.
3. Three vials of Antiserum: dissolve contents of each vial in 2ml of bidistilled water.

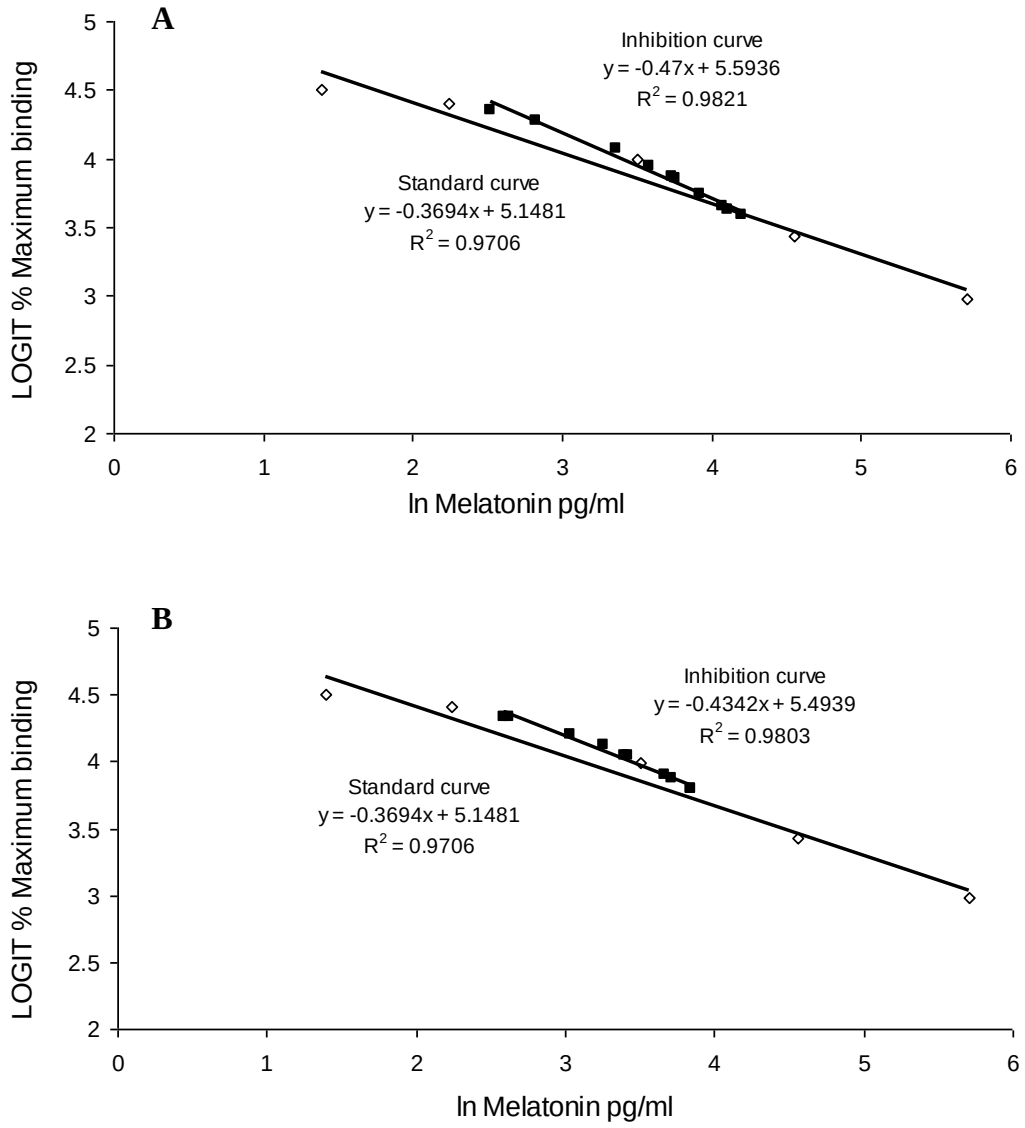

Figure 2.2 Parallelism of an inhibition curve obtained from a serial dilution (1:10) of pooled night time extracts of a) Nile tilapia (*O. niloticus niloticus*) and b) African catfish (*C. gariepinus*) with the Melatonin Elisa standard curve (IBL, Hamburg, Germany). The curves have been linearised using the logit transformation, with the x-axis denoting the natural log of the melatonin content in the standards. When inhibition plot was compared to the standard curve (t tests), no significant difference between the slopes of the plots was found. This validated that the melatonin measured in the samples was immunologically similar to the standards.

4. Three vials of melatonin-Biotin: dissolve contents of each vial in 2ml of assay buffer.
5. Six vials of standard A-F: dissolve contents of each vial in 2ml of bidistilled water.
6. Two vials of control serum 1 and 2: dissolve contents of each vial in 2ml of bidistilled water.
7. One vial of enzyme conjugate (250 µl): contains of anti-biotin-alkaline phosphatase in TRIS buffer with stabilizer, dilute 1:81 with Assay Buffer.
8. P-nitrophenyl phosphate (PNPP) substrate buffer (30ml): contains of diethanolamine, ready to use.
9. Nine tablets of PNPP Substrate: dissolve every 3 PNPP Substrate tablets in 8 ml of PNPP substrate buffer.
10. One bottle of PNPP stop solution (10ml): contains 1 N NaOH with 0.25 M EDTA.
10. Three pieces of adhesive foil.

Melatonin assay procedure

Preparation of column

1. Place one extraction column for each sample to be extracted into polystyrene tubes (12x75mm).
2. Add 2x 1ml of methanol (100%) to columns, centrifuge for one minute at 200x g.
3. Add 2x1ml of bidistilled water to columns, centrifuge for one minute at 200x g.

Preparation of samples

4. Place each column into correspondingly marked tubes.
5. Add 0.5ml of standards, controls and samples to columns, centrifuge for one minute at 200 x g.

6. Add 2x 1ml of 10% methanol to columns, centrifuge for one minute at 500x g.
7. Place the columns into clean correspondingly marked polystyrene tubes.
8. Add 1ml of methanol (100%) to columns, centrifuge for one minute at 200x g.
9. Remove columns from tubes, evaporate the methanol to dryness and reconstitute the samples with 0.15ml of bidistilled water, Vortex for at least one minute.

Assay procedure

10. Pipet 50 µl of each extracted standards, controls and samples into the wells.
11. With a multipipette add 50 µl melatonin-biotin and 50 µl of antiserum into each well and shake the plate carefully.
12. Seal the plate with adhesive foil and incubate overnight (14-20h) at 2-8 °C.
13. Wash each well three time with assay buffer. Remove the assay buffer and invert the plate and remove any remaining liquid by tapping on clean paper.
14. With a multipipette pipet 150 µl of enzyme conjugate into the wells.
15. Seal the plate with adhesive foil and incubate for 120 minute at room temperature on an orbital shaker (500U/min).
16. Wash each well three time with assay buffer. Remove the assay buffer and invert the plate and remove any remaining liquid by tapping on clean paper.
17. Using a multipipette, add 200 µl of PNPP substrate solution into each well.
18. Incubate at room temperature for 20-40 minute on an orbital shaker (500U/min).
19. Using a multipipette, add 50 µl PNPP stop solution into each well to stop the substrate reaction. Gently shake the plate for mixing the contents.
20. Read the optical density at 405nm (reference wave length 600-650nm) with a microtitre plate reader within 60 minute after stopping.

Calculation of results

The concentrations of the standards (abscissa, logarithmic) are plotted against their corresponding optical density (ordinate, linear) on a graph paper. The concentration of the samples can be read directly from this standard curve by using their average optical density. Any sample reading greater than the highest standard, should be diluted with assay buffer and reassayed.

2.3.2 Testosterone and 17-β Oestradiol Assay

The levels of testosterone (T) and 17-β Oestradiol (E2) present in blood plasma were measured using a radioimmunoassay adapted from Duston and Bromage (1987). Except for the use of specific antibodies, standards and radiolabels, the same procedure for both hormones was used as follows:

Steroid Extraction

Extraction of steroid from the plasma was required prior RIA analyses. Steroid was extracted by suspending the organic phase in a solvent (ethylacetate) as follows:

1. Add 200µ of plasma samples to 3ml polypropylene tubes (LP3P).
2. Add 1ml ethyl acetate to each tube and stopper.
3. Spin tubes on a rotary mixer for 1 hr.
4. Centrifuge tubes at 1500 rpm, for 10 minutes at 4°C.

Samples extracted can be assayed immediately or stored at 4°C.

Buffer

Glass volumetric flask was used to prepare the buffer by dissolving the following chemicals in 250ml of nanopure water at 50°C for 30 minutes:

4.44g disodium hydrogen phosphate

2.91g sodium dihydrogen phosphate

2.25g sodium chloride

0.25g gelatine

Buffer was made fresh for every assay and stored at 4°C.

Radiolabel

The tritiated testosterone label [(1,2,6,7-^{3}H) testosterone] and oestradiol label [(2,4,6,7-^{3}H) oestradiol] was supplied by Amersham Company, in 250 µCi quantities with specific activity of 70-105 Ci/mmol. An intermediate solution was prepared in 20ml glass vials by diluting 20µ of the primary stock in 2ml absolute ethanol and then stored at -20°C until used. The working solution then was made from the intermediate solution. 90µl testosterone intermediate solution or 80µl estradiol intermediate solution was diluted in 10ml assay buffer to give an approximate activity 20,000dpm/100 µl.

Antibody

The preparation of both anti-testosterone and anti-17-β Estradiol are similar. Antibodies for both steroids were supplied freeze-dried by ABD Serotec Company Ltd., reconstituted in 1 ml of assay buffer. Antibodies were stored at -20°C as 100µl aliquots until the assay. A single 100µl aliquot was diluted in 9.9 ml buffer.

Standards

Stock standard solutions of testosterone and estradiol were supplied by Sigma-Aldrich Company Ltd. The stock solution (100ng/ml) was prepared by dissolving 1mg of testosterone or estradiol in 10ml of absolute ethanol and stored in glass vial at -20 ºC until required. A working solution (10 ng/ml) was prepared by diluting 100µl of stock solution

in 0.9ml of absolute ethanol and used to produce a standard serial dilution curve ranging from 1000pg to 1.95pg testosterone in 100µl absolute ethanol or 250pg to 0.488pg estradiol in 100µl absolute ethanol. All standards were produced in duplicate for testosterone or estradiol, 1000pg or 250pg respectively per tube standard were prepared from 100µl of working solution with subsequent standard being prepared from a series of 1:1 serial dilutions as follow:

Tube No.	standard (µl)	+ ethanol (µl)	standard amount
1&2	100	none	1000(T) or 250(E_2)
3&4	100	100	500(T) or 125(E_2)
5&6	100 of 3&4	100	250(T) or 62.5(E_2)
7&8	100 of 5&6	100	125(T) or 31.25(E_2)
9&10	100 of 7&8	100	62.5(T) or 15.625(E_2)
11&12	100 of 9&10	100	31.3(T) or 7.812(E_2)
13&14	100 of 11&12	100	15.6(T) or 3.9(E_2)
15&16	100 of 13&14	100	7.8(T) or 1.953(E_2)
17&18	100 of 15&16	100	3.9(T) or 0.976(E_2)
19&20	100 of 17&18	100	1.95(T) or 0.488(E_2) (Remove 100µ from each tube)
21&22	None	100	0
23&24	None	100	NSB

Assay protocol

1. 200µ extract sample was added to each duplicate LP3P tube
2. The standard curve was prepared as described above which included 4 tubes containing only absolute ethanol; and duplicate tubes for zero steroid level and duplicate tubes to show any non-specific binding (NSB).
3. All tubes were dried in a vacuum oven at less than 35°C for approximately 45minutes then the tubes were cooled to 4°C.
4. 100µl antibody solution was added to all tubes except NSB's (to these were added 100µl of buffer).

5. 100µl of intermediate solution label (^{3}H-Testosterone or ^{3}H-Oestradiol) was added to all tubes, the tubes then were vortexed and incubated at 4°C for 18 hrs.
6. 0.48g dextran coated charcoal was dissolved in 100 ml buffer and stirred on ice for 30 minutes.
7. 500 µl of charcoal suspension was added to all to remove any unbound radioactivity, then tubes were vortexed and incubated at 4°C for 15 minutes.
8. The charcoal was then taken out of suspension by centrifuging all tubes at 2000rpm at 4°C for 15 minutes.
9. 400µl supernatant was transferred to 6ml polyethylene scintillation vials, then 4 ml of Ultima Gold scintillation fluid was also added.
10. All scintillation vials were vortexed and counted for 10 minutes using Packard 1900 TR Liquid Scintillation Analyzer (Canberra Packard Ltd, Packard instrument company).

Aliquots of pooled rainbow trout plasma were used as quality controls (QCs) and the intra- and inter-specific assay coefficients of variation were 3.7 % and 2.9 %, respectively, for the testosterone assays and 6.3 % and 5.5 %, respectively, for the estradiol assays.

Calculation of results

The readings of unknown samples showed the concentration of testosterone or 17-β Estradiol per tube in Pg. These values were subsequently converted to Pg.ml^{-1} by exporting the results to an Excel sheet and using the following formula:

$$\left(\frac{Pg\ per\ tube}{400}\right) \times 700 = \text{Pg of T or } E_2 \text{ per 200µl of extract (Equation 1)}$$

$$\left[\frac{(\text{Equation 1})}{200} \times 1200\right] \times 5 = \text{Pg. ml of T or } E_2$$

2.3.3 Locomotor activity recording

The purpose of this technique is to determine or sample the overall locomotor activity observed in groups of fish or individuals exposed to various conditions and produce a representative result. Results obtained do not represent the absolute or total activity displayed by fish, but only provide a representative snapshot of the activity over a period of time. Such activity recording systems have been used in several other fish species including Japanese sea catfish (Kasai *et al*, 2009), Nile tilapia (Vera *et al*., 2009), zebrafish (Olmeda and Sanchez-Vazquez, 2009) and seabream (Vera *et al*., 2006).

The system

Locomotor activity of catfish was registered using infrared photocell sensors (E3Z-D67, OMRON, China). Photocells were connected to a motherboard (USB-1024HLS, Measurement Computing, USA) connected to a computer. Every time a fish interrupted the infrared light beam it produced an output signal that was recorded and stored in the computer in 10 minute bins using specialized software (DIO98USB, University of Murcia, Spain).

Locomotor Activity Monitoring

An infrared photocell was placed in the front wall of each aquarium. Three different positions were first tested to optimise and validate the assessment of locomotor activity in catfish which have a very different behaviour to most previously studies species: top, middle and bottom layers of the tank. A total of nine aquaria were used for this purpose, three per photocell position (triplicated). Each tank was provided with one catfish (155±6g) to estimate the ability of the system to detect low activity levels. Animals were acclimatized to a 12:12 h LD cycle for two weeks and then their locomotor activity was

monitor for one full week. At the end of the trial, the mean daily total activity of catfish was 27±7, 55±3 and 78±5 registers/10min for top, middle and bottom placement of the photocell, respectively. However, the locomotor activity of fish monitored with photocells set in the middle position showed a clearer daily rhythm under a LD cycle, in comparison to the activity registered by photocells placed at the top and bottom positions (Figure 2.3). Such middle location placement of the photocells was therefore adopted during the present experiments. Additional optimisation/validation steps were performed prior to the start of the trials to set up the infrared beam range of detection.

Data analysis

The raw data were analysed using the integrated package for chronobiology analysis "El Temps" (A. Díez-Noguera, Universitat de Barcelona, Spain, 1999). Activity data were plotted as actograms, which is the graphical display of a time series along two time axes and double plots to represent the activity patterns. The duration of a cycle (or predicted duration of a cycle) determines the length of each plot line. Successive cycles (days) are plotted on successive lines. In each line, values may be plotted in digital format (all-or-none data points) or analog format (using compressed Y axes). The horizontal lines in each actogram represent 24-h periods; the vertical marks indicate bouts of activity per 10 min. Successive days are plotted from top to bottom.

Under constant conditions, the actograms showed free-running rhythms. The period length (*tau*) of this rhythm was determined by chi square periodogram analysis (Sokolove and Bushell, 1978) at a confidence level of 95%. The periodogram method provides information about the significant periods as well as the percentage of variance (PV) explained by the rhythm using the mean activity level as a threshold. The mean activities of catfish were exported to Microsoft Excel to estimate the total daily activity for each tank

and the percentage of activity displayed under both the photo- and scotophase. The mean activity of catfish was then represented as waveforms.

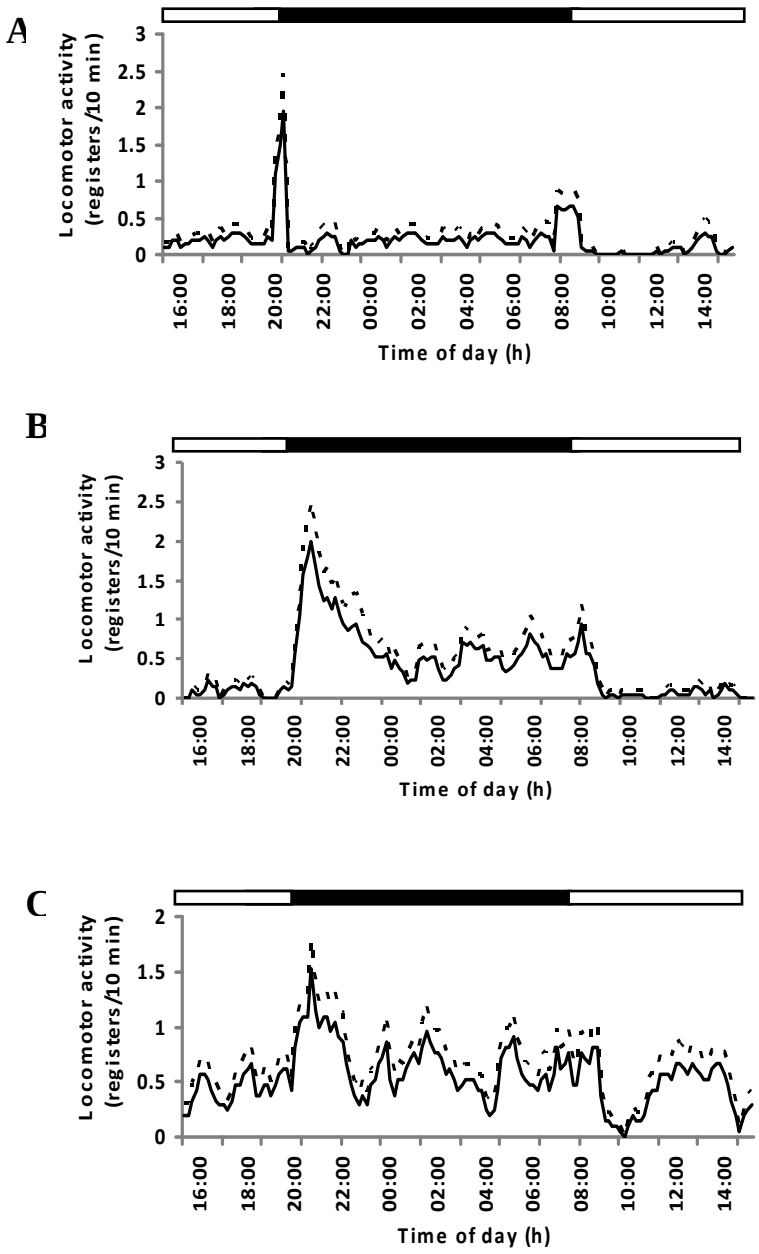

Figure 2.3 Average daily locomotor activity observed in *C. gariepinus* using different location of photocells: top (A), medial (B) and bottom (C). Data are expressed as mean of the three tanks (thick line) + S.E. (dotted line). Horizontal bar above the graph represents day (in white) and night (in black) hours.

CHAPTER 3

PAPER I: EVIDENCE FOR DIFFERENTIAL PHOTIC REGULATION OF PINEAL MELATONIN SYNTHESIS IN TELEOSTS

The following manuscript was compiled from data from six different species of teleosts. The current author was involved in the design of the experiments, sampling and analyses and supplied the data related to African catfish. He also contributed to the sampling of the Nile tilapia experiment. Co-authors provided data from the other species (rainbow trout, Atlantic salmon, European seabass, Atlantic cod and Nile tilapia). The submitted manuscript was written in collaboration between all authors.

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EVIDENCE FOR DIFFERENTIAL PHOTIC REGULATION OF PINEAL MELATONIN SYNTHESIS IN TELEOSTS

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Running title: Photic regulation of pineal melatonin in teleosts

Keywords: Ophthalmectomy; melatonin; teleosts; circadian axis; pineal gland

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ABSTRACT

The aim of this study was to compare the circadian control of melatonin production in teleosts. To do so, the effects of ophthalmectomy on circulating melatonin rhythms were studied along with *ex vivo* pineal culture in six different teleosts. Results strongly suggested that the circadian control of melatonin production could have dramatically changed with at least three different systems being present in teleosts when one considers the photic regulation of pineal melatonin production. Firstly, salmonids presented a decentralized system in which the pineal gland responds directly to light independently of the eyes. Then, in seabass and cod both the eyes and the pineal gland are required to sustain full night-time melatonin production. Finally, a third type of circadian control of melatonin production is proposed in tilapia and catfish in which the pineal gland would not be light sensitive (or only slightly) and required the eyes to perceive light and inhibit melatonin synthesis. Further studies (anatomical, ultrastructural, retinal projections) are needed to confirm these results. *Ex vivo* experiments indirectly confirmed these results, as while the pineal gland responded normally to day-night rhythms in salmonids, seabass and cod, only very low levels were obtained at night in tilapia and no melatonin could be measured from isolated pineal glands in catfish. Together, these findings suggest that mechanisms involved in the perception of light and the transduction of this signal through the circadian axis has changed in teleosts possibly as a reflection of the photic environment in which they have evolved in.

INTRODUCTION

Photoperiodism in all vertebrates relies upon a "central circadian axis" comprising the retina, suprachiasmatic nucleus of the hypothalamus (or comparable brain region) and pineal complex, which have been shown to be involved in the control and regulation of circadian and circannual rhythms (Collin *et al*., 1989; Falcon, 1999). There is extensive literature describing the gross structure and examining the potential role performed by these individual components, in particular the pineal, in non-mammalian vertebrates; however, there is limited work considering the system as a whole and discussing its interaction (Ekstrom and Meissl, 1997). Common to all vertebrates is the fact that the circadian axis is based around a circadian pacemaker mechanism fed entraining light signals from photoreceptors that are then turned into neuroendocrinological signals that subsequently transmit this information to target tissues that then determine the physiological response (Korf *et al*., 1998; Simonneaux and Ribelayga, 2003). In mammals the indoleamine melatonin released into the plasma by the pineal gland, accurately reflects night period and it is shown to regulate many of the above-mentioned rhythms by targeting receptors in the hypothalamic region of the brain (Simonneaux and Ribelayga, 2003; Falcon *et al*., 2007). In non-mammalian vertebrates it has been suggested that circulating melatonin can be produced by solely the pineal, or the retina can provide a contribution (Ekstrom and Meissl, 1997; 2003; Steele *et al*., 2006; Wright *et al*., 2006). Although there has been much work focused on melatonin as it is the main endocrine signal shown to be regulated by photoperiod (Max and Menaker, 1992; Randall *et al*., 1995), its role in regulation of physiological rhythms such as reproduction remains unclear in teleosts (Ekstrom and Meissl, 1997; Bromage *et al*., 2001).

Importantly, there is a strong indication that the control of pineal activity has changed dramatically during phylogeny, as a response to 500 million years of evolution to the

diverse environments occupied by vertebrates during that time (Falcon, 1999; Mayer *et al*., 1997). In mammals, previous studies have demonstrated through ophthalmectomy (Underwood *et al*., 1990; Yamazaki *et al*., 1999) that photoentrainment is exclusively mediated by retinal photoreceptors and as such pineal photoreceptors have lost their direct light sensory abilities in comparison to lower vertebrates, reducing their role solely to a melatonin secretory gland (Ekstrom and Meissl, 2003). However, unlike mammals, in all teleosts species studied so far, to our knowledge, as in birds, *in vitro* studies have shown that the pineal gland was directly photosensitive (Max and Menaker, 1992; Randall *et al*., 1995; Falcon, 1992; Migaud *et al*., 2006). Such reports came from studies mainly performed in temperate fish species but also two tropical species, the goldfish *Carassius auratus* (Iigo *et al*., 1991) and zebrafish *Danio rerio* (Cahill, 1996). In summary two forms of circadian organization have been previously suggested in fish relating to melatonin secretion by the pineal gland (Falcon, 1997; Ekstrom and Meissl, 1997; 2003; Falcon *et al*., 2007): a) salmonids, a group of fish characterized by a directly light sensitive pineal, without pacemaker activity (no melatonin rhythm appears under constant darkness and a light entrained rhythm is observed under LD) , and b) all other fish studied, in which the pineal organ is a true circadian light sensitive pacemaker (melatonin displays a free running circadian rhythm under DD and a light entrained rhythm under LD). However, these two models are only based on the pineal gland and do not consider the potential integrated role of the retinas as is seen in higher vertebrates. Because there is a natural tendency to generalise results that one finds in a given fish species to the whole teleost phylogenic class (Falcon *et al*., 2007), the hypothesis tested in the present study was that due to the variety of environments inhabited by fish, from temperate to tropical or freshwater to deep seawater, and high divergence demonstrated in fish physiology regarding biological rhythms in terms of feeding behavior and locomotor activity (diurnal

vs. nocturnal) and reproductive strategies (iteropare *vs.* continuous spawner), it is unlikely that one unique circadian organization (retina-pineal gland network) exist in fish. The objective of this study was, therefore, firstly to compare the effects of ophthalmectomy on melatonin production in a diverse range of teleosts from temperate to tropical latitudes. Secondly, these results were confirmed through *ex vivo* pineal gland culture. Finally a comparison of light transmission through the cranium was measured in all species studied.

MATERIALS AND METHODS

Fish and facilities

Fish species, origin and mean weight of populations used during the experiments are presented in Table 1. Experiments have been performed in a number of rearing systems depending on the species. Three main facilities owned by IoA were used: flow through freshwater rearing tanks at Niall Bromage Freshwater Research Facility (NBFRF) for Rainbow trout (*Onchorhynchus mykiss*), flow through sea water tanks at Machrihanish Marine Environmental Research Laboratories (MERL) for Atlantic salmon (*Salmo salar*), European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) and recirculating tank systems at the Tropical Research Facilities for Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarius gariepinus*). All fish populations were reared under ambient temperature and photoperiodic regimes (simulated natural photoperiod, 56°N 3°W, range 6-18hr photophase) except for tilapia and catfish which were held at constant temperature (27 ± 1^{o}C) and photoperiod (12L:12D). All experiments were carried in accordance with the Animal (Scientific Procedures) Act 1986, UK.

Experiment 1*: in vivo* ophthalmectomy

Fish were acclimated to a 12L:12D photoperiod for at least 2 weeks before surgery. The fish were anaesthesised using a 1:20,000 concentration of 2-phenoxyethanol solution (0.2mL/L, SIGMA). The membrane around the eye was cut out, the eye lifted and the optic nerve sectioned. A drop of a 3/1 w/w mix of Orahesive powder (ConvaTec, Ref 25535, Squibb & Sons Ltd., UK) and cicatrin antibiotic (The Wellcome Foundation Ltd., London) was applied to the eye socket. No mortalities were observed. Two days following the ophthalmectomy procedure, fish were captured and immediately killed by lethal anaesthesia in a 2-phenoxyethanol solution (1mL/L, SIGMA) and then blood sampled at day (12.00h) and night (04.00h) over two consecutive days (n=4-8 depending on species and sampling). No sham operation could be performed due to limitations in fish number and restrictions placed by our local ethical review committee. Nocturnal blood samples were taken in red dim light with the head of the fish covered.

Experiment 2: *Ex vivo* pineal gland culture

Fish from same origin than used for *in vivo* experiments (Table 1) were acclimatised to a 12L: 12D photoperiod and standard rearing temperature (10 ± 1°C for salmon, trout, cod and sea bass and 27 ± 1°C for tilapia and catfish) during a 2 week period. The pineal culture system consisted of a continuous flow through system regulated by a peristaltic pump at a flow rate of 1.5 ml of culture medium / hour and a fraction collector automatically collecting samples every hour after passing through the culture chambers (Migaud *et al*., 2006). The culture media (Sigma, ref: R8755) was supplemented with HEPES sodium salt (Sigma, ref: H3784, 4.77g/l) as a pH regulator with the pH adjusted to 7.4 and penicillin-streptomycin (10mg/l) and Fungizone (5mg/ml) to avoid bacterial and fungal development. Medium was replaced every day. Immediately after their capture, fish

were killed by lethal anaesthesia in a 2-phenoxyethanol solution (1mL/L, SIGMA). Fish were sampled during the day period and pineal glands removed using a dissecting microscope, washed with culture medium, placed in incubating chambers and then exposed *ex vivo* to the same photoperiod and temperature regime. Dissection of the pineal glands was adapted for each species depending on size, skull thickness, exact location and overall ease to sample. In trout and salmon, due to the relative large size of the pineal, the fish head was sectioned laterally below the brain which was then lifted to access the pineal gland. Whereas in cod, tilapia, catfish and sea bass the pineal gland was accessed dorsally by opening the skull around the pineal window. Once in the culture system, pineal glands were maintained for two complete LD cycles. Pineal glands were illuminated by custom made light boxes with dichroic halogen bulbs characterized by an emission spectrum equivalent to a 4700ºK Black Body radiator (Solux, 4700K CRI 99, 10° spread, USA) providing a light intensity of approximately 12watts/m^2 at the pineal level during the day (measured by a single channel light sensor, Skye instruments, UK). Only selected media samples were analysed (2-3/day-night periods depending on species) for melatonin levels corresponding to 4, 8 and 12hrs of each day or night period. At the end of the culture period the pineal glands were removed from the culture chambers and cells viability was checked. To do so, the pineals were stained with 0.2% trypan blue (BDH Merck Ltd. UK.) in phosphate buffer and observed under x100 magnification using an Olympus CH light microscope (Olympus Optical Co., London, UK.).

Experiment 3: Cranial light transmission

Fish origin and mean weight are presented in Table 1. Results obtained for salmon and sea bass were previously published (Migaud *et al*., 2006). All fish were killed by a lethal dose of anesthetic and then decapited. The cranium was dissected and tissue underneath the

skull removed to access the pineal window (the overlying dermal tissue was left intact) and transmission measurements performed immediately. The same lighting system as that used in the *ex vivo* experiment was used in this study. The light box was placed at a standardised distance (26cm) from the dissected cranium. Light intensity from the light source was checked prior to measurement for all species. Light measurements were carried out using a spectroradiometer equipped with a fiber optic cable and cosine corrector (EPP2000c Stellarnet Inc., USA, calibrated to National Physics Laboratory UK standard light sources) placed directly behind the pineal window. To study the differential penetration of light of different spectrum, visible spectrum was divided in seven equal narrow bandwidths using bandpass interference filters (Melles Griot Photonics Component Group) characterised by a FWHM (Full Width Haft Maximum) of 80nm (centre wavelengths: 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61nm). Differences in relative transmittance between filters were corrected by the use of neutral density filters in order to balance light intensity at 5 watts/m^2, $1.6x10^{15}$photons/sec/cm^2. Readings were recorded in watts/m^2 (400-740nm) and transformed into a percentage of full relative illumination passing through the pineal window.

Melatonin assay

Blood and *ex vivo* media samples were stored at - 70°C until assayed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). All standards and samples were assayed in duplicate. Intra-assay coefficient of variation were 5.5% (n=4) and inter-assay coefficient of variation were 9.4% (n=3). The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard was 3pg/ml. Pooled rainbow trout plasma with a melatonin content of approximately 250

pg/ml, sampled during the night, was used to check the reproducibility of measurements between assays, i.e. for quality control.

Statistical analysis

In vivo data (experiment 1) were analysed by a nested ANOVA using a General Linear Model (GLM) with treatment and time as tested factors (replicate nested within treatment). When comparing mean melatonin levels *ex vivo* (experiment 2, 4-6 pineal glands/species, 2-3 day-night periods, 2-3 samples analysed/period) and penetration of the light through the pineal window (experiment 3), statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by Tukeys multiple comparison test. Data are expressed as mean + SEM. No replicate effects were observed and as such data were pooled. All statistical tests were carried out with Minitab v14.1. The minimum level of significance was set at $P \leq 0.05$.

RESULTS

No significant differences in melatonin profile and levels were observed in ophthalmectomised fish as compared to intact fish in both Atlantic salmon and rainbow trout (Fig. 1a-b). However, in ophthalmectomised sea bass and cod melatonin levels were significantly lower at night as compared to intact fish except in sea bass during the second night (Fig. 1 c-d). With regards to Nile tilapia and African catfish, night plasma melatonin increase was suppressed in ophthalmectomised fish with levels remaining comparable to basal day levels (Fig. 1e-f). Relative to night levels in controls, plasma melatonin in ophthalmectomised trout and salmon was unchanged (≥100%), reduced to 40-60% in sea bass and cod and below 20% in tilapia and catfish (Fig 2).

When trout, salmon, sea bass or cod pineal glands were exposed to a 12L:12D cycle, rhythmic melatonin production were observed with low day levels (below 100pg/ml in rainbow trout and Atlantic cod and below 500pg/ml in Atlantic salmon and sea bass) and high night-time levels (mean levels from 2500 to 3700pg/ml in trout, salmon and cod and 1200pg/ml in sea bass, Table 2). Melatonin synthesis and release from Nile tilapia pineal glands was very low at night (15.9 ± 2.8pg/ml), however, a day night rhythm was still observed although levels were below the assay sensitivity threshold (day levels of 0.6 ± 0.4pg/ml). Numerous attempts to culture catfish pineal glands were performed under various conditions (fish history, pineal removal, medium, temperature) but no melatonin production above threshold of assay sensitivity was measured in response to a LD cycle. When comparing all species for the relative melatonin synthesis and release in the culture medium at night by the pineal gland expressed as a percentage of plasma melatonin, a clear difference was observed in tilapia (plasma melatonin equivalent to 660% of *ex vivo* melatonin released by a pineal gland) as compared to the other species (between 3 and 11%). Similarly, when considering day levels, tilapia plasma melatonin concentrations were equivalent to 1522% of what is produced by a pineal gland as opposed to <32% in all the other species.

Light penetration through the pineal window in the 6 species was studied (Fig. 3). A significantly higher percentage of light (ambient spectrum recreated by the use of day light bulbs) penetrated the tilapia pineal window (8.23 ± 0.58%) relative to sea bass (4.28 ± 0.26%), cod (3.21 ± 0.12%), trout (2.57 ± 0.10%), salmon (2.23 ± 0.16%) and catfish (1.05 ± 0.09%) (Fig. 3a). Penetration was directly related to wavelength with longer wavelengths having a greater penetrative ability (Fig. 3b). Penetration of light in tilapia remained significantly higher than all other species at wavelength >550nm.

DISCUSSION

The rhythmic melatonin signal remains a highly conserved circadian output across all vertebrates and reflects the perception of the prevailing photoperiod. However, the circadian control of melatonin production by the pineal gland has considerably evolved. In higher vertebrates this system is highly compartmentalised (Morse *et al*., 2002) which contrasts with that of lower vertebrates and invertebrates that possess a network of independent oscillatory components (Tamai *et al*., 2003). Many studies have focused on characterising the function of the pineal organ in fishes (e.g. Falcon, 1999; Max and Menaker, 1992; Gern *et al*., 1992; Molina *et al*., 1996; Cahill, 1996). However, research into circadian biology to study the pineal gland as part of an entire system/network within the lower vertebrates has been sparse by comparison with that in mammalian and invertebrate models.

The current results bring further evidence from melatonin studies that suggest mechanisms involved in the light perception and transduction through the central circadian axis would have radically changed in teleosts species probably reflecting the environment in which they have evolved in. To date, only two kinds of circadian organization have been proposed *i.e.* salmon *vs.* other teleosts (Ekstrom and Meissl, 2003, Falcon, 2007). It is presently suggested that a third organization could be at work in teleosts based on the photic control of melatonin production by the eyes and pineal gland. First, in salmonids, represented by salmon and trout in this study, the circadian melatonin rhythms and amplitude of the levels produced were not affected by the ophthalmectomy. A similar bilateral ophthalmectomy operation in goldfish (Kezuka *et al*., 1992) did not significantly affect plasma melatonin levels as well. This confirms in these species the pineal gland is light sensitive and does not require input from the eyes to control rhythmic melatonin production (Ekstrom and Meissl, 1997; Kezuka *et al*., 1992). Such a system could be

considered as not specialized with pineal cells both perceiving light and producing melatonin. This also confirms that melatonin produced by the eyes in such species would not contribute to plasma levels. In fact, melatonin synthesis by fish retina was shown in certain cases (species and season dependent) to be high during the photophase (Underwood *et al*., 1990; Falcon *et al*., 2003; Besseau *et al*., 2006) as opposed to higher vertebrates where retinal melatonin synthesis is enhanced in the scotophase as in the pineal gland (Simonneaux and Ribelayga, 2003; Cahill and Besharse, 1991). Such phase shift differences between pineal and retinal melatonin production could be due to different functional roles with melatonin from the pineal gland providing a reliable endocrine indicator of the day/night cycle (Falcon, 2007) while melatonin from the eyes could be involved in the paracrine protection and adaptation of the retina (Falcon *et al*., 2003; Besseau *et al*., 2006; Iuvone *et al*., 2005).

A different circadian system could be at work in seabass and cod as ophthalmectomy resulted in a significant decrease of night time production of melatonin. Such results are in accordance with previous reports in seabass (Oshima *et al*., 1989) as well as birds (Brandstatter, 2003; Jimenez *et al*., 1995) and amphibians (Wright *et al*., 2006). In all these species, findings suggest that both the eyes and the pineal gland are required to sustain full amplitude melatonin rhythms meaning that light perceived by the eyes could regulate melatonin synthesis by the pineal gland probably through neural projections into the brain (Jimenez *et al*., 1995; Yanez and Anadon, 1998). In fish, studies have shown that three different types of pinealocytes (true and modified photoreceptors and pinealocytes) co-exist in the lamprey or pike (Ekstrom and Meissl, 2003), although it is thought that pinealocytes are the evolved form of the true pineal photoreceptors; in mammals only pinealocytes remain (Ekstrom and Meissl, 2003; Malpaux *et al*., 2001). It is not known whether these different forms co-exist in both sea bass and cod, but if this were the case it

could explain how light perceived by the retina may influence pineal activity. Further studies are clearly needed to characterize this network.

The situation in tilapia and catfish appeared very different from all other teleosts studied and suggests, for the first time, the existence of a possible third kind of circadian system in which the pineal gland would not be light sensitive or far less sensitive than previously studied teleost species. Furthermore, the results suggest these species would also not contain an independent circadian pacemaker as following bilateral ophthalmectomy, night-time melatonin rise was shown to be fully abolished with basal levels maintained as during the day. *Ex vivo*, the tilapia pineal gland displayed rhythmic melatonin production. It is very unlikely however that the levels recorded (20pg/ml/h) could explain blood levels observed in the species, especially as it has been shown in higher vertebrates that melatonin produced by the pineal gland is also directly released in the cerebrospinal fluid (CSF) through the pineal recess (Tricoire *et al*., 2002) resulting in levels twenty times as high in the CSF as in blood (Skinner and Malpaux, 1999). Although no *ex vivo* melatonin production was observed in catfish after many attempts and cell viability confirmed, no definitive conclusions can be made as such results could still relate to the difficulty of extracting the gland in this species. As such, these results would suggest for the first time a mammal-like circadian organization in terms of the photic control of melatonin production in at least two teleost species in which the system would be more specialized, with the eyes involved in light perception and the pineal gland reduced to a slaved secretory gland. However, one fundamental difference with mammals remains, that being the lack of an apparent independent circadian pacemaker which would drive the melatonin production in the absence of the eyes. Interestingly, another circadian organisation also relying on retinal photoreception has been suggested in a more primitive fish species, the hagfish, *Eptatretus burgeri* (Menaker *et al*., 1997; Kabasawa and Ooka-

souda, 1989). Further studies are clearly needed to confirm the existence of such systems with especially the characterization of the anatomy and ultrastructure of the pineal gland in relation to retinal neural projections. The same is true for the sea bass and cod as the present results clearly imply that in all four species retinal and/or deep brain photoreception may contribute, i*n vivo,* to the control of melatonin production. But, to date, to our knowledge, no direct connection between the retina and the pineal gland has been clearly identified in teleosts.

It is recognised that the effects of post-surgery stress on melatonin synthesis following ophthalmectomy may raise concerns. However, the present results obtained in salmonids and sea bass match the findings of previous studies (Mayer *et al*., 1997; Bayarri *et al*., 2003) where in some cases (Bayarri *et al*., 2003), samples were taken two weeks post-surgery as opposed to 48 hours in the present study. This could thus confirm that post-surgical stress would not affect melatonin production and secretion. It appears then unlikely that results obtained in the remaining species studied could have been influenced by post-surgery stress while results in salmon and sea bass appeared not to be.

To understand whether the circadian system at work could be related to the perception of light by the pineal gland, light transmittance through the cranium was investigated. Clear differences were observed between species with the lowest overall light transmittance in catfish (1%) and the highest in tilapia (>8%). Furthermore, light transmittance is clearly dependent on the spectral content of the light with longer wavelength penetrating the cranium more efficiently. Interestingly, irrespective of pigmentation, trout, salmon and catfish showed a similar profile of transmittance across the visible spectrum but tilapia was characterized by a much higher penetration than the other species for spectra ≥ 650nm (>14% *vs*. <6% in the other species). Together, these results are surprising as both tilapia and catfish would appear to have similar circadian control of

melatonin production. It has been suggested (Menaker *et al*., 1997) that the inherent advantage of localised (decentralised) photoreception (and regulation) as seen in salmonids, sea bass and cod in the present study, is lost with evolution in an environmental niche with weak environmental entraining signals. In such a habitat multiple oscillators bring the risk of generating conflicting messages, and a more centralised system is favoured, such as that in mammals. In fact, only the most sensitive photoreceptors in the most exposed tissues that can receive enough light to generate a response would remain during evolution (Menaker *et al*., 1997). Such a hypothesis is further strengthened by the apparent lack of photic sensitivity in catfish pineal gland, which could be an adaptation to the very low light transmittance of this species chosen habitat. However, it is difficult at this stage to explain how and why the pineal gland in tilapia, although exposed to more light than all the other teleost species studied, would not directly respond to light or only slightly. It is possible however that the ancestral line was earlier subjected to such a selection pressure (e.g. nocturnal existence) which forced the circadian adaptation apparent today, as has been proposed for mammals.

The circadian axis in fish thus appears to be a very interesting system to study evolution within a single vertebrate class. While some teleosts have a fully integrated "circadian axis" without pacemaker activity within the pineal gland (salmonids, Fig. 4a), in others the light sensitive pineal gland has become increasingly dependent on retinal (and possibly deep brain) photoreception (sea bass and cod, Fig. 4b) to such an extend that in some cases (tilapia and catfish, Fig. 4c) the pineal gland could have lost its light sensitivity and become reliant on retinal (and possibly deep brain) photoreception alone. This would clearly suggest that a shift has occurred within teleosts towards a compartmentalized "circadian system", similar to what is seen in mammals (Fig. 4). Importantly, the location and role of circadian pacemakers within these systems has yet to be characterized. As

previously stated (Kusmic and Gualtieri, 2000), the differences in circadian organisation that one finds among the vertebrates are to a large extent the consequence of rapid adaptation to particular photic niches into which groups have been pushed by a variety of unrelated selection pressures. Fish have undoubtedly evolved during a very long period to very diverse environments. And importantly, if these adaptations have been dictated by numerous factors (e.g. temperature, water level, food availability, predation…) it can be suggested that the circadian systems have been mainly shaped by the light signal (Menaker *et al*., 1997). The diversity of circadian system suggested in the present study is at first glance closely related to the phylogeny of the fish species studied. However, findings in catfish clearly showed that phylogeny may be a little too simplistic as although catfish could be considered as primitive as salmonids (subdivision of the Ostariophysi) (Helfman *et al*., 1997), a comparable circadian system to tilapia was suggested by the present data. Further studies on species across the animal kingdom will certainly help to understand the evolution of the circadian control of melatonin and particular attention should be paid to the environmental history in which species have evolved to better define the role this has played in shaping this key regulatory system.

Acknowledgements

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Table 1. Origin and mean weight of each fish species used in the three experiments.

Species		**Origin**	**Experiment 1: Ophthalmectomy**	**Experiment 2: Pineal *in vitro***	**Experiment 3: Light transmission**
Common	**Latin name**				
Rainbow trout	*Oncorhynchus mykiss*	▪ Glen Wyllin, all female population (spring 05) ▪ NBFRF[1]	150 ± 19 g	86 ± 13 g	600 ± 151 g
Atlantic salmon	*Salmo salar*	▪ Howietoun Fisheries (March 05) ▪ MERL[2]	114 ± 12 g	193 ± 37 g	1850 ± 250[4]
European sea bass	*Dicentrarchus labrax*	▪ Llyn Aquaculture (spring 03) ▪ MERL	660 ± 123 g	152 ± 22 g	609 ± 100 g[4]
Atlantic cod	*Gadus morhua*	▪ MMF[3] (spring 05) ▪ MERL	63 ± 8 g	140 ± 42 g	932 ± 129 g
Nile tilapia	*Oreochromis niloticus*	▪ Tropical Facilities at IOA (2005) ▪ Red strain from the tilapia Reference Collection	208 ± 56.1 g	216 ± 71.0 g	523 ± 150 g
African catfish	*Clarias gariepinus*	▪ Tropical Facilities at IOA (spring 2005)	160 ± 27 g	420 ± 85 g	

[1] Niall Bromage Freshwater Research Facilities, IoA; [2] Machihanish Environmental Research Laboratories, IoA; [3] Machrihanish Marine Farms (cod hatchery); [4] salmon and sea bass used for the light transmission experiments were respectively originated from Marine Harvest Lochairlort Research Station and the Instituto de Acuicultura de Torre de la Sal in Spain.

Table 2. Comparisons between melatonin levels in the plasma and produced by pineal glands in culture. Levels are expressed as mean ± SEM with n representing the number of animals sampled and number of pineal glands, respectively, for *in vivo* (plasma) and *in vitro* (pineal culture) experiments.

Species	**Plasma (pg/ml)**		**Pineal culture (pg/ml/hour)**		**Relative melatonin production in plasma /pineal**	
	Night-time	**Day-time**	**Night-time**	**Day-time**	**Night**	**Day**
Rainbow trout	116.4 ± 9.5 n = 12	19.1 ± 2.1 n = 12	3706.7 ± 69.3 n = 4	60.0 ± 4.6 n = 4	3.14%	31.9%
Atlantic salmon	291.6 ± 10.7 n = 10	11.8 ± 2.5 n = 10	2536.2 ± 53.3 n = 6	405.0 ± 71.6 n = 6	11.5%	2.9%
European sea bass	43.8 ± 2.6 n = 10	5.9 ± 1.1 n = 10	1207.1 ± 46.6 n = 4	383.8 ± 31.7 n = 4	3.6%	1.5%
Atlantic cod	112.0 ± 11.4 n = 12	9.6 ± 1.3 n = 12	2563.4 ± 99.5 n = 6	86.2 ± 4.1 n = 6	4.4%	11.4%
Nile tilapia	105.1 ± 8.2 n = 8	8.8 ± 1.5 n = 8	15.9 ± 2.8 n = 6	0.6 ± 0.4 n = 6	660.5%	1522.4%
African catfish	47.0 ± 2.9 n = 12	5.6 ± 0.5 n = 12	-	-	-	-

Figure 1. Effect of ophthalmectomy (Eye X) on *in vivo* plasma melatonin levels in comparison to intact fish (control) in rainbow trout (a), Atlantic salmon (b), sea bass (c), Atlantic cod (d), Nile tilapia (e) and African catfish (f). Values are expressed as mean ± SEM (3-7 individuals/sampling point). Superscripts denote significant differences (GLM, $p<0.05$) and numbers sampling size.

Figure 2. Summary of the relative percentage of night time melatonin levels in ophthalmectomised fish as compared to control fish in all species studied. Values are expressed as mean of n=3-7 individuals over two night periods.

Figure 3. Percentage of white artificial light (a, Solux bulb) and narrow bandwidth light at 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61nm (centre wavelengths)(b, Solux bulb + bandpass interference filters) through rainbow trout (n=4), Atlantic salmon (n=7), sea bass (n=6), Atlantic cod (n=4), Nile tilapia (n=6) and African catfish (n=6) pineal windows. Superscripts denote significant differences between species for a given light treatment.

Figure 4. Suggested evolution of the regulation of pineal melatonin synthesis by the circadian axis in teleosts. In addition to the two types of circadian organisation already proposed in fish (a and b), a third type could exist where pineal light sensitivity would be dramatically reduced (c). The regulation of pineal activity would have thus evolved from an independent light sensitive pineal gland, without pacemaker activity, as seen in salmonids (a); to an intermediary state where the pineal gland remains light sensitive and could possess a circadian pacemaker, but is also regulated by photic information perceived by the retina as seen in seabass and cod (b); to reach a more advanced system closer to higher vertebrates where light sensitivity of the pineal gland would be significantly reduced and its melatonin synthesis activity primarily regulated by a circadian pacemaker (unknown location) entrained by photic information perceived by the retina (c).

Figure. 1

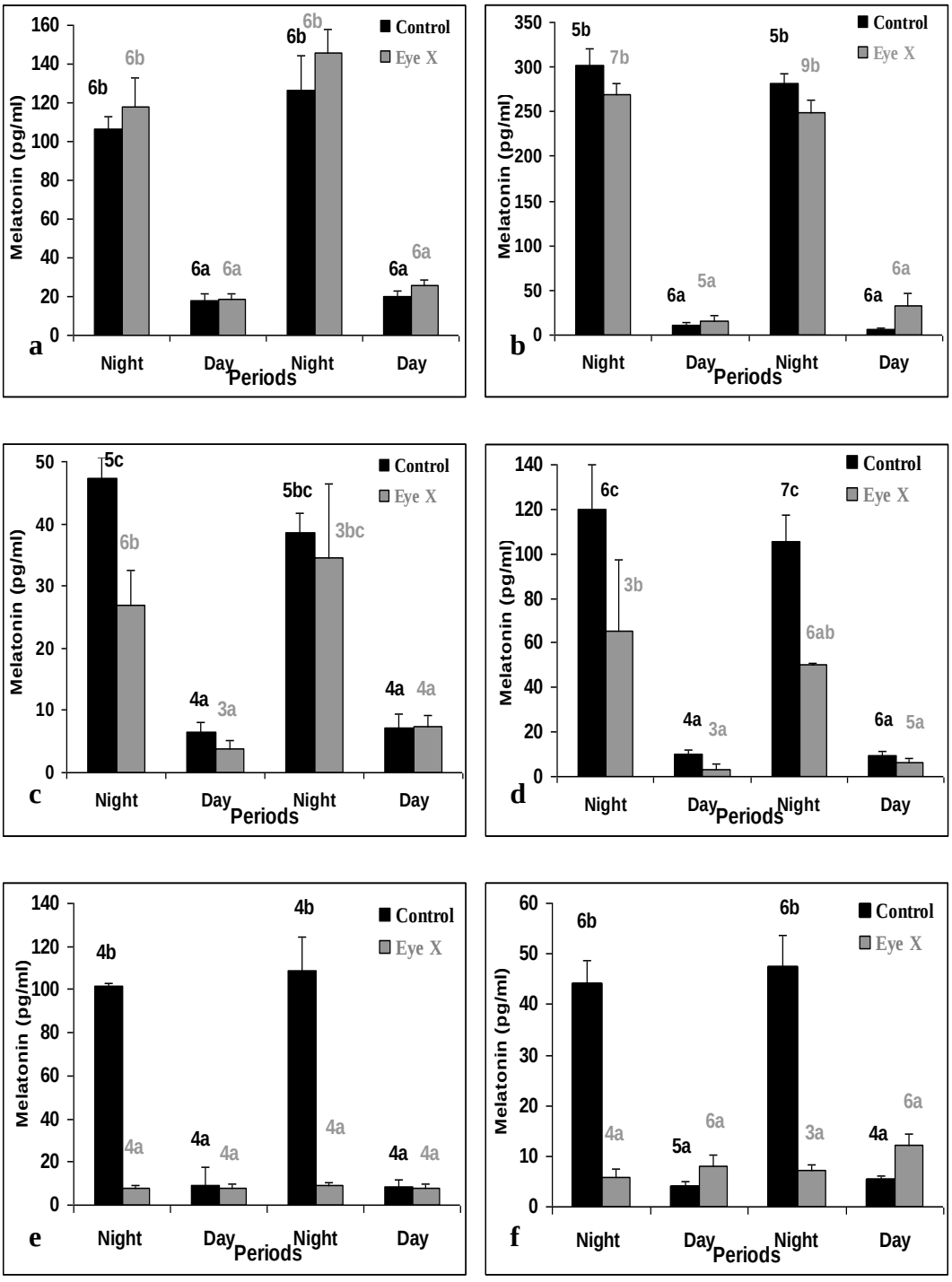


Figure. 2

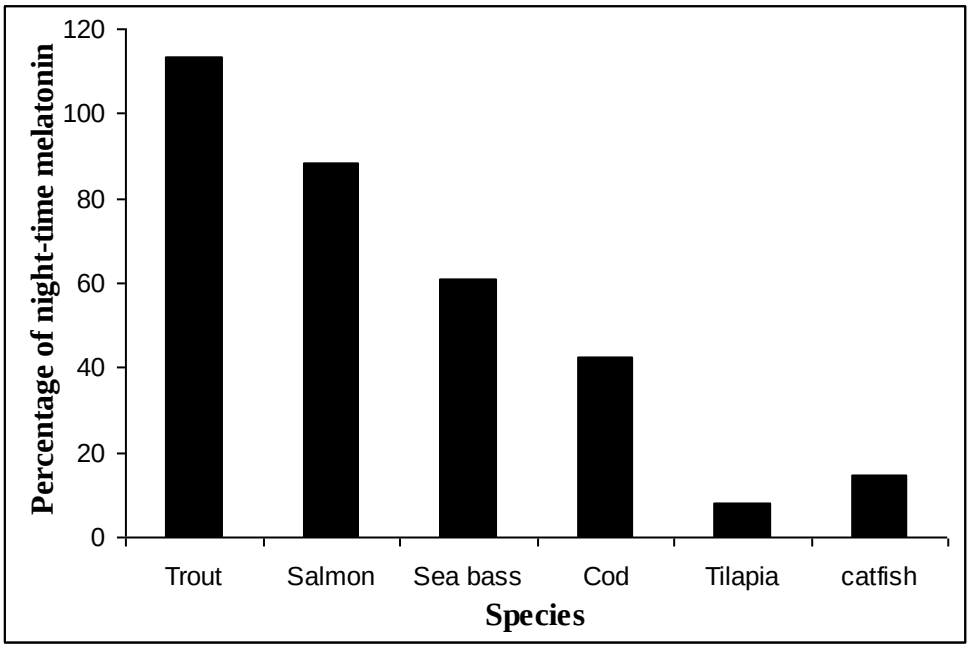
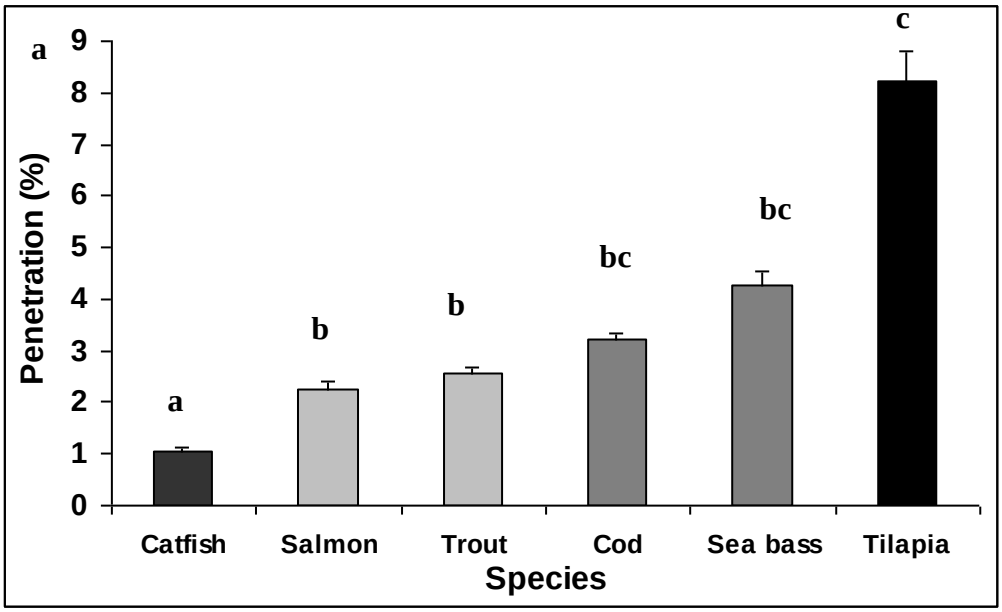

Percentage of night-time melatonin
120
100
80
60
40
20
0
Trout
Salmon
Sea bass
Cod
Tilapia
catfish
Species

Figure. 3


a
b
b
bc
bc
c
Penetration (%)
0
1
2
3
4
5
6
7
8
9
Catfish
Salmon
Trout
Cod
Sea bass
Tilapia
Species

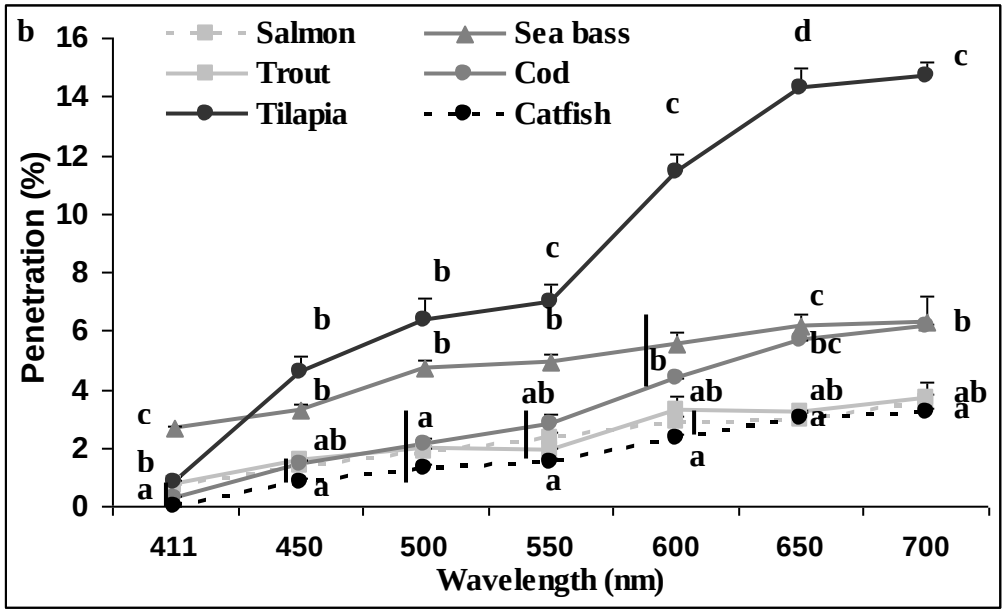
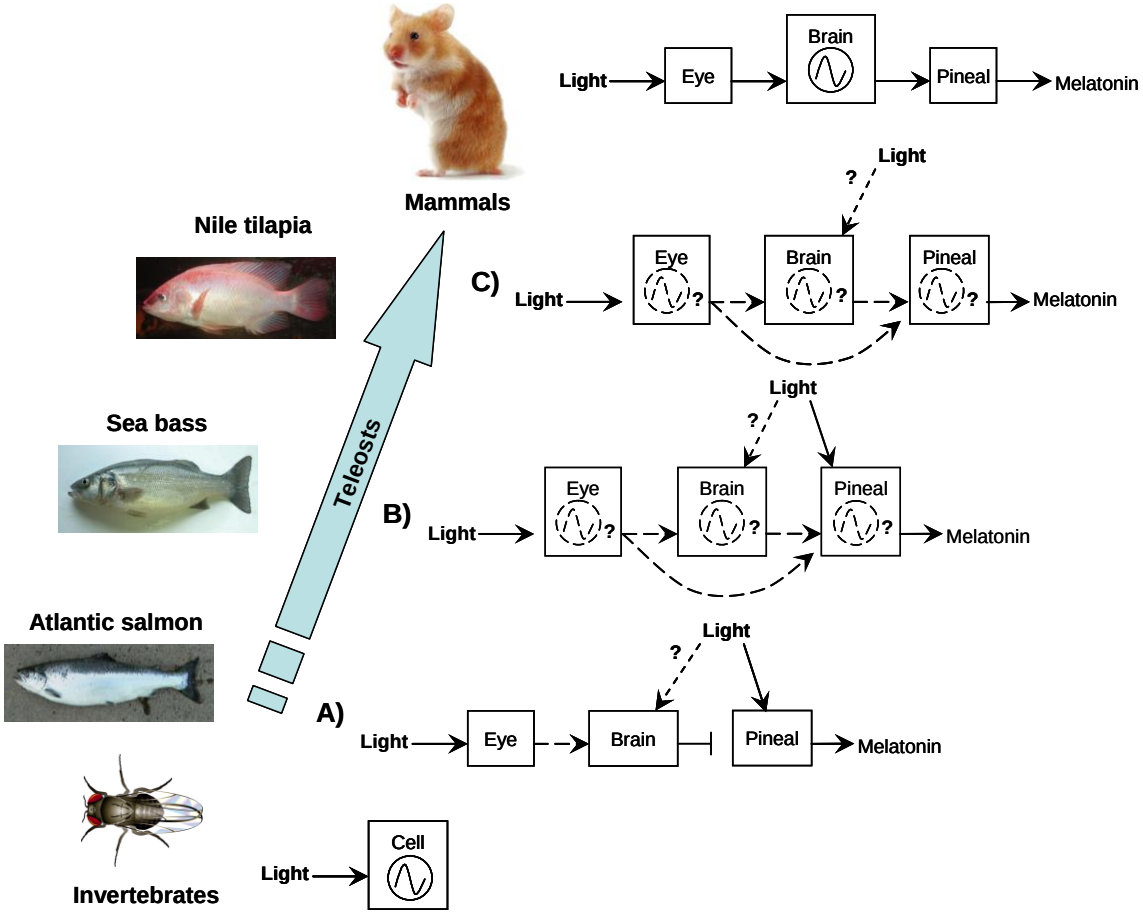

b
Salmon
Sea bass
Trout
Cod
Tilapia
Catfish
Penetration (%)
0
2
4
6
8
10
12
14
16
411
450
500
550
600
650
700
Wavelength (nm)

Figure. 4


Mammals
Light
Eye
Brain
Pineal
Melatonin
Nile tilapia
C)
?
Light
Eye
Brain
Pineal
Melatonin
Sea bass
Teleosts
B)
Light
Eye
Brain
Pineal
Melatonin
Atlantic salmon
A)
Light
Eye
Brain
Pineal
Melatonin
Invertebrates
Light
Cell

CHAPTER 4

<u>PAPER II:</u> CLOCK CONTROLLED ENDOGENOUS MELATONIN RHYTHMS IN NILE TILAPIA (*OREOCHROMIS NILOTICUS NILOTICUS*) AND AFRICAN CATFISH (*CLARIAS GARIEPINUS*).

The two first authors contributed equally to this manuscript (experimental design, sampling, data analyses and writing). Co-authors provided guidance and proof reading of the submitted manuscript.

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CLOCK CONTROLLED ENDOGENOUS MELATONIN RHYTHMS IN NILE TILAPIA (*OREOCHROMIS NILOTICUS NILOTICUS*) AND AFRICAN CATFISH (*CLARIAS GARIEPINUS*).

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Endogenous melatonin rhythms

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ABSTRACT

The purpose of this work was to investigate the circadian melatonin system in two tropical teleost species characterised by different behavioural habits, Nile tilapia (diurnal) and African catfish (nocturnal). To do so, fish were subjected to either a control photoperiod (12L:12D), continuous light (LL) or darkness (DD) or a 6L:6D photoperiod. Under 12L:12D, plasma melatonin levels were typically low during the photophase and high during the scotophase in both species. Interestingly, in both species melatonin levels significantly decreased prior to the onset of light, which in catfish, reached similar basal levels to those during the day, demonstrating that melatonin production can anticipate photic changes probably through circadian clocks. Further evidence for the existence of such pacemaker activity was obtained when fish were exposed to DD, as a strong circadian melatonin rhythm was maintained. Such an endogenous rhythm was sustained for at least 18 days in Nile tilapia. A similar rhythm was shown in catfish although DD was only tested for four days. Under LL, the results confirmed the inhibitory effect of light on melatonin synthesis already reported in other species. Finally, when acclimatised to a short photo-cycle (6L:6D), no endogenous melatonin rhythm was observed in tilapia under DD, with melatonin levels remaining high. This could suggest that the circadian clocks cannot entrain to such a short photo-cycle. Additional research is clearly needed to further characterise the circadian axis in teleost species, identify and localize the circadian clocks and better understand the environmental entrainment of fish physiology.

INTRODUCTION

Melatonin is known to be a biological time keeping hormone or "zeitgeber" which is entrained by light and displays circadian and circannual rhythms in vertebrates (Menaker *et al.* 1997). These rhythms can also be self-sustained and are under the control of circadian clocks (Falcon 1999; Fukada and Okano 2002; Holzberg and Albrecht 2003; Falcon *et al.* 2007). As such melatonin seems to play a major role in synchronising many behavioural (locomotor, feeding, shoaling, and migration activities) and physiological (growth, reproduction, immunity) processes across the animal kingdom. Key components of the circadian system (photoreceptors involved in the light reception, clock mechanisms that regulate the rhythms and neuroendocrine regulation of physiological functions) have been investigated and characterized in mammals (Malpaux *et al.* 2001; Herzog and Tosini 2001; Simonneaux and Ribelayga 2003). However, in teleosts, circadian organization and clock controlled rhythms are still poorly characterized with studies focusing on very few species such as zebrafish *Danio reiro* (Cahill 2002; Vallone *et al.* 2005; Lopez-Olmeda *et al.* 2006; Carr *et al.* 2006; Ziv and Gothilf 2006). Furthermore, no clear pathway between the hormone melatonin and the seasonality of fish physiology has been demonstrated in fish (Mayer *et al.* 1997; Falcon *et al.* 2007) as opposed to higher vertebrates (Arendt 1998). This is due to the inconsistency of the results reported so far which could be partly explained by the numerous factors which have been shown to affect melatonin production in fish (light, temperature, size, age and previous photoperiod entrainment), highlighting the complexity of the melatonin system in fish and the diversity of experimental procedures used (melatonin administrated by injection, in feed or in water, pinealectomy) (Mayer *et al.* 1997; Falcon *et al.* 2007). Ultimately, these conflicting findings could also result from the highly divergent nature of the systems at work in fish which might have

evolved due to the multitude of environments they inhabit (Hardeland *et al*. 2006; Migaud *et al*. 2007).

Circadian rhythms are a conserved feature observed from photosynthetic prokaryotes to mammals (Menaker *et al.* 1997; Ekstrom and Miessl 2003). At the core of any circadian rhythm is a network of autonomous endogenous oscillators, also called biological clocks or circadian pacemakers, which in the case of mammals feed information to a master clock found in the Suprachasmatic Nucleus (SCN), synchronizing their physiology to the photic conditions (Foulkes *et al.* 1997; Vígh *et al.* 2002; Holzberg and Albrecht 2003; Iuvone *et al.* 2005). Importantly, in mammals, photo-entrainment is exclusively mediated by retinal photoreceptors and as such pineal photoreceptors have lost their direct light sensory abilities (Ekstrom and Miessl 2003). In teleosts, research has predominantly focused on temperate, annual breeding species such as salmonids, pike *Esox lucius* and sea bass *Dicentrarchus labrax* (Iigo *et al.* 1998; Bayarri *et al.* 2002; Bayarri *et al.* 2003). Results to date in these species have suggested a more decentralized organization in fish compared to that found in other vertebrates, where the pineal gland is light sensitive and independent of the SCN (or similar structure still to be found) or eyes (retina) and may contain, depending on the species, an endogenous oscillator that can sustain *in vitro* melatonin rhythms (Zachmann *et al.* 1992a; Cahill 1996; Okimoto and Stetson 1999b; Iigo *et al.* 2004). However, a recent study suggested a different type of circadian organisation in Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*), characterised by a pineal gland which is not light sensitive or far less sensitive than in previously studied teleost species and no independent circadian pacemaker regulating melatonin production (Migaud *et al*. 2007). Studies from pineal glands in culture performed in both temperate and tropical teleosts have commonly demonstrated rhythmic melatonin production under light and dark (LD) periods (Gern and Greenhouse 1988; Kezuka *et al.* 1989; Iigo *et al.* 1991; Migaud *et*

al. 2006). However, some species such as Nile tilapia and African catfish seem to be exceptions to this generalised model, where melatonin production was shown to rely on photic information perceived by the eyes while in sea bass and cod, both the eyes and the pineal gland are required to sustain full amplitude melatonin rhythms (Bayarri *et al.* 2003; Migaud *et al.* 2007). This clearly illustrates the diversity of adaptations present in fish. Intrapineal oscillators, capable of self-sustaining melatonin rhythms *in vitro* in the absence of light stimuli, have been found in numerous species including pike, *Esox lucius* (Falcon *et al.* 1989), goldfish, *Carassius auratus* (Kezuka *et al.* 1989; Iigo *et al.* 1991), white sucker, *Catostomus commersoni* (Zachmann *et al.* 1992b), zebrafish (Cahill 1996), sailfin molly, *Poecilia velifera* (Okimoto and Stetson 1999a, b), golden rabbitfish, *Siganus guttatus* (Takemura *et al.* 2006), ayu, *Plecoglossus altivelis* (Iigo *et al.* 2004) and sea bass (Bolliet *et al.* 1996; Ron 2004; Bayarri *et al.* 2004a; Migaud *et al.* 2006). However, no such endogenous circadian system have been shown to exist in salmonids (Gern and Greenhouse 1988; Migaud *et al.* 2006; Iigo *et al.* 2007) and common dentex, *Dentex dentex* (Pavlidis *et al.* 1999).

Such endogenous rhythms are clearly entrained by circadian clocks which have not yet been fully characterised in fish. In higher vertebrates, the molecular basis of the circadian clock has been shown to consist of feedback loop mechanisms involving a number of clock genes (mainly BMAL, Clock, Per's, Cry's) entrained by light which maintain and synchronise self-sustained rhythms (Zordan *et al.* 2001; Stehle *et al.* 2003; Iuvone *et al.* 2005). Understanding these endogenous rhythms in fish is still in its infancy. However, teleosts could provide very useful models in the field of chronobiology, not only for their plasticity but also for their diversity.

The present studies were carried out on two different tropical species occupying different niches and displaying different reproductive and feeding strategies: the Nile tilapia, an

omnivorous batch spawner fish with diurnal habits, and the African catfish, a carnivorous seasonal breeder with nocturnal habits (Bromage and Roberts 1995). Furthermore, photoperiodic manipulations have recently been shown to exert effects on growth performances, sexual maturation (timing of spawning, fecundity) and fry survival in both species (Appelbaum and McGeer 1998; Ridha and Cruz 2000; Campos-Mendoza *et al.* 2004; Almazan-Rueda *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006) although the mechanisms by which photoperiod act on reproduction are still unknown. Therefore, to better understand the basis of such photoperiodic physiological effects, this work aimed to investigate circadian endogenous melatonin rhythms. To do so, a series of trials were carried out, to firstly confirm circadian melatonin rhythms, secondly examine endogenous melatonin rhythms under constant photic conditions - continuous light (LL) and darkness (DD) and determine whether these rhythms are circadian in nature and thirdly to determine the effects of short photo-cycles on the entrainment of these endogenous melatonin rhythms.

MATERIALS AND METHODS

Mixed sex red Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*) (mean weight ranging from 150-200 g) were obtained from the tropical aquarium facilities at the Institute of Aquaculture, University of Stirling. All fish were raised from first feeding under 12L:12D conditions and were acclimated for two to three weeks in the experimental rearing systems prior to the start of the experiments to 12L:12D or 6L:6D photoperiod. In all experiments, to exclude feed as an environmental input variable fish were fed to satiation with commercial trout pellets (Standard Expanded, Skretting, Cheshire, UK) delivered continuously throughout the 24 h period with automatic feeders (Fish-mate F14 Pet-Mate, Surrey, UK). In all experiments under a 12L:12D photoperiod,

lights were switched on at 08:00 h and off at 20:00 h. Similarly, lights for 6L:6D where switched on at 08:00 h and then switched off and on at 6 hour intervals. For each species experiments were done in closed water recirculation systems (27 ± 1 ^{o}C) as previously described (Campos-Mendoza *et al.* 2004) unless stated otherwise. Nitrate, nitrite, ammonia and pH were monitored throughout the experiments with aquarium water quality kits (C-Test kits, New Aquarium Systems, Mentor, USA) and remained within safe limits. In all experiments, fish were either anesthetized (0.1-0.15 g/l) or killed by a lethal dose (0.5-0.8 g/l) of benzocaine solution (SIGMA, Poole, UK) and blood sampled by venipuncture of the caudal vein using heparinised syringes. Rearing tanks were lit using standard 60 W GLS bulbs (CPC, Leeds, UK) providing a light intensity of approximately 0.75 Wm^{-2} at the water surface (measured by a single channel light sensor, Skye instruments, Powys, UK). Extreme care was made to the experimental lighting regimes and sampling to avoid potential light pollution. In all experiments light meter readings showed no detectable penetration of external light into the tank system. However, during all experiments the main laboratory lights were left on constantly to prevent the possibility of any background photoperiod affecting the system (except during night time sampling when lights were switched off to have access to the fish under DD and sampling was performed using a red dim light). Furthermore the rearing systems for tilapia and catfish include 4 and 8 light-proof individual compartments with two tanks in each, respectively. Tank size is 40x120x40 cm (200 L) and 46x46x41 cm (86 L) for tilapia and catfish, respectively. Fish under a given experimental photoperiodic treatment can therefore not be entrained to the regime of the other compartments. All trials were carried out according to international ethical standards (Touitou *et al*. 2006).

Experiment 1: Diel plasma melatonin profiles in Nile tilapia and African catfish.

To determine the diel plasma melatonin profile, fish (n=10 and 5 individuals at each sampling point for tilapia and catfish, respectively) exposed to 12L:12D photoperiod were sacrificed and blood sampled during two consecutive light periods at 14:00 h, 19:00 h (first day) and 09:00 h, 14:00 h (second day) and every two hours (tilapia) or every three hours (catfish) during the scotophase. In this trial Nile tilapia were held constant at 24 ± 1 ^{o}C due to heater malfunction during acclimation and sampling. A follow up trial was then performed to determine whether both species could anticipate the onset of light by reducing melatonin production before the lights were switched on (i.e. dawn). To do so, fish (n=5) were sacrificed and blood sampled during night (02:30, 05:30, 06:15, 07:00 and 07:45 h) and day (08:30 and 14:30 h).

Experiment 2: Endogenous melatonin rhythms in Nile tilapia exposed to LL and DD conditions.

The effects of both LL and DD regimes on plasma melatonin levels were studied firstly to confirm the inhibitory effect of LL on melatonin production and determine the profile of return to normal melatonin levels and secondly to establish whether or not circadian rhythmic melatonin production remained in fish exposed to DD for 18 days. For the LL trial, a total of 32 mixed sex tilapia were placed in 8 tanks (4 fish/tank, isolated by Perspex sheets with flow through holes). Fish from each tank were blood sampled every 12 hours (mid-photophase or subjective photophase at 14.00 h and mid-scotophase or subjective scotophase at 02:00 h). Fish from the three first tanks were randomly selected and first blood sampled under anaesthesia during the 12L:12D ambient photoperiodic regime (day-night-day) after which continuous light treatment began in all tanks (day two of the trial). Fish were returned to a 12L:12D photoperiod on day nine. The sampling regime consisted

in blood sampling fish under anaesthesia from all tanks once (corresponding to 8 sampling points, randomised design, fish were then allowed to recover in aerated water and returned to their tanks) and then sacrificing fish from each tank (same order) for a second blood sampling (sampling 9 to 16 from days 8 to 12). For the DD trial, a similar approach was taken although in this case, 44 fish in total (n=4) were exposed to DD for 18 consecutive days.

Experiment 3: Endogenous melatonin rhythms in African catfish exposed to 12L:12D, LL and DD conditions.

The aims of this study were similar to the previous experiment performed on tilapia except that the duration of exposure to LL and DD was shorter (4 days), a batch of fish was exposed in parallel to a 12L:12D (control) photoperiod and the experimental design differed as described below. A total of 180 mixed sex catfish were stocked in 6 tanks (30 fish/tank) corresponding to the three experimental photoperiodic regimes in duplicate (control, LL and DD). Sampling consisted in sacrificing 3 fish per tank (6 per treatment) every 12 hours during the middle of the photophase or subjective photophase at 14:00 h and scotophase or subjective scotophase at 02:00 h by lethal anaesthesia and blood sampling. Each fish was thus only sampled once.

Experiment 4: Circadian plasma melatonin rhythm in Nile tilapia and African catfish under DD

The aim of this experiment was to determine whether the endogenous melatonin rhythms previously observed in experiment 2 and 3 under DD (for tilapia and catfish, respectively) was circadian in nature. To do so, fish of both species (previously acclimated to 12L:12D) were subjected to DD for three consecutive days before performing a 24 h sampling. Four

fish of each species were blood sampled every 4 hours during the subjective photophase at 14:00, 18:00 h (first day) and 10:00, 14:00 (second day) and subjective scotophase at 22:00, 02:00, 06:00 h.

Experiment 5: Endogenous melatonin rhythms in Nile tilapia exposed to DD and previously acclimated to a 6L:6D photo-cycle

This short term trial was designed to test the oscillator capacity to synchronize to a short photo-cycle and entrain rhythmic endogenous melatonin production under DD in tilapia. Mixed sex fish were acclimated to a 6L:6D photoperiod for two weeks before being exposed to DD. Sampling (n=4) took place during 3 consecutive subjective photo-cycles under DD, at the middle of the subjective photophase (14.00 h) and scotophase (02.00 h). A control photophase sample was taken before placing fish under DD.

Melatonin assay

Blood samples were centrifuged at 1200 x g for 15 min at 4^0C (Jouan CT422, Buckinghamshire, UK) and plasma stored at -70 ^{0}C until analysed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). Prior to the analyses, the kit has been validated by confirming the parallelism between serial dilutions of night-time pooled plasma from both species to the standard curve (data not presented). All standards and samples were assayed in duplicate. The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard, was 3 $pgml^{-1}$. Intra-assay coefficient of variation was 5.5% (n=4) and inter-assay coefficient of variation was 9.4% (n=3). Pooled rainbow trout plasma with a melatonin content of 211.6 ± 2.3 $pgml^{-1}$, sampled during the night, was used to check the reproducibility of measurements between assays, i.e. for quality control.

Statistical analysis

All data was analysed using MINITAB® Release 14.13 (Minitab Ltd., UK). When necessary data was transformed using the natural logarithm to conform to normality and homogeneity of variance (Kolmogorov-Smirnov and Bartlett's tests). Melatonin levels were analysed using a General Linear Model (Zar 1999) followed by Tukey's post-hoc tests to identify significant differences. In the case of the replicated trial (experiment 3), data was pooled as no significant differences between duplicates was observed. Data is expressed as mean ± S.E.M values. Significant differences were determined at $p \leq 0.05$.

RESULTS

A clear diel rhythm was observed in Nile tilapia (Fig. 1a) and African catfish (Fig. 1b) with basal levels (<20 pg ml^{-1} and <10 pg ml^{-1}, respectively) during the photophase and high melatonin levels (>45 pg ml^{-1} and >30 pg ml^{-1}, respectively) during the scotophase. In tilapia, peak melatonin levels were observed 2 hours after the light was switched off and were maintained (plateau) until the last scotophase sampling point (06:00 h) after which levels returned to basal levels in the following day sampling points (09:00 and 14:00 h) (Fig. 1a). Similarly, melatonin levels in catfish remained basal during the photophase (14:00 and 19:00 h), then significantly increased 30 minutes after the start of the scotophase (20:30 h) to peak 3 hours (23:30 h) later and remain high at the following sampling point (02:30 h) (Fig. 1b). Thereafter, melatonin significantly decreased at the last scotophase sampling point (05:30 h) before returning to basal photophase levels after the light was switched on (08:30 and 11:30 h). Scotophase melatonin levels (sampling points from 02:30 h to 07:45 h) in Nile tilapia were significantly reduced although basal levels were only reached after the onset of photophase at 08:30 h and 14:30 h (Fig. 2a). On the other hand, catfish melatonin levels were shown to decrease significantly during the

scotophase 1 hour prior to the start of the photophase (07:00 h) and reached basal levels shortly before the start of the photophase at 08:00 h (Fig. 2b).

In experiment 2, following a control (12L:12D) melatonin profile, the LL regime was shown to fully suppress scotophase melatonin production in tilapia, with plasma levels remaining basal (<20 pg ml^{-1}) throughout the LL period (Fig. 3a). Once fish were returned to a 12L:12D photoperiod, plasma melatonin levels increased to approximately 50% (42.9 ± 3.3 pg ml^{-1}) of normal night time plasma melatonin levels during the first dark period (day 10). Melatonin production and day-night profiles were fully restored during the following photo-cycle (days 10-11). Exposure to DD did not affect circadian melatonin rhythms which were maintained at basal levels (<20 pg ml^{-1}) during the subjective photophase and at significantly higher levels during the subjective scotophase (40-80 pg ml^{-1}). This rhythm was maintained throughout the 18 days of the DD regime (Fig. 3b). With exception of photophase/scotophase levels in day 14, all other scotophase or subjective scotophase levels were found to be significantly higher than the previous photophase or subjective photophase levels.

In catfish, normal day and night melatonin fluctuations were observed in fish exposed to a control 12L:12D photoperiod (Fig 4a). When LL was applied, melatonin levels remained basal with no significant differences until a photo-cycle cycle was restored at which point melatonin significantly increased (Fig. 4b). However, when DD was applied, a robust daily rhythm of significantly lower amplitude (mean subjective scotophase levels of 26.0 ± 0.6 pg ml^{-1}) to that observed under a control 12L:12D regime (mean subjective scotophase levels of 48.8 ± 3.2 pg ml^{-1}) was maintained (Fig. 4c). When fish were then exposed to a control photo-cycle (day 5-6), melatonin levels significantly increased and returned to normal scotophase levels (as control).

When sampled on day 3 under DD, Nile tilapia and African catfish showed the same temporal profile of melatonin production to that observed under normal conditions (12L:12D). Nile tilapia melatonin levels were basal (<20 pg ml^{-1}) during the subjective photophase (14:00 and 18:00 h, Fig. 5a) after which levels significantly increased during the subjective scotophase by 22:00 h and peaked (73.8 ± 4.3 pg ml^{-1}) by 02:00 h. Mean levels then started to decrease by 06:00 and 10:00 h (subjective photophase) reaching basal levels (<20 pg ml^{-1}) by 14:00 h. In a similar way, catfish subjective photophase levels were basal on the first day (14:00 and 18:00 h, Fig. 5b) but then showed an early tendency to increase towards the onset of the subjective scotophase. Significant levels were observed at the first sampling point under the subjective scotophase (22:00 h) and peaked also at 02:00 h (32.5 ± 3.5 pg ml^{-1}). Mean levels then started to decrease by 06:00 h and reached basal levels at 10:00 and 14:00 h.

When Nile tilapia was acclimated for two weeks to a short 6L:6D photo-cycle, a normal photophase (low)-scotophase (high) melatonin profile was observed (not presented). Thereafter, no circadian melatonin rhythm was observed in fish exposed to DD, with plasma melatonin concentrations remaining significantly higher (circa 40 $pgml^{-1}$) than basal levels during two subjective photo-cycles (Fig. 6).

DISCUSSION

Seasonal breeders rely on environmental factors such as photoperiod to synchronize their physiology (Arendt 1998; Pevet 2003). In teleosts, this has been thoroughly documented in temperate species which are exposed to marked seasonal changes of day-length and temperature (Falcon *et al.* 2007). Recently, a number of reproductive and growth performance studies have shown that tropical teleosts such as Nile tilapia and African catfish can also be responsive to photoperiodic changes (Campos-Mendoza *et al.* 2004;

Almazan-Rueda *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006). However, importantly, the role of melatonin and circadian endogenous rhythms in these tropical species had not been previously studied.

The present results firstly showed a similar diel plasma melatonin profile in Nile tilapia and African catfish to that previously reported in most vertebrate species (Reiter 1988; Cassone 1990; Mayer *et al.* 1997; Pavlidis *et al.* 1999; Hardeland *et al.* 2006; Migaud *et al.* 2006). Although still to be demonstrated in fish, these typical photophase (low) - scotophase (high) circadian plasma melatonin fluctuations may provide both species, as in other vertebrates, with daily and calendar time which entrain the endogenous time keeping system. Interestingly, the present study showed a significant decrease in plasma melatonin levels in both species, more so in catfish which reached basal day levels before the start of the photophase. These observations clearly suggest the involvement of a clock controlled system of melatonin secretion which is capable of anticipating the next photophase period. If so, the output (melatonin) of this system is likely to be regulated by arylalkylamine-N-acetyltransferase (AANAT) or hydroxyindole-O-methyl-transferase (HIOMT) synthesis at the transcriptional and/or translational level as previously suggested in other species (Klein *et al.* 1997; Falcon *et al.* 2001; Appelbaum *et al.* 2005). Indeed, light has been shown to regulate the expression of several circadian clock genes (i.e. *Per*2) and photoreceptor conserved elements (PCEs) that are capable of regulating E-box and promoter regions of genes such as AANAT which will ultimately affect the rate and production of melatonin synthesis (Klein *et al.* 1997; Zordan *et al.* 2001; Pando *et al.* 2001; Appelbaum and Gothilf 2006).

In order to further characterise the circadian control of melatonin production in tilapia, melatonin rhythms under constant photic conditions and its entrainment were investigated. Constant photoperiod (i.e. LL/DD) has commonly been used to describe rhythmic

melatonin production in many vertebrates species including teleosts (Gern and Greenhouse 1988; Falcon *et al.* 1989; Kezuka *et al.* 1989; Okimoto and Stetson 1999b; Migaud *et al.* 2006; Takemura *et al.* 2006). In the current study constant LL regimes resulted in a clear suppression of day-night plasma melatonin rhythms in both species, as already reported in many other fish species (Falcon *et al.* 2007). Interestingly, following 7-8 days under LL, normal rhythmic melatonin production was restored during the second night period in fish exposed to day-night cycles. The initial surge (50% of full night-time melatonin) might be explained by a desensitisation of the melatonin production system after having been suppressed for seven complete days under LL. On the other hand, when exposed to DD, a strong endogenous melatonin rhythm was maintained for 18 days (duration of DD exposure) in tilapia previously acclimatised to a 12L:12D photoperiod. A similar endogenous rhythm was also able to sustain itself for at least 4 days (duration of DD exposure) in African catfish. Importantly, results also demonstrated that melatonin rhythm in both species exposed to DD for three days was circadian (i.e. cycling over approximately 24 hours). However, from these trials it is not possible to determine whether there was a phase-shift in the circadian rhythm later on as a single sampling point was performed in the middle of the subjective photophase/scotophase throughout the DD trials. Furthermore, if full amplitude of melatonin oscillations were shown in tilapia under DD, melatonin levels during mid subjective scotophase (02:00 h) were only 50-60 % of normal scotophase levels under 12L:12D in catfish. It is difficult at this stage to explain these findings however, it is possible that the oscillator driving the endogenous melatonin production rhythm might be 'desenzitised' during prolonged exposure to DD resulting in a near 50% output signal. These results raise interesting questions as to whether these robust clock controlled melatonin rhythms may eventually dampen and/or free run or continue.

In support to the present results, many *in vitro* studies suggested that intrapineal oscillators exist in fish (Bolliet *et al.* 1996) with the exception of salmonids (Iigo *et al.* 2007) in which the pineal gland would either not contain such oscillators or these would not control melatonin production (still to be demonstrated). However, *in vivo* data on endogenous melatonin rhythms are clearly lacking in fish with only very few species studied (Randall *et al.* 1995; Pavlidis *et al.* 1999; Kazimi and Cahill 1999; Bayarri *et al.* 2004b; Migaud *et al.* 2006; Vera *et al.* 2007; Oliveira *et al.* 2007). A recent *in vivo* study performed on temperate fish species (sea bass, Atlantic salmon and Atlantic cod) has shown that when acclimatised to a 12L:12D photoperiod at two different temperatures (10 and 18^0C) and thereafter exposed to DD, no circadian endogenous melatonin rhythm was maintained, with levels remaining as high as during night-time (Migaud *et al.* unpublished). These results obtained in sea bass and Atlantic cod appear to not support previous *in vitro* findings obtained in the same species (Bolliet *et al.* 1996; Ron 2004) in which endogenous melatonin rhythms from isolated pineal glands were reported although rearing temperatures were different than in the *in vivo* trials (24 and 20-22^{0}C for sea bass and cod respectively). This could therefore highlight the importance of studying the circadian system as a whole (*in vivo*). Finally, it could be hypothesized that such strong endogenous rhythms in tropical species may reflect an adaptation to the rather steady photic environment they inhabit as compared to the strong seasonal variations experienced by temperate species. Two main differences were observed between tilapia and catfish; a clearer melatonin anticipation to photic changes and a reduced melatonin amplitude under DD as compared to 12L:12D in catfish. It is difficult at this stage to determine whether these differences are related to specific behavioural adaptations.

The present studies also reported interesting results on the entrainment of the endogenous system to short photo-cycles. Indeed, when acclimated to a short 6L:6D photoperiod, no

melatonin rhythms were observed under DD in tilapia with levels remaining high although only two subjective photo-cycles have been studied. This suggests that, irrespective of its location (pineal, retina and/or brain), the endogenous melatonin oscillator was either a) not able to entrain to such short photo-cycles or b) able to entrain but the coupling with the output (melatonin) became dissociated resulting in constant high plasma melatonin levels when subsequently exposed to DD. Further studies with longer exposure to DD are needed to determine if free running melatonin rhythms occur as observed in humans (Foster and Kreitzman 2005).

In the last decade or so understanding of the molecular bases of circadian clock mechanisms has substantially progressed and has been shown to involve transcriptional and translational feedback loops involving a highly conserved set of "clock genes" across vertebrates (Iuvone *et al.* 2005). It could thus be hypothesised that this circadian clockwork requires a minimal time/lapse of integration of environmental signals for the gene expression of the positive and negative components (transcriptional/translational factors) and the synthesis and activation of the final products (protein, metabolite and molecular signals) to ultimately entrain the physiology of the animal. Thus, according to this hypothesis, the 6L:6D photoperiod under which fish were acclimatized in this study could be too short for the circadian clock to entrain an endogenous melatonin rhythm output that would continue under DD conditions, as observed in fish previously acclimatised to a 12L:12D photoperiod. These results could thus explain why eggs from broodstock subjected to the same 6L:6D photoperiod were shown to be not viable as compared to control 12L:12D (Biswas *et al.* 2005). However, these preliminary results can only suggest that the entrainment of the melatonin rhythm may have been affected by the previous acclimation to 6L:6D as only four samplings over two subjective 6L:6D photo-cycles were performed. In order to conclude that the entrainment is truly disturbed, further studies in a

range of teleosts species raised under various short photo-cycles and sampled over longer periods under DD are needed to confirm such a hypothesis and determine the critical minimal period required for the system to be entrained.

Taken together these results further enhance our knowledge of light perception and circadian rhythmicity in tropical teleosts and show the potential for these species to become interesting models in chronobiology. Irrespective of their localisation which still needs to be determined, these studies have demonstrated the presence of circadian melatonin oscillators which can anticipate daily photic changes and maintain strong circadian rhythmic melatonin production under darkness. However, the results have shown that short photo-cycles appear to disrupt these endogenous melatonin rhythms, possibly by affecting the transcriptional-translational feedback loops of the circadian clock which might not be able to entrain over such short periods. Further studies are needed to confirm this hypothesis and better characterise the circadian axis in fish.

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FIGURE LEGENDS

Figure 1. Circadian plasma melatonin profile in a) Nile tilapia (n=10) and b) African catfish (n=5). All values shown are mean ± SEM. Letters indicate significant (p<0.05) difference between sample points. Open and filled boxes indicate photophase and scotophase respectively.

Figure 2. Anticipatory decrease of plasma melatonin levels prior to light onset in a) Nile tilapia (n=5) and b) African catfish (n=5). Values shown are mean ± SEM. Letters indicate significant (p<0.05) difference between sampling points. Open and filled boxes indicate photophase and scotophase respectively.

Figure 3. Plasma melatonin profile of Nile tilapia subjected to a) LL and b) DD regimes. Values shown are mean ± SEM (n= 4). Letters indicate significant (p<0.05) difference between sampling points. Symbols (*) indicate significant differences with previous sampling point. Open and filled boxes indicate photophase and scotophase respectively.

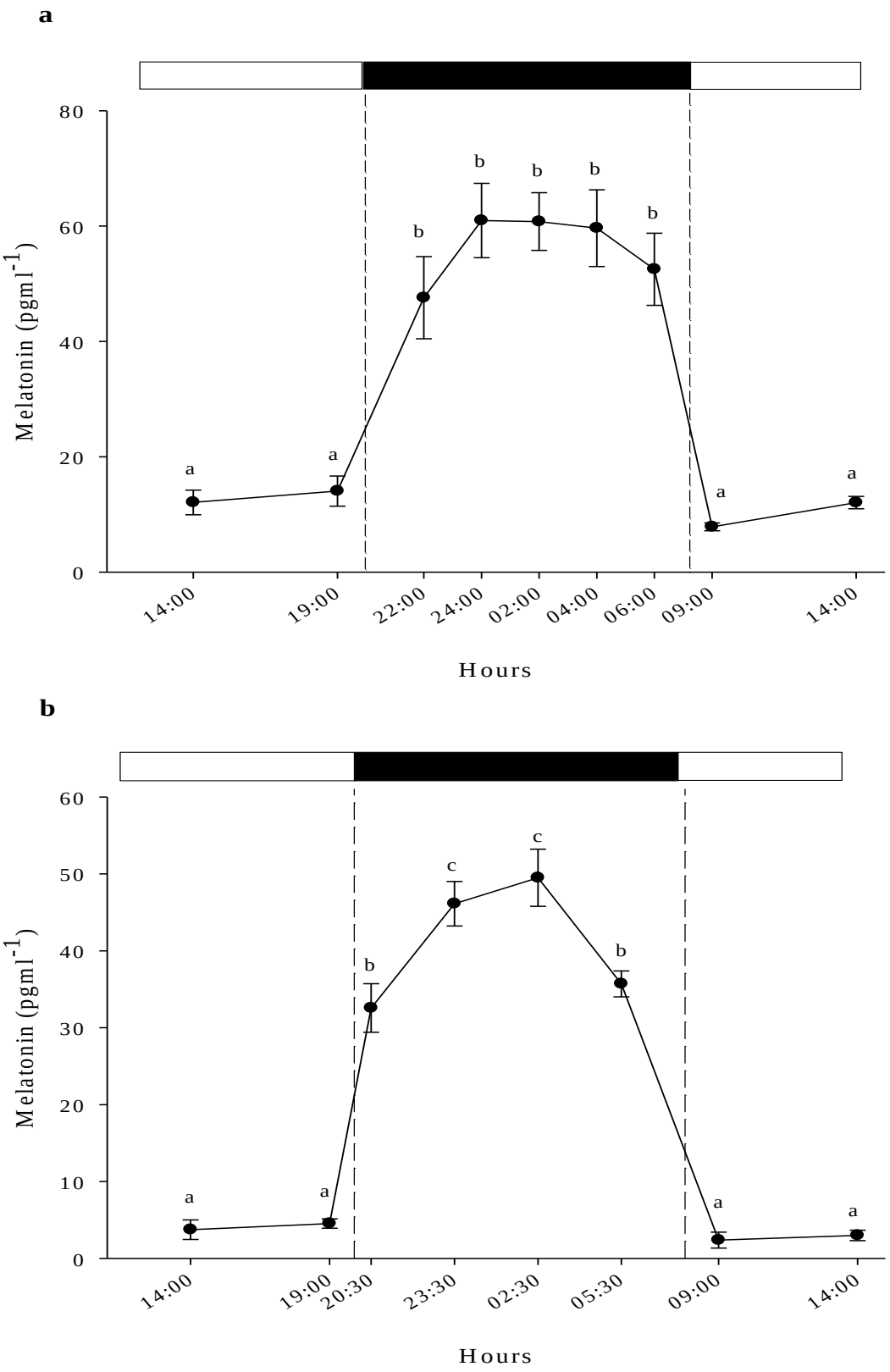
Figure 4. Plasma melatonin profile of African catfish subjected to a) 12L:12D, b) LL and c) DD regimes. Values shown are mean ± SEM (n= 6). Letters indicate significant (p<0.05) difference between sampling points. Open and filled boxes indicate photophase and scotophase respectively.

Figure 5. Circadian plasma melatonin profile in a) Nile tilapia and b) African catfish on the third day under DD. All values shown are mean ± SEM (n=4). Letters indicate significant (p<0.05) differences between sample points. Grey boxes indicate the subjective photophase periods and darker filled box the subjective scotophase period.

Figure 6. Melatonin levels of Nile tilapia acclimatised for two weeks to a 6L:6D photoperiod and sampled before and under DD for 2 following subjective day-night cycles. Each point represents the mean ± SEM (n= 4). Letters indicate significant (p<0.05)

differences between sampling points. Open and filled boxes indicate photophase and scotophase respectively.

Figure 1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Melatonin (pgml-1)
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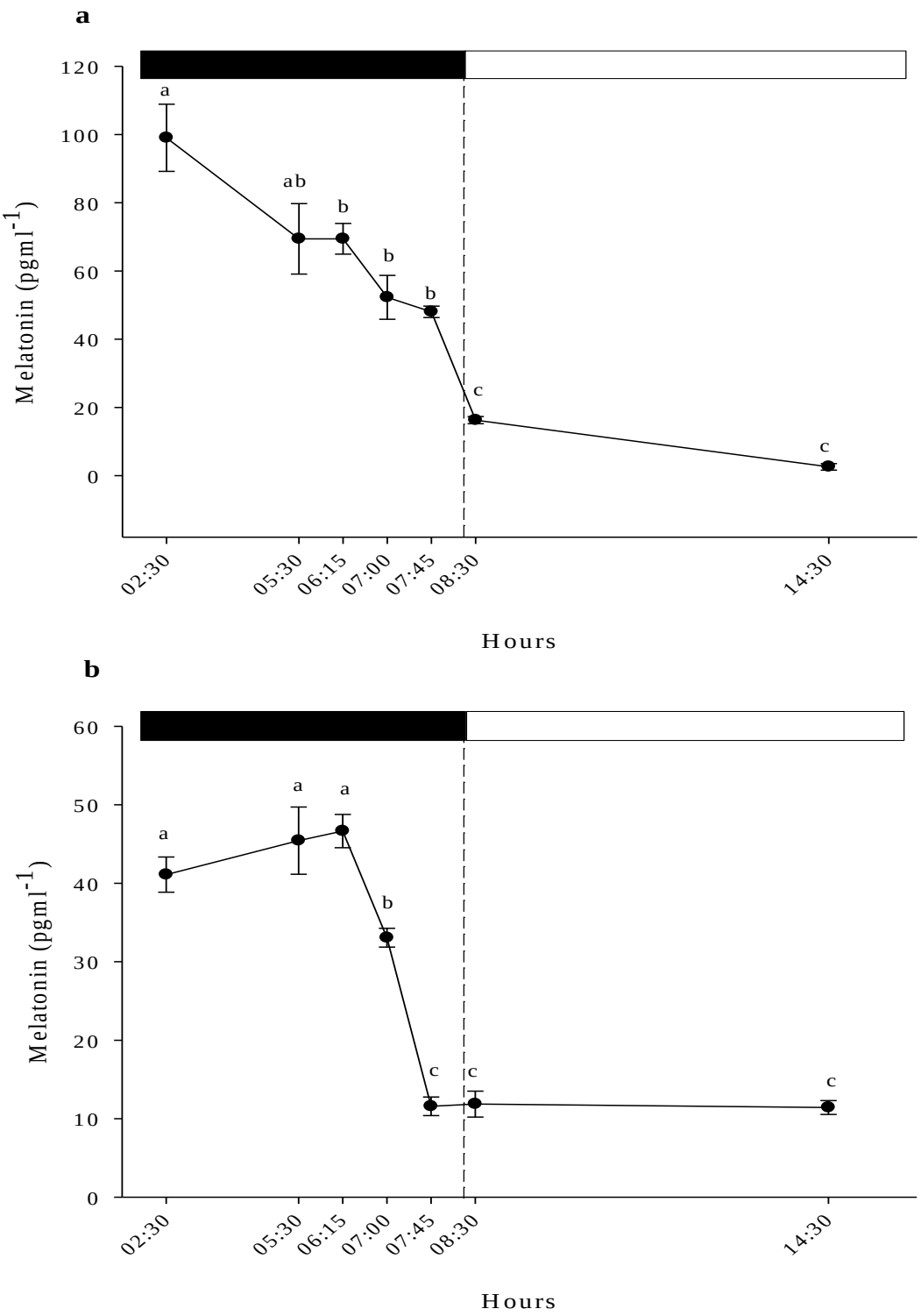
Figure 2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Melatonin (pgml-1)
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Figure 3

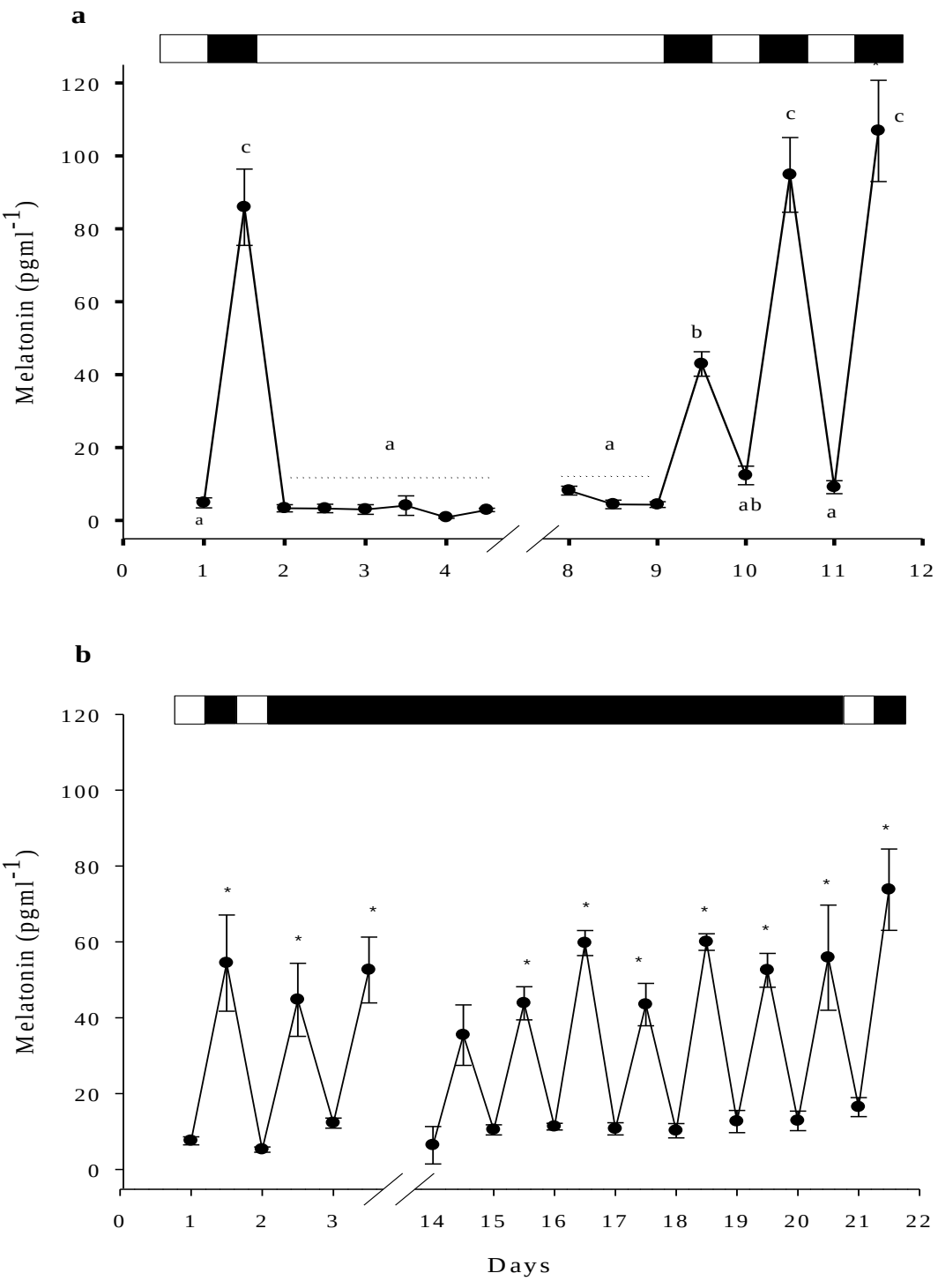
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Figure 4

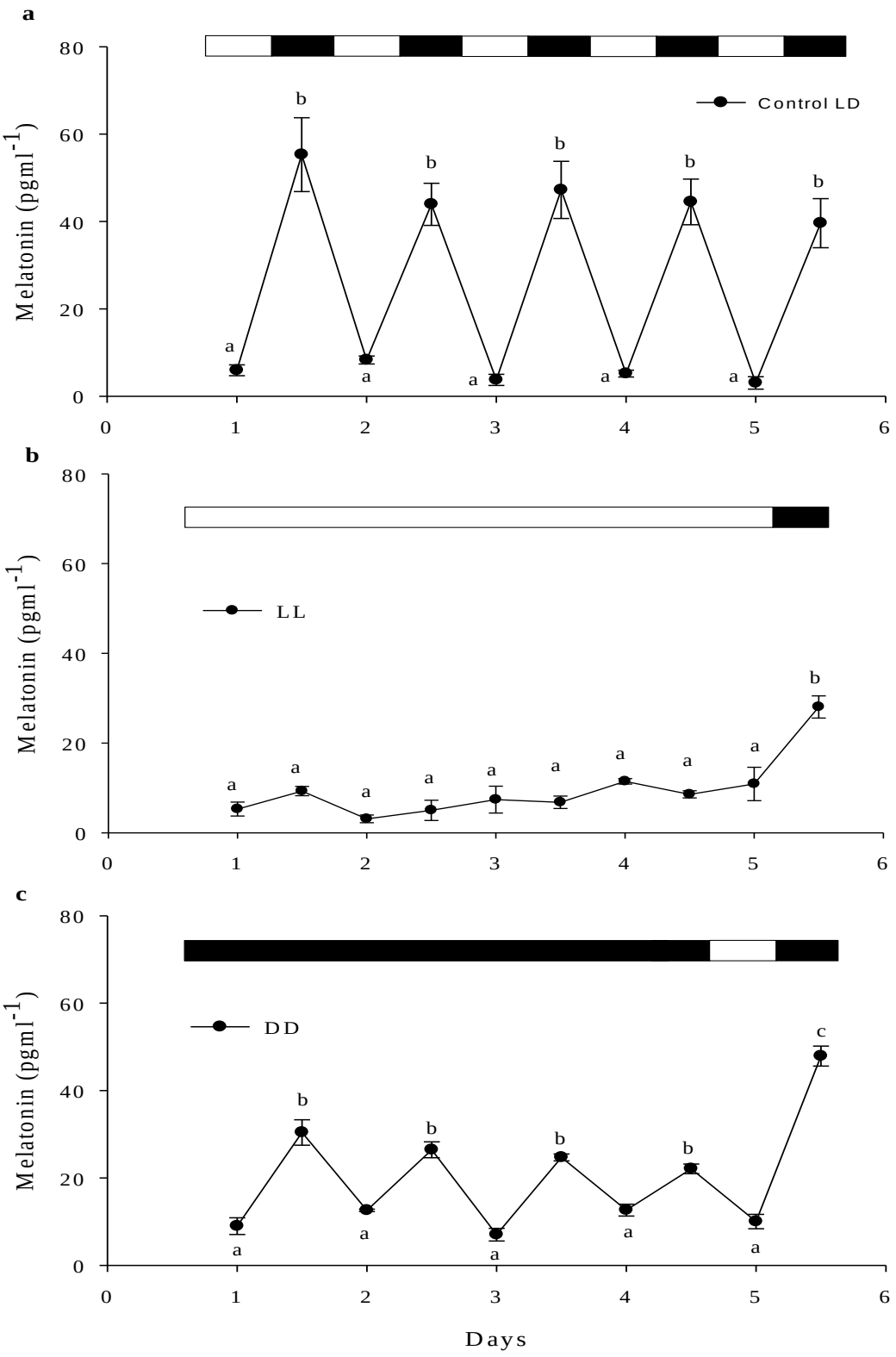
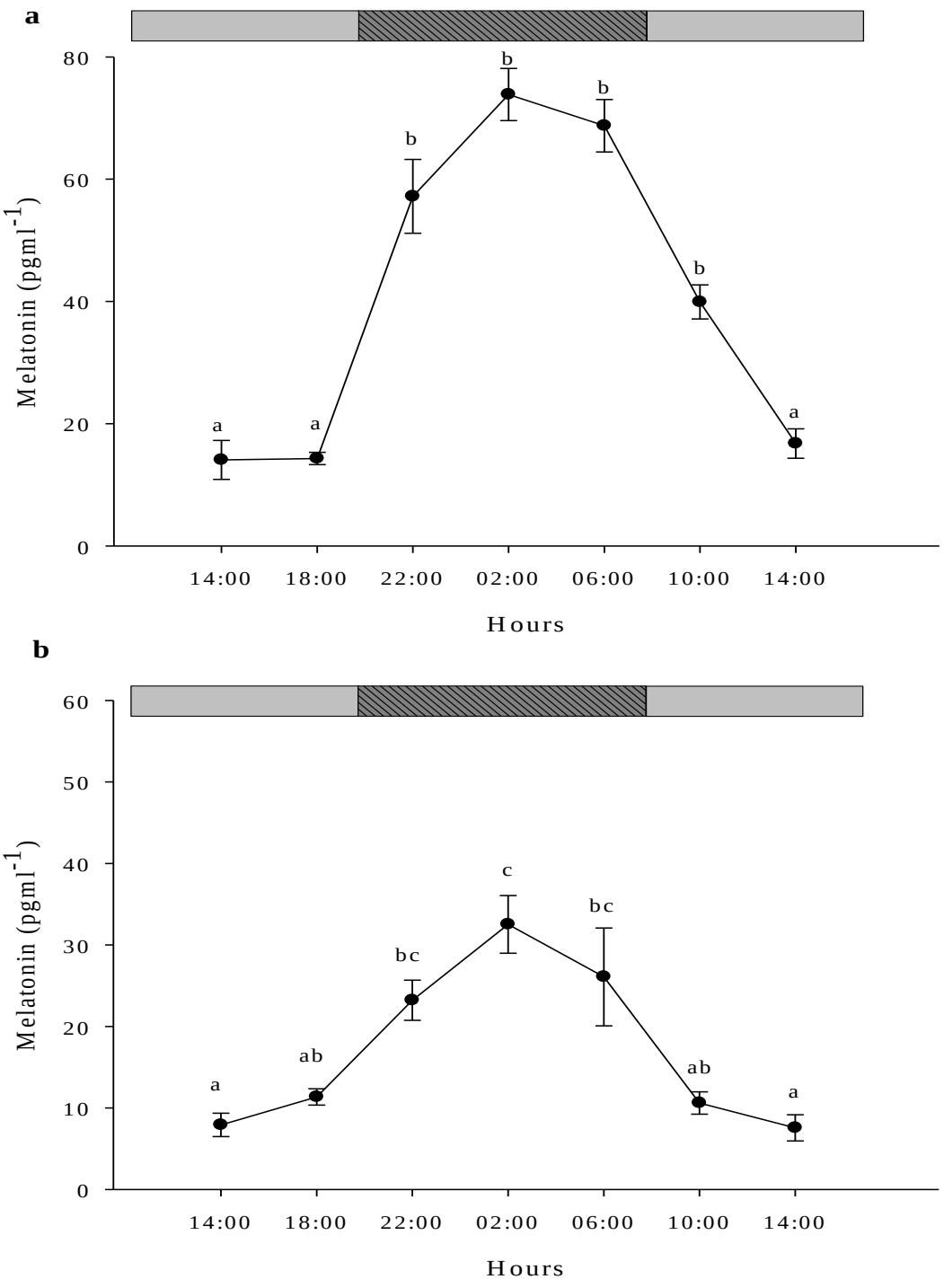


Figure 5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Melatonin (pgml-1)
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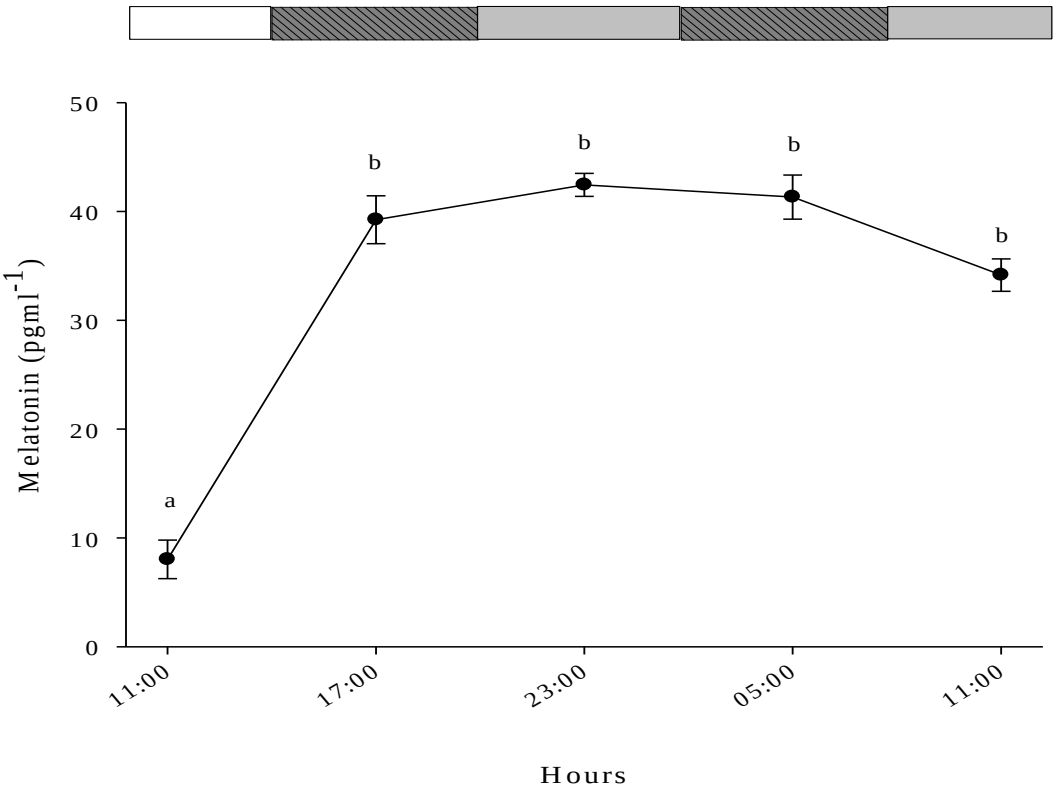
Figure 6


50
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10
0
Melatonin (pgml-1)
a
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b
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b
11:00
17:00
23:00
05:00
11:00
Hours

CHAPTER 5

PAPER III: CIRCADIAN RHYTHMS OF LOCOMOTOR ACTIVITY IN THE AFRICAN CATFISH, CLARIAS GARIEPINUS

The following manuscript was compiled and written in full by the author. The co-authors assisted with the experimental design and with the guidance and proof reading of the submitted manuscript.

Manuscript status: Ready for submission in the relevant peer reviewed journal.

CIRCADIAN RHYTHMS OF LOCOMOTOR ACTIVITY IN THE AFRICAN CATFISH, CLARIAS GARIEPINUS.

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Running title: Circadian rhythms in catfish

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ABSTRACT

The effects of stocking density on locomotor activity of African Catfish *C. gariepinus* under different light regimes were the aim of this study. To investigate this, *C. gariepinus* were stocked at different densities (1, 5 10 fish/ tank) then the locomotor activities of the fish were recorded under light-dark cycle LD, constant light (LL), dark (DD), and LD reversed (DL). Fish reared individually or in groups of 10 displayed a nocturnal rhythm under LD cycle, where fish kept in groups of 5 were considered as arrhythmic. When fish were subjected to DD, all tanks with medium, high and only 50% of tanks in low stocking densities showed circadian rhythmicity under DD with free-running of an average period (tau) from 21.4 to 24.9 h for low density or mean t*au* 23.3 ± 0.4 h and 24.3 ± 0.5 h, for medium and high respectively. Under LL, activity levels decreased significantly in comparison with levels observed under LD and DD. However, 25%, 50% and 75% of tanks with low, medium and high densities respectively showed free-running rhythms under LL. When the photocycle was reversed (DL), most of the tanks in low and high stocking densities (75% in both densities) showed a gradual resynchronisation to the new phase and transient cycles of activity were observed within 2-3 days, whereas medium stocking densities displayed an arrythmic activity pattern. The results suggest that stocking density of fish affected the display of rhythmicity and the intensity of activity levels, indicating that social interactions may have an important influence on behavioural patterns in this species.

Keywords: Circadian rhythms, locomotor activity, African catfish, photoperiod.

1. INTRODUCTION

Animals usually synchronize their biological rhythms to the environmental cycles, resulting in the display of daily behavioural patterns in locomotor and feeding activity (Favreaua, 2009; Eriksson, 1978). The daily light-dark (LD) cycle is the main circadian synchronizer in vertebrates (Tabata *et al*.,1988) and it entrains most physiological functions, especially reproduction, growth, and daily locomotor and feeding activities (Mayer *et al.*, 1997; Falcon *et al.*, 2007). Fish have been classified into diurnal, nocturnal or crepuscular species depending on the time of day in which their activity is displayed (Iigo and Tabata, 1996). However, in some cases the classification is not clear. Thus, some individuals of a given species can be active at different phases of the LD cycle whereas others can shift their activity patterns along their life cycle (from diurnal to nocturnal and *vice versa*) (Iigo and Tabata, 1996; Eriksson, 1978; Reebs, 2002; Favreaua, 2009). Behavioural rhythms can be resynchronized from one season to another depending on environmental changes e.g. daylength (Muiler, 1978), light intensity (Eriksson, 1978), temperature (Fraser *et al*, 1993), food availability (Sanchez-Vázquez *et al*., 1996) and intraspecific activities (e.g. migration, spawning, parental activities) (Metcalfe & Steele, 2001, Vera *et al*., 2006).

In vertebrates, biological rhythms can be classified according to their periodicity: circadian (approx. 24 h), infradian (> 24 h) and ultradian (< 24 h) (Reebs, 2002). To demonstrate the existence of circadian rhythmicity, animals are usually reared under constant environmental conditions. In the absence of external cues the biological rhythms then free run with a period (*tau*) close to 24 h but not exactly. In fish, these rhythms eventually can damp after a few days (Pittendrigh & Daan, 1976; Naruse & Oishi, 1994). The existence of free running rhythms can also be tested by phase-shifting the environmental light-dark cycle and investigating the appearance of transient cycles of

activity. On the first day following the shift the activity starts at the same time of light or dark and then the activity is slowly resynchronized day after day to the new phase of the light-dark cycle (Favreaua, 2009; Reebs, 2002).

Many studies have investigated activity rhythms in fish however locomotor activity studies on catfish species are scarce. Results obtained to date have shown the existence of diel rhythms when fish were reared under LD cycles, with activity peaking during the dark phase as shown in Japanese sea catfish *Plotosus lineatus* (Kasai, 2009), channel catfish, *Ictalurus punctatus*, (Goudie *et al*., 1983), sea catfish, *Arius felis* (Steele, 1984), Japanese catfish, *Silurus asotus* (Tabata *et al*., 1991), European catfish, *Silurus glanis* (Boujard *et al*., 1995), Brazilian cave catfish, *genus Taunayia* (Trajano & Menna-Barreto, 2000) and Indian catfish, *Clarias batrachus* (Ramteke, *et al*, 2009). This probably reflects adaptations to their natural habitat as most Siluridae teleosts are typical bottom-dwellers. However, under constant environmental conditions, different results are obtained among species. The circadian rhythms of cave catfish for example were shown to disappear immediately after shifting the photoperiod from LD to continuous darkness (DD) and a weak free-run rhythmicity was observed (Trajano and Barreto, 2000; Trajano *et al*., 2005). Similar results were found on the blind catfish, *Synodontis nigriventris* (Kabasawa, 1986), and sea catfish (Steele, 1984). In contrast, a strong free-running activity was recorded under DD and continuous light (LL) in the Japanese and channel catfish (Goudie *et al*., 1983; Tabata *et al*., 1991) even in animals previously ophthalmectomised, demonstrating that the circadian oscillator regulating the endogenous rhythmic activity is not located in the eyes in this species.

Circadian activity rhythms under LD cycle have also been reported in a wide range of fish species. Clear locomotor activity patterns are observed in some fish species: e.g. zebrafish, *Danio rerio* (diurnal) and tench, *Tinca tinca* (nocturnal) (Herrero *et al*, 2003;

Hurd *et al* 1998) whereas a large inter-individual variation of daily activity has been reported in other fish, such as tilapia *O. niloticus* (Vera *et al*., 2009) and goldfish *Carassius auratus* (Iigo and Tabata, 1996). Generally activity patterns in fish show a strong plasticity, especially in freshwater species, probably due to the relative instability of their environment (Reebs, 2002).

The objective of this study was 1) to describe the daily activity rhythms of *Clarias gariepinus* reared under an LD cycle, 2) elucidate the existence of endogenous control on these activity rhythms under constant light conditions (DD and LL) and after phase-shifting the LD cycle and 3) test the influence of stocking density on activity rhythms.

2. MATERIALS AND METHODS

Fish and housing

Male and female *C. gariepinus* (175.5±16 body weight, 64 fish) were obtained from stock bred and held at the main tropical aquarium of the Institute of Aquaculture at the University of Stirling, where the present study was carried out. The system consisted of six glass tanks (54cm × 46cm × 46cm, 114L) placed in three light proof compartments (two tanks/compartment). These units were subdivided using black plastic sheets with small holes to allow water exchange, giving a total of 12 experimental tanks. The walls of all aquaria were also covered with black plastic sheets to prevent interactions between individuals that could affect the activity records.

Animals were acclimatized to a 12:12 h LD cycle for two weeks before the start of the experiment. Light was provided by 60 watts light units (Lampways Triple Plus 60 Watt Pearl Bulb, United Kingdom) placed above tanks giving a light intensity at the water surface of 0.6 $W \cdot m^{-2}$ (equivalent to approximately 100 lux) during the photophase. Photoperiod was controlled by digital timer clocks. Each tank was provided with a constant

flow of well aerated freshwater. Water (27°) and ambient air temperature (27-30°C in sealed compartments) were kept constant. Water quality (nitrate, nitrite, ammonia, and pH) were monitored throughout the experiments with aquarium water quality kits (C-Test kits, New Aquarium Systems, Mentor, Ohio, USA) and remained within safe limits.

Experimental design

The experiment consisted of testing three different stocking densities in quadruplicate: 1 (low density), 5 (medium density) and 10 (high density) fish/tank. Following acclimation, fish activity was registered under the same LD cycle for three weeks to characterize their daily activity rhythms before exposing them to DD conditions for 2 weeks in order to study circadian rhythmicity. Thereafter, fish were resynchronized to the LD cycle for 2 weeks and exposed to LL for 2 additional weeks. Then fish were returned to a LD cycle for 2 more weeks before exposing them to DL. Finally, fish were resynchronized to the LD cycle for 2 weeks. Fish were fed at random times during the photophase every 2 days to avoid feeding from acting as a potential synchronizer.

Locomotor Activity Monitoring

To record the locomotor activity each aquarium was equipped with one infrared photocell (E3Z-D67, OMRON, China) placed in the centre of the front glass wall. Such activity recording systems have been used in several other fish species including Japanese sea catfish (Kasai *et al*, 2009), Nile tilapia (Vera *et al*., 2009), zebrafish (Olmeda and Sanchez-Vazquez, 2009) and seabream (Vera *et al*., 2006).

Previously, the position of the photocell had been optimised through pilot testing of locomotor activity. Photocells were connected to a motherboard (USB-1024HLS, Measurement Computing, USA) connected to a computer. Every time a fish interrupted the

infrared light beam it produced an output signal that was recorded and stored in 10 minute bins using a specialized software (DIO98USB, University of Murcia, Spain).

Data analysis

Locomotor activity records were analyzed using chronobiological software (El Temps®, Prof. A. Díez-Noguera, University of Barcelona, Spain). The period length (tau) of free-running rhythms was determined by periodogram analysis at a confidence level of 95%. Data analysis was performed with the aid of Microsoft Excel.

3. RESULTS

- Daily locomotor rhythms under LD cycles

Under an LD cycle, the stocking density influenced the daily activity pattern. Fish reared individually or in groups of 10 displayed a nocturnal rhythm with percentages of locomotor activity under the scotophase of 81±7.2% and 65±2.2% (Table 1) respectively characterised by a peak of activity during the LD switch. However, the catfish kept in groups of 5 were considered as arrhythmic with only 58.5±1.8% of locomotor activity (Table 1) displayed during the night and two peaks of activity during both shift from light/dark and vice versa (Fig.1A, B, C).

- Circadian rhythms under DD and LL regimes

When fish were subjected to DD, 50% of fish reared individually showed free-running rhythms of locomotor activity from either the second or the seventh day, although these self-sustained rhythms damped after 3-4 days (Fig. 2A). The period of the endogenous rhythm (*tau*) ranged from 21.4 to 24.9 h (Table 2). All tanks with medium and high stocking densities showed circadian rhythmicity under DD. However, 75% of tanks

showed circadian rhythmicity from the second day and 25% of tanks started from the sixth (tanks with 5 fish) or seventh day (tanks with 10 fish). Circadian rhythms were sustained for 5-9 days (tanks with 5 fish) and 5-7 days (tanks with 10 fish) (Fig. 2B) after which rhythms disappeared. Mean t*au* were 23.3 ± 0.4 h and 24.3 ± 0.5 h, respectively (Table 2).

Under LL, activity levels decreased significantly in comparison with levels observed under LD and DD (Table 3). After 7 days under LL only 1 out of 4 low density tanks (1 fish/tank) showed free-running rhythms during 3 days (*tau* = 23.7 h) (Fig. 3A). Two out of the 4 medium density tanks (5 fish/tank) showed self-sustained activity rhythms after 6 days under LL. Rhythms free-ran for 5 days with a *tau* ranging from 24.1 to 25.2 (Table 2) (Fig. 3B). Finally 3 out of the 4 high density tanks (10 fish/tank) showed circadian rhythmicity from the third (two tanks) or ninth day (one tank). This free running activity was maintained for approximately 5 days all three tanks (Fig. 3C), with a *tau* ranging from 23.7 to 25.2 (Table 2).

- **Circadian activity under phase-shifted LD cycle (DL)**

When the photocycle was reversed (DL), most of the tanks with low and high stocking densities (75% in both densities) showed a gradual resynchronisation to the new phase characterised by the existence of transient cycles of activity for 2-3 days (Fig. 4). Interestingly all medium stocking densities tanks did not resynchronise to DL and displayed an arrythmic activity pattern (Fig. 4B).

Overall, tanks with low stocking density showed the lowest total activity levels while both medium and high density tanks displayed similar activities. However, when the number of fish is taken into account, then the medium density tanks displayed significantly higher activity than high or low density tanks under all photoperiods (LD, DD, LL and DL) (Table 3). In average, individual activity under LD/DD/DL was 55-65, 260-390 and 150-

160 respectively for low, medium and high density tanks whereas activity was reduced to 13, 121 and 69 under LL (Table 3).

4. DISCUSSION

Catfish nocturnal activity has been reported in numerous species (Tabata et al, 1989; Trajano and Menna-Barrelo, 1996; Trajano and Menna-Barrelo, 2000). The present study confirmed that African catfish is mostly active at night and interestingly that the activity level can vary depending on the stocking density. Furthermore, this species displays circadian endogenous activity under continuous darkness suggesting the existence of circadian oscillators that control activity rhythms in the absence of day/night cycle.

The locomotor activity profile under an LD cycle showed a peak of activity in catfish starting just before light switch off (approximately half hour) and lasting for two hours. This clearly indicates that catfish can anticipate the night period through internal clocks that are not localised nor identified yet in any fish species. Similar findings have been reported on catfish in melatonin profile under LD cycle (Martínez-Chávez *et al*., 2008). This peak of activity was not influenced by the density of fish. Then, the locomotor activity gradually decreased to relatively steady levels (still higher than during the day) maintained throughout the night thereafter in the low and medium stocking density treatments. In these latter treatments, activity then increased slightly towards the end of the night period and dropped suddenly during the first hour following the light onset. However, in the medium density treatment, activity in the middle of the night was similar to that during the day with the exception of a second peak starting approximately 1 hour before light onset.

Many locomotor activity studies performed in fish have shown the existence of free running rhythms when exposed to constant photic conditions (Kasai *et al*, 2009;

Pittendrigh & Daan, 1976; Naruse & Oishi, 1994; Tabata *et al*, 1989). Typically, fish can show these rhythms for several days before losing rhythmicity (e.g. becoming arrhythmic). This has already been shown in two species of Japanese catfish; *Plotosus lineatus* (Kasai, 2009) and *Silurus asotus* (Tabata et al, 1989). In the present study, all fish from the medium and high density tanks exhibited self-sustained activity rhythms under DD for up-to 7-9 days whereas only 50% of the fish reared individually showed such a circadian rhythmicity. These results support previous studies which demonstrated the existence of an endogenous clock controlling melatonin production in the absence of LD signal (DD) in the African catfish and Nile tilapia (Martínez-Chávez *et al*., 2008). However, the clock mechanisms controlling activity rhythms and hormonal synthesis are unknown to data and might differ.

When animals were exposed to LL, the daily total activity sharply decreased in all tanks, although free-running rhythms were still observed in all experimental groups. In this case the percentage of tanks showing endogenous rhythmicity also depended on the fish density: 25%, 50% and 75% of tanks with low, medium and high density, respectively. These differences among fish reared individually or in groups could be explained by the existence of social interactions, which can affect locomotor and feeding behaviour in fish (Kavaliers, 1980). In some species such as catfish or tilapias, locomotor activity can be altered when they are kept individually or in small groups, as animals become more active to defend their territory. However, when the number of fish is increased, fish form shoals and the time and energy spent in confrontations decrease (Hecht *et al*., 1997; Hossain *et al*., 1998). Moreover, the period of the circadian rhythm differed between fish reared individually and those kept in groups as suggested by the present study (Favreaua, 2009). This phenomenon has been previously reported in Atlantic killifish (*Fundulus heteroclitus*) when they were studied in groups of 5 or 25. When animals were maintained in small

groups they behaved as isolated individuals and only occasionally formed loose aggregation, whereas big groups functioned as an interacting social unit or shoal (Kavaliers, 1980). Concerning other catfish species, European catfish showed an arrhythmic activity pattern when they were reared individually, but in groups of three or more, feeding and locomotor rhythms were strictly nocturnal and stable (Boujard, 1995). In the present study, single fish showed lower levels of activity and were at the bottom of the tank most of the time. In contrast, fish in groups of 5 and 10 were very active.

Under constant light conditions, the existence and duration of free-running activity rhythms clearly differs among catfish species. In the Japanese catfish, strong circadian rhythmicity was described under both DD and LL, even in ophthalmectimised individuals, suggesting that the pacemaker entraining this rhythm must be located in another anatomical structure, most likely centrally in the brain (Tabata *et al*., 1991). In the channel catfish, similar results were found (Goudie *et al*., 1983). However, in other catfish species such as cave catfish, blind upside-down catfish and sea catfish, the circadian rhythms disappeared immediately after shifting the LD to DD (Steele, 1984; Kabasawa, 1986; Trajano and Barreto, 2000; Trajano *et al*., 2005). Nevertheless, the existence of weak circadian locomotor rhythmicity under constant conditions is a common feature among many teleost species. In goldfish, only 57% of experimental individuals showed self-sustained locomotor rhythmicity under DD and LL (Iigo and Tabata, 1996; Sanchez-Vázquez *et al*., 1996). Similarly, only 5 out of 30 Atlantic salmon (*Salmo salar*) showed circadian locomotor activity rhythms under DD, and none under LL (Richardson and McCleave, 1974; Thorpe, 1988) whereas 50% of pink salmon (*Oncorhynchus gorbuscha*) were rhythmic under LL (Godin, 1981).

Another way to test the existence of endogenous rhythmicity is to phase-shift the LD cycle and record transient cycles of activity. When fish require several days to synchronise

their rhythms to the new phase it can be concluded that there is endogenous control on the display of locomotor activity. However, if fish resynchronise within a day, it may indicate that the behavioural rhythms are mainly driven by exogenous factors (Btinning, 1973; Tabata *et al*., 1989). In the present study, when the LD cycle was reversed to DL a gradual resynchronization was observed in 75% of catfish reared individually or in groups of 10 fish, suggesting the existence of an internal pacemaker in this species. The results are similar to findings obtained in the Japanese catfish which needs 1-2 transient cycles to fully adapt to the new phases of LD (Tabata *et al*., 1988; 1989). This latter species was shown to display strong circadian locomotor activity under DD and LL.

To conclude, the present study clearly showed that catfish is a nocturnal species under an LD cycle and suggests that its locomotor activity rhythms can be controlled by an internal circadian clock, since circadian rhythmicity was observed under constant conditions. Furthermore, the stocking density of fish affected the display of rhythmicity and the intensity of activity levels, indicating that social interactions may have an important influence on behavioural patterns in this species.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the University of Stirling Tropical Aquarium for providing the fish and helping with the daily management and Dr Javier Sanchez-Vazquez for providing the system to study locomotor activity. Financial support was provided by the Saudi Arabian Government (studentship to Sami Al-Khamees) as well as by “Fundación Séneca” and “Agencia Regional de Ciencia y Tecnología” for the postdoctoral grant awarded to L.M. Vera.

Table 1: Percentage of catfish activity under LD cycles in low, medium and high density tanks (n=4) during the scotophase. Fish showed nocturnal (N) or arrhythmic activity (A).

Tank	**Treatment**	**% Activity during the scotophase**
1	Low density	95 N
2		84 N
3		87N
4		61 A
5	Medium density	63 A
6		59 A
7		58 A
8		54 A
9	High density	66 N
10		65 N
11		71 N
12		60 A

Table 2: Free-running rhythms period (*tau*) in catfish under DD and LL in low, medium and high density tanks. NS: non significant.

Tank	**Treatment**	***Tau* value (h)**	
		DD	**LL**
1	Low density	21.4	NS
2		NS	23.7
3		24.9	NS
4		NS	NS
5	Medium density	22.1	NS
6		24.1	NS
7		22.9	25.2
8		24.1	24.1
9	High density	24.5	24.3
10		23	25.2
11		25.7	NS
12		24.1	23.7

Table 3: Mean daily total (top) and relative (bottom) activity of catfish in low, medium and high density tanks exposed to LD, DD, LL and. Values are expressed as mean ± S.E (n=4 tanks). Superscripts denote significant differences.

Fish density	**Mean daily total and relative activity (registers/10 min)**		
	LD	**DD**	**LL**
Low	65.21 ± 4.8 a	57.13±4.4 a	13.49±2.9 a
Medium	1950.47±147.3 b 390±29.46	1330.81±217.7 b 266±43.54	609.33±172.4 b 121±34.48
High	1586.17±136.3 c 158±13.63	1501.01±129.6 c 150±12.9	691.65±140.8 c 69±14

FIGURE LEGENDS

Figure 1. Average daily locomotor activity observed in *C. gariepinus* kept under low (A), medium (B) and high (C) stocking densities under laboratory conditions. Data are expressed as mean of the four tanks (thick line) + S.E. (dotted line). Horizontal bar above the graph represents day (in white) and night (in black) hours.

Figure 2. Locomotor actograms (left column) and their corresponding periodogram (right column) for *C. gariepinus* kept on low (A), medium (B) and high stocking densities (C) under constant darkness conditions (DD). The period of the free-running rhythm (*tau*) is indicated above the periodogram. Actogram are double-plotted for better visualization. Horizontal bar at the top of the actograms represents light regimes.

Figure 3. Locomotor actograms (left column) and their corresponding periodogram (right column) for *C. gariepinus* kept on low (A), medium (B) and high stocking densities (C) under constant light conditions (LL). The period of the free-running rhythm (*tau*) is indicated above the periodogram. Actogram are double-plotted for better visualization. Horizontal bar at the top of the actograms represents light regimes.

Figure 4. Locomotor activity of *C. gariepinus* from three tanks under low (A), medium (B) and high stocking densities (C). Locomotor activity recorded first for 21 days under LD cycle, before exposing them to DD conditions for 2 weeks then every time fish were resynchronized to the LD for 2 weeks before exposed to LL and DL for 2 weeks. Horizontal bar at the top of the actograms represents light regimes.

Figure 1

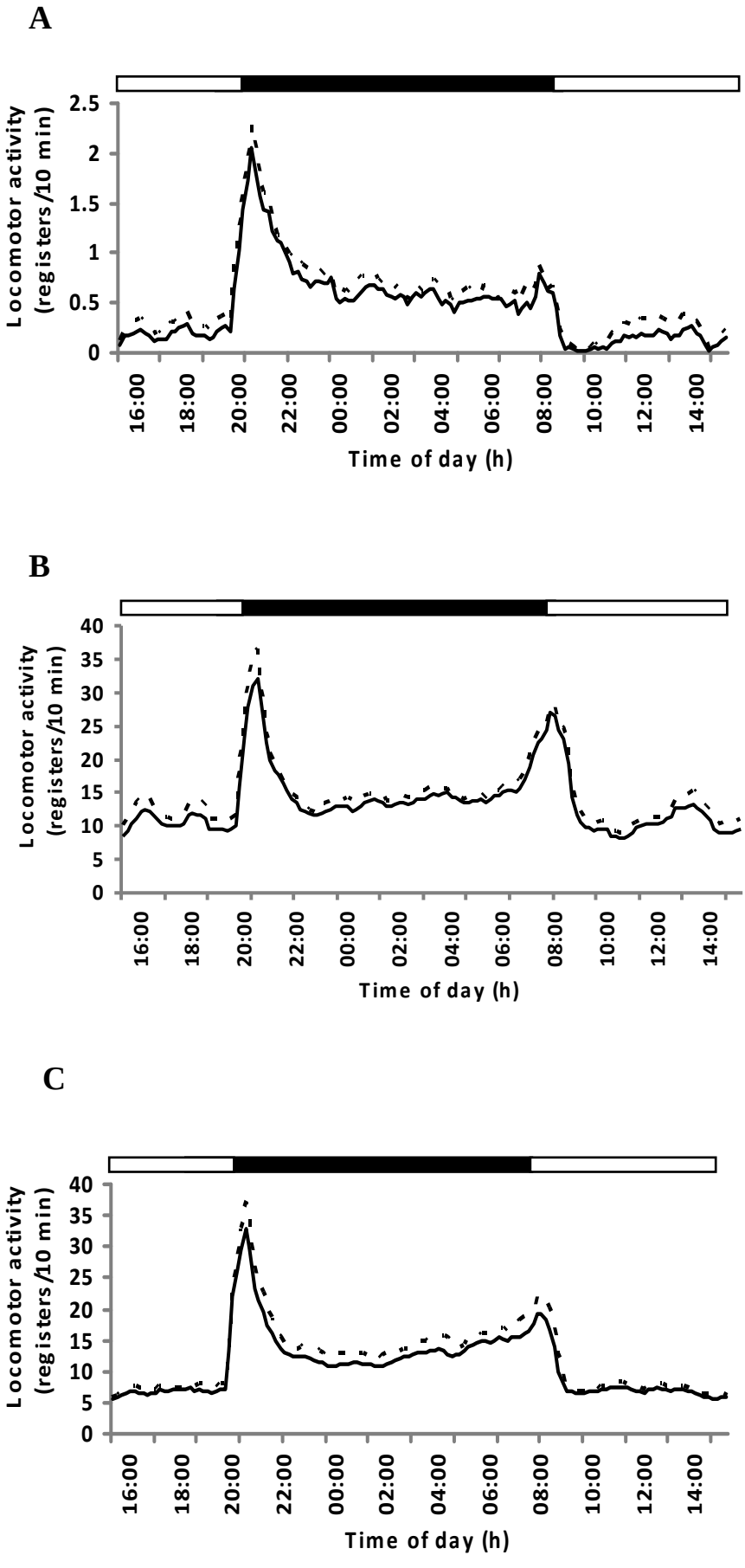
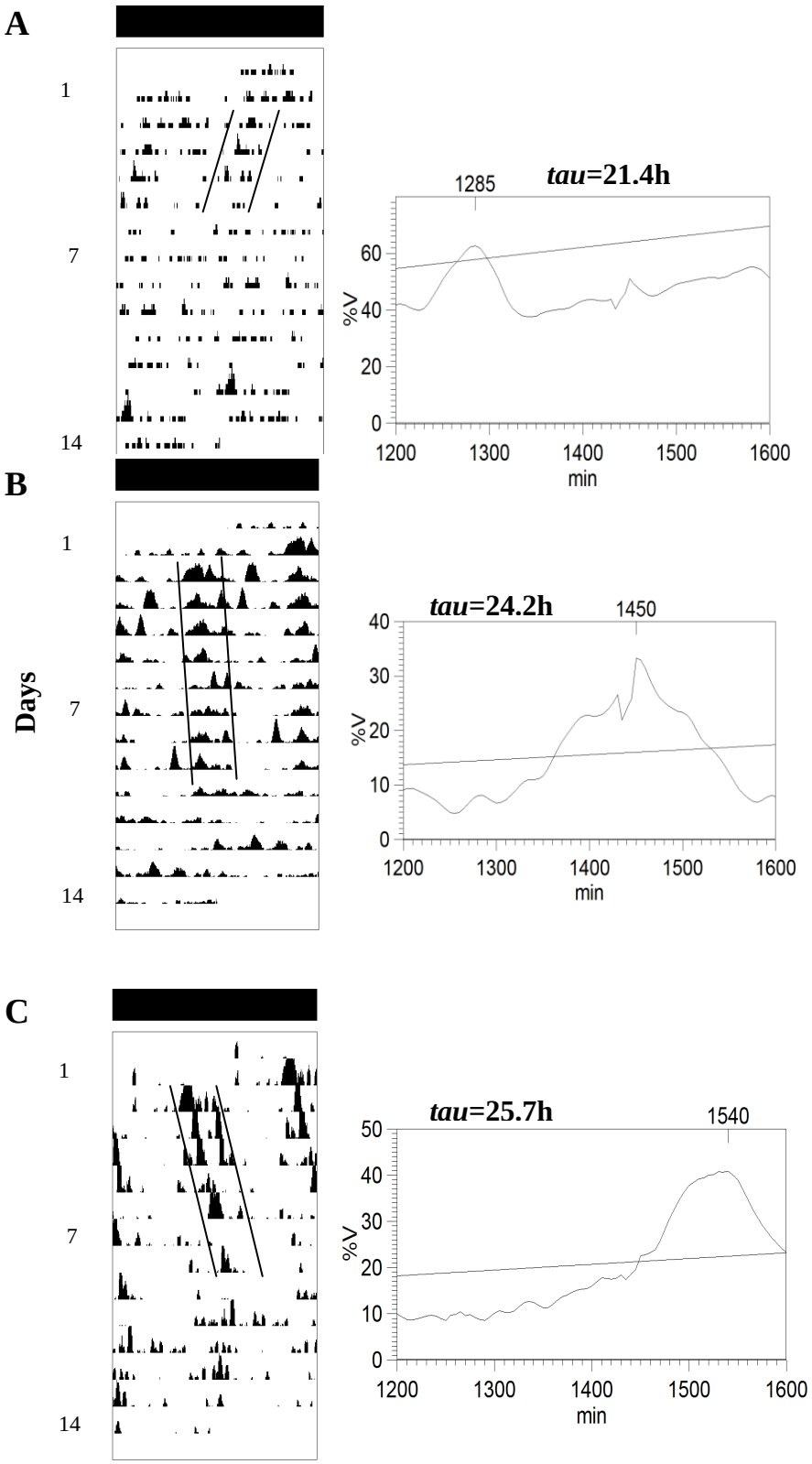

A
Locomotor activity (registers/10 min)
2.5
2
1.5
1
0.5
0
16:00
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
14:00
Time of day (h)
B
Locomotor activity (registers/10 min)
40
35
30
25
20
15
10
5
0
16:00
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
14:00
Time of day (h)
C
Locomotor activity (registers/10 min)
40
35
30
25
20
15
10
5
0
16:00
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
14:00
Time of day (h)

Figure 2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7
14
1285
tau=21.4h
60
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%V
1200
1300
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B
Days
1
7
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tau=24.2h
1450
40
30
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10
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%V
1200
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1400
1500
1600
min
C
1
7
14
tau=25.7h
1540
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40
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%V
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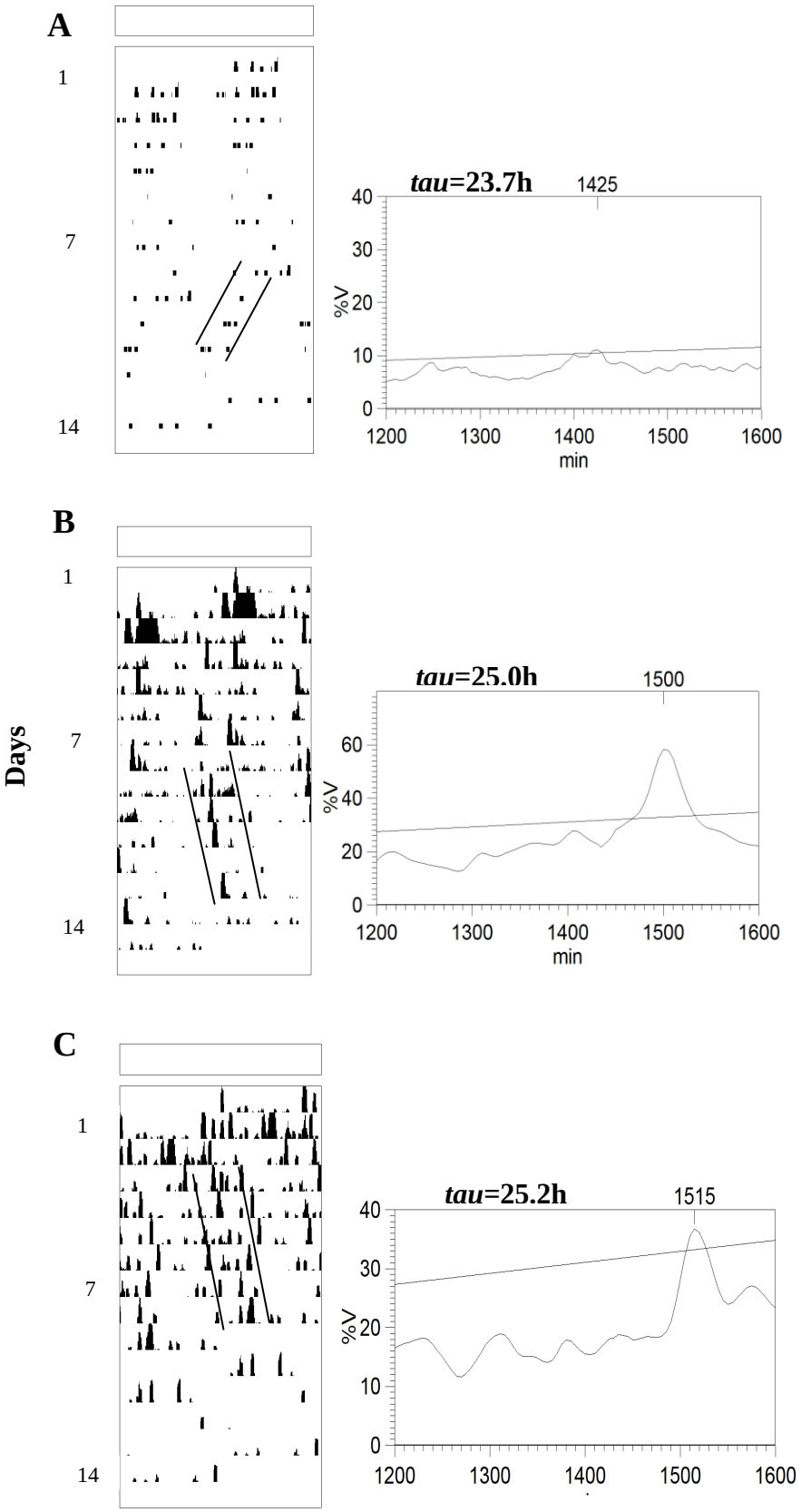
1500
1600
min

Figure 3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tau=23.7h
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1300
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Days
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7
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tau=25.0h
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%V
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1500
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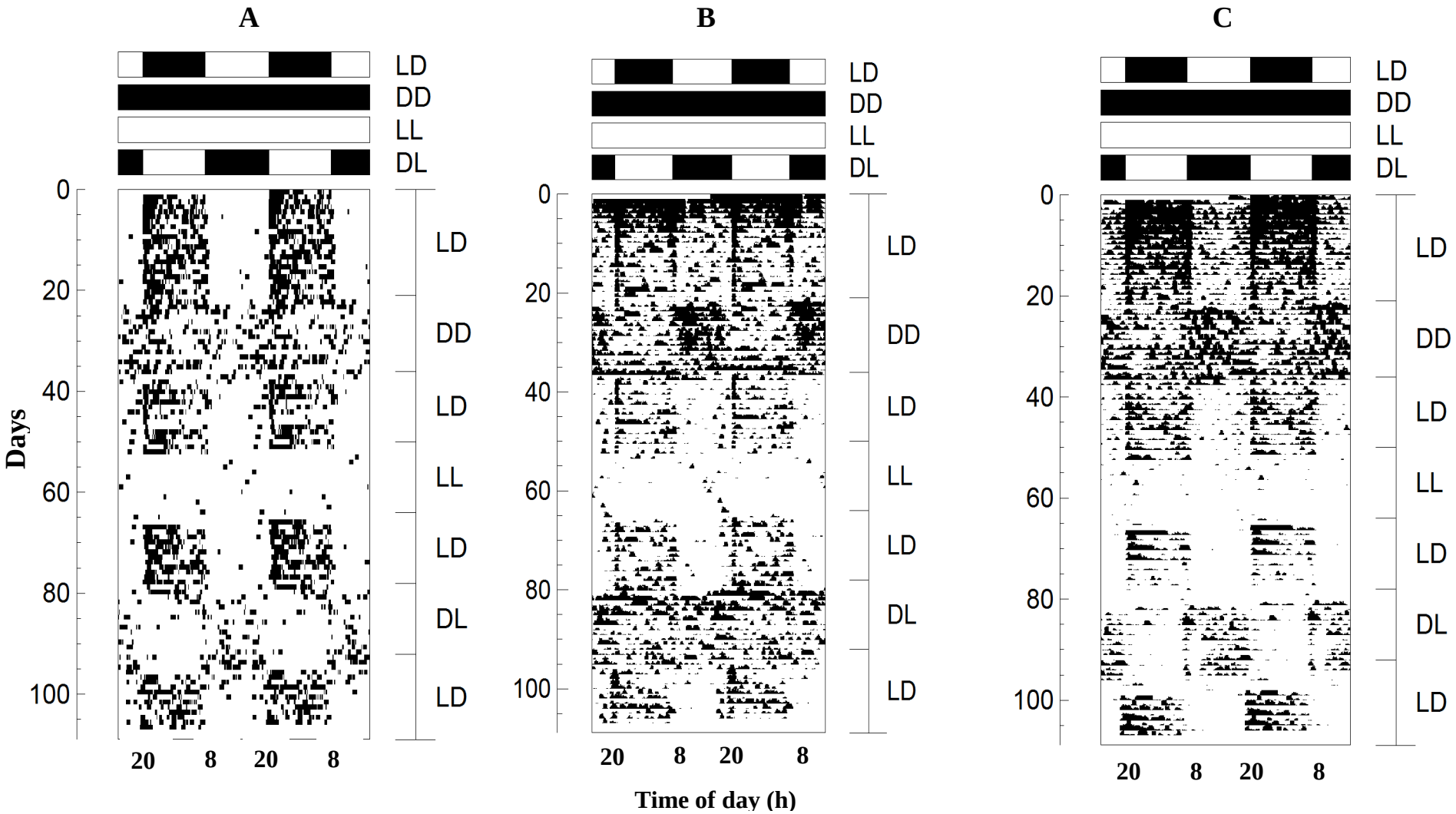
Figure 4


A
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C
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DD
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DL
0
20
40
60
80
100
Days
LD
DD
LD
LL
LD
DL
LD
20
8
20
8
Time of day (h)

CHAPTER 6

PAPER IV: EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH, SEX STEROID, PRECOCIOUS MATURATION AND HATCHING RATES IN AFRICAN CATFISH (*C. GARIEPINUS*)

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EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH, SEX STEROIDS, PRECOCIOUS MATURATION AND HATCHING RATES IN AFRICAN CATFISH (*C. GARIEPINUS*).

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Running title: Photoperiod effects on growth and maturation in African catfish

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ABSTRACT

The aims of the study were to determine the effects of photoperiod on the growth and survival of African catfish, *Clarias gariepinus*, up to first maturation and to test whether different light regimes can impact on early maturation and associated hormonal changes as well as fecundity, egg diameter and hatching rates. To do so, newly hatched larvae were exposed to two different photoperiodic treatments (12L:12D or LL) until 90 days post hatch (DPH). Then, fish under 12:12 photoperiod were shifted randomly to one of the following photoperiodic treatments in duplicated tanks: 6L:18D, 6L:6D, LL and 12L:12D (control), and fish under LL were shifted to either 12L:12D and LL (control). The experiment was run until 360 DPH. Finally, fish from the 6L:18D, 6L:6D, LL and 12L:12D treatments were hormonally induced and stripped in order to assess egg quality. A range of parameters were monitored to assess growth and feeding (weight, length, condition, specific growth rate, feed consumption, feed efficiency), survival, reproductive status (gonadosomatic index, histological gamete staging, plasma steroid levels) and reproductive performance (fecundity, egg diameter, hatching). No significant weight and length differences between fish reared under either 12:12 or LL photoperiods from hatching to 120 DPH were observed. Large mortality rates were recorded at this stage in both treatments (40% at 120 DPH) due to aggression and cannibalism. Thereafter, photoperiod had a significant effect on growth performance with fish (females and males) under LL showing a 25 to 40% reduction as compared to all other treatments. This was related to significantly lower food consumption from 150 DPH onwards. However, no major effects of photoperiod were observed on sexual development with fish all maturing under all treatments although slight differences in the timing of key gametogenesis stages were observed. Interestingly fish under the LL treatment appeared to reach the final reproductive stages earlier than fish under the other treatments despite their lower growth

rate. Differences between treatments were also observed in mortality rates with an overall mortality, smaller than at the juvenile stage, ranging from 6 to 11% between 120 and 330 DPH. Finally, no major differences were observed in egg quality and reproductive performance except for egg diameter that was larger in egg batches from the LL fish. Overall, this study confirms previous findings published in other catfish species on the effects of extended daylength on growth performances in African catfish that might be related to stress response or other light induced behavioural alterations. Photoperiod did not appear to play a major role in the entrainment of reproduction in this tropical species, nordid temperature which was maintained constant, suggesting that other genetic or clock controlled mechanisms may be at work.

Keywords : photoperiod, *Clarias gariepinus*, growth, food consumption, survival, reproduction

1. INTRODUCTION

While a number of environmental factors are involved as possible proximate cues of fish physiology including photoperiod, temperature, rainfall, food supplies and pheromones, it is the seasonally changing pattern of daylength that is the main signal entraining the onset of reproduction in most temperate teleost species. Photoperiodic manipulation is therefore routinely used in fish farming throughout the production cycle to manipulate and regulate a number of key physiological functions (hatching growth, broodstock spawning, growth, smoltification and early maturation) (Bromage, 1995; Boeuf and Le Bail, 1999; Purchase *et al*., 2000, Bromage *et al*., 2001; Randall *et al*., 2001; Rodriguez *et al*., 2001; Biswas and Takeuchi, 2002 and Gines *et al.*, 2003). Long day photoperiod regimes have been shown to be a key signal to enhance growth rate and/or delay sexual maturity in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*), sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) (Boeuf and Le Bail, 1999, Oppedal *et al*., 1999, Jonassen *et al*, 2000, Simensen *et al*., 2000, Kissil *et al*., 2001, Randall *et al*., 2001, Rodriguez *et al*., 2001 and Gines *et al*., 2004). Growth stimulation under such photoperiod is thought to be driven by either improved feed efficiency or direct stimulation of the growth hormone axis in juvenile fish (Taylor *et al*., 2006; shan *et al*., 2008; Mc Cormick *et al*.,1995) and suppression or delay of sexual maturation resulting in the diversion of energy to somatic growth instead of gonadal development in adult fish (Boeuf and Le Bail, 1999 and Gines *et al*, 2004; Fitzpatricka *et al*., 2006; Abdul Razak, 1999; Cal *et al*., 2006). In physiological terms, early sexual maturity results in reduced growth and consequently poor feed conversion which mean higher feed costs and lower profitability for fish farms (Rad *et al*., 2006).

Most studies performed over recent decades have focused on a wide range of temperate species (e.g. salmonids, flatfish, gadoids, cyprinids) with little research so far on sub-tropical or tropical teleosts (Bromage *et al*., 2001). The effects of photoperiodic manipulation on reproduction in these species are not well understood (Campos-Mendoza *et al*., 2004; Rad *et al*., 2006). In the Nile tilapia (*Oreochromis niloticus*) long day photocycle (18L:6D) has been shown to stimulate seed production with specifically an increase in fecundity, a shortening of inter-spawning intervals (Ridha & Cruz 2000; Campos- Mendoza *et al*., 2004) and higher mean final weight, whereas gonadosomatic index (GSI) and oocyte size were significantly smaller in fish maintained under continuous light regime (LL) than those of 20L:4D, 18L:6D and control treatments (Rad *et al*., 2006).

The use of a 12-14 h photophase, at a temperature of 25-29°C, was shown to be optimal for natural spawning in the same species (Biswas *et al*, 2005). Galman *et al* (1988) found that extending the light phase during the winter months (December-February) using artificial light can help to spread out the production of red tilapia fry all year round. However, Ridha and Cruz (2000) reported that fish exposure to long days and high light intensity (18L:6D with 2500 lux) displayed higher fecundity and better synchronisation of spawning in Nile tilapia (*Oreochromis niloticus*) compared with short days (12L:12D; 15L:9D) and low light intensity (500 lux). Other studies have reported effects of photoperiod on reproduction of tropical species. In goldfish, long photoperiod (16L:8D) can stimulate gonadal development as opposed to short photoperiod (12L:12D) (Stacey *et al*., 1979; Razani *et al.*, 1989). In Indian carp, *Catla catla*, long photoperiods (16h) can be used to advance testis development, while short days (8h) applied during the prespawning phase of the reproductive cycle can suppress maturation together with maturation-associated growth reduction and deterioration in fish growth (Bhattacharyya *et al*., 2005).

In African catfish, spawning is highly seasonal and cued by a decrease of temperature during the rainfall season followed by an increase in temperature and daylength during the summer which suggest that ovarian recrudescence (puberty or onset of gonadal development) could be entrained by these two factors (Bruton 1979a; Haylor, 1992). However, in enclosed laboratory conditions, once fish have reached puberty, then no seasonal annual rhythm in gonadal activity can be seen as a result of the constant high temperature (25-28 °C) and 12L:12D photoperiod exposure (Richter *et al*., 1987a). Female broodstock remain all year long at postvitellogenic gonadal stages and recycle oocyte batches through atresia and intense ovogenesis (Bromage and Roberts, 1995; Richter *et al*., 1987a). These fish can be hormonally induced and spawn at any time of the year which make *C. gariepinus* a very good experimental species to study reproductive physiology. Previous studies have shown that both photoperiod and temperature can alter gonadal activity of catfish species such as *Heteropneustes* ***fossillis*** and *Clarias batrachus*, although temperature appears to be the most important factor (Vasal and Sundararaj, 1976; Sundararaj and Vasal, 1976; Young, 1990). In catfish species, long day photoperiod can stimulate early spermatogenesis in the testis as shown in *C. batrachus* (Singh, 1998) or stimulate final oocyte maturation as shown in *Mystus tengara* (Guraya *et al*., 1976). However, photoperiod and light intensity regimes can also affect behaviour, growth rate and survival as shown in *C. gariepinus* (Britz and Pienaar, 1992; Appelbaum and Mcgeer, 1998; Appelbaum and Kamler, 2000; Almazan, 2004; Adewolu, 2008). These studies suggested that light can act as a stressor in catfish which are known to be nocturnal fish and growth rate would be reduced as daylength increase.

In view of the little available literature on the environmental regulation of African catfish physiology (e.g. growth and reproduction), it is clear that further studies are needed. The objectives of the present study were therefore (1) to determine the effects of

photoperiod manipulation on the growth and survival of *C. gariepinus*; (2) to test whether different light regimes can impact on early maturation and associated hormonal changes as well as hatching rates, fecundity and egg diameters.

2. MATERIALS AND METHODS

2.1 Incubation and experiment system

Hatching and experimental rearing systems were housed inside the main tropical aquarium facilities at the Institute of Aquaculture, University of Stirling. Both systems were used closed water recirculation with constant temperature (27±1°C) and photoperiod (12L:12D). Water quality was monitored weekly (nitrate, nitrite, ammonia, and pH) with kits (C-Test kits, New Aquarium Systems, Mentor, Ohio, USA) and levels remained within safe limits throughout the trial. The hatching system was composed of two fibreglass raceways (210 x 54 x 10 cm, 113L) light proofed with heavy plastic sheets. Light was provided by two light units (60 watt bulbs, CPC, Leeds, UK) fixed above each tank giving a light intensity of 0.6 watts/m^2 (equivalent to approximately 100 lux) at the water surface. The on growing experimental system included six light-proof individual compartments each housing two fiberglass tanks (46 x 46 x 41 cm, 86 L). Light was provided by a lamp placed above each tank (12 watts bulb, CPC, Leeds, UK) and light intensity at the water surface was 0.22 watts/m^2 (equivalent to approximately 53 lux). In both systems, light was controlled via timers to set up different photoperiods. Throughout the trial, extreme care was made to the experimental lighting regimes and sampling to avoid potential light pollution. Both systems were checked for light-proofness using a single channel light sensor (Skye instruments, Powys,UK) calibrated to UK standard.

2.2 Experiment design

For clarity, the experiment was split into three sections: 1) Effects of photoperiod (12L:12D and LL) on egg development and growth performance (up to 90 days post hatch, DPH) in juvenile catfish. 2) Effects of photoperiodic regimes on growth and first reproductive cycle in adult catfish and 3) Effects of photoperiodic regimes on broodstock fecundity, egg diameter and catfish larvae hatching rate.

2.2.1. Effects of photoperiod (12L:12D and LL) on egg development and growth performances (up to 90 days post hatch, DPH) in juvenile catfish

The aim of this trial was to test whether photoperiod could alter early egg and larvae development and influence growth performances of catfish juveniles. Two adult females (450±26g) from stock fish bred and held at the tropical aquarium facilities at the Institute of Aquaculture (University of Stirling, UK) were used. Fish were anaesthetized with 1% Benzocaine solution (SIGMA, Poole, UK) and induced to ovulate by injection of 0.5ml/kg body weight of Ovaprim (Syndel, Vancouver, Canada; 1 mL containing 20mg salmon GnRHa and 10 mg domperidon) as a standard method to induce breeding in *C. gariepinus* (Senoo, 2002). Fish were kept individually in 25L circular tanks at 27±1°C for 8-9h before stripping was performed into a glass bowl. Two male (670±37g) broodstock were sacrificed, gonads dissected and milt collected. After mixing the eggs and milt for about 1min, water was added. Fertilised eggs were split into two groups which were incubated under either 12L:12D or LL photoperiods. Eggs from both groups were spread onto a 0.5mm plastic mesh. Light was switched on at 08:00 h and off at 20:00 h for the 12L:12D photoperiod. The eggs hatched within 2 days, and yolk sac resorption lasted four days. Then larvae were fed to satiation with artemia for one week and gradually with powder of commercial trout pellets (Standard Expanded, Skretting, Cheshire, UK) three times a day

for one month. Larvae weight and length were monitored during this period at hatching, 7, 15 and 30 days post-hatching (DPH). Then, 2400 mixed sex larvae were randomly transferred to the experimental rearing system composed of 12 tanks (200 larvae/tank): 8 tanks under 12L:12D and 4 tanks under LL photoperiod regimes (duplicated trial). Ten fish from each tank were monthly sacrificed for weight and length measurements.

2.2.2 Effects of photoperiodic regimes on growth and first reproductive cycle in adult catfish

The aim of the experiment was to test the effects of different photoperiod manipulations on growth and maturation in catfish previously reared under either standard 12L:12D photoperiod or continuous light (LL). Fish used were previously acclimated to either 12L:12D or LL (see section 2.2.1). At 120 DPH, fish under 12L:12D photoperiod were randomly exposed to one of the following: 6L:18D, 6L:6D, LL and 12L:12D (control), and fish under LL were shifted to either 12L:12D and LL (control), all treatments were tested in duplicate. Light was switched on at 08:00 h and off at 20:00 h under a 12:12 photoperiod. Similarly, lights for 6:6 and 6:18 were switched on at 08:00 h and then switched off and on at 6 h intervals for 6:6 and at 14:00 h for 6:18. At this stage fish were fed to satiation with commercial trout pellets (Standard Expanded, Skretting, Cheshire, UK) three times a day. Food consumption was determined weekly for each tank. To do so, each tank was allocated a container with a known amount of food that was then weighed at the end of each 7 day period. 40 fish/treatment (20 fish/ replicated tank) were randomly sampled monthly for weight and length, and ten fish/treatment (5 fish/ tank) were sacrificed for blood samples and gonadal histology with a minimum of four males and females per treatment. In all sampling, fish were either anesthetized (0.1–0.15 g/l) or killed by a lethal dose (0.5–0.8 g/l) of benzocaine solution (SIGMA, Poole, UK). All

sampling was performed from 08:00-14:00 during the light phase for all treatments. Blood was sampled by venipuncture of the caudal vein using heparinized syringes, and the plasma was separated by centrifugation and stored at -70°C for later steroid hormonal analyses (testosterone and oestradiol) using radioimmunoassays according to Duston and Bromage (1987). Due to small fish size at the early stages of the study it was not possible to confirm the effects of the experimental photoperiodic regimes on melatonin profiles. However, previous studies performed in the present research programme have already shown the effects of daylength on melatonin secretion in the African catfish (chapters 3 and 4).

The gonads of sacrificed fish were weighed to determine gonadosomatic index (GSI) and sampled gonad tissues were fixed in 10% neutral buffered formalin for histological examination. The fixed gonad samples were embedded, sectioned and stained as described by Davie (2005). Three serial sections were then taken from embedded tissues at 5µm thickness, using a microtome, and placed on consecutive slides.

Gonadal development was determined histologically using a bifocal microscope (Olympus Optical Co., London, UK). The developmental stages of testes and ovaries were visually determined for each fish. Spermatozoa classification was based on the histological criteria adapted from Babiker and Ibrahim (1979). Oocytes were classified by developmental stage adapted from Coward and Bromage (1998). Female gonads were assigned to the atresia stage when numerous atretic follicles could be found on the three histological slides obtained from one ovary. Gonadosomatic index measurements (GSI) were carried out for all sacrificed fish using the following formula: GSI = (wet gonad weight (g) x wet body weight-1 (g)) x 100.

Survival was estimated by recording the daily mortalities. All experimental fish were counted in all experimental units at 120 DPH and at the end of the experiment. This

allowed confirmation of the mortality assessment made on a daily basis and determination of potential errors due to the pronounced cannibalistic behaviour of the species (missing fish). Only 0.75 to 1.5% of the fish were missing in each tank at 120 DPH and 0 to 0.5% at the end of the experiment. Unfortunately, no distinction between mortality and cannibalism could be made as daily inspection of the tanks allowed not onlyremoval of intact dead bodies but also of remaining body parts (even cranial bone).

Weight and length data over the 120-330 DPH period used to determine condition factor (K), feed conversion efficiency (FCE%) and specific growth rate (SGR), using the following formulae:

$$K = 100 \text{ X } (W/L^3)$$

where, W = wet body weight (g) and L=body length (cm).

$$FCE\ (\%) = 100 \text{ X [wet weight gain (g)/dry feed intake (g)]}$$

$$SGR = 100 \text{ X } (LnW_2 - LnW_1) / \text{time (days)}$$

where, W1 and W2 indicate the initial and final weight (g), respectively.

2.2.3 Effects of photoperiodic regimes on broodstock fecundity, egg diameter and catfish larvae hatching rate.

The aim of the experiment was to check the quality of eggs originating from broodstock held under different photoperiods. To do so, eggs were stripped from female broodstock produced in experiment 2.2.2. A hatching system with closed water recirculation (27±1°C) was built with 12 plastic tanks (28 x 18 x 6cm, 3L). Due to the limited number of tanks in the hatchery system, only three broodstock fish from 4of the photoperiodic treatments were used and then the trial was repeated according to the same procedure. In each run, three adult females from each of the following treatments,

12L:12D, LL, 6:6 and 6:18, were weighted and measured. Fish were anaesthetized and induced to ovulate as previously described.

Each fish was stripped individually into a petridish, and the total egg weight was measured. Seven samples of 1g each were taken from each egg batch to assess hatching rates (in triplicates), determine fecundities (in triplicate) and measure egg diameter (single). Fertilisation was performed in the first three petridishes with 1ml of milt obtained from dissected testes of 4 male broodstock (743±42g) sacrificed from stock fish in the facilities. After mixing the eggs and milt for about 1min, 4ml of water was gently added. Fertilised eggs were then spread onto 0.5mm small plastic mesh and each egg group was incubated individually in a tank under a 12L:12D light regime. Following hatching (1-3 days post-fertilisation), larvae were killed by a lethal dose of benzocaine and counted to determine hatching rates. Fecundity was determined by counting eggs in the next three samples taken from each egg batch. Finally, egg diameter was assessed in the last sample by measuring 30 oocytes/egg batch. Image analysing software (ImageProPlus, Media Cybernetics Inc., MD, USA) was used to measure the long and short axis of 30 leading oocytes / replicate / treatment / sample point (mean values are presented).

Relative fecundity was calculated using the following equation:

RF= TF /W

Where: RF : Relative fecundity (Eggs/g)

TF: Total fecundity (number of the fresh eggs)

W: Female body weight (g)

2.5 Statistical analyses

Statistical analyses were carried out using SPSS program for Windows (v. 15.0) and Minitab (v. 16.0). Data concerning males and females were analyzed separately. Feed

conversion efficiency (FCE) and specific growth rate (SGR), hatching rate, relative fecundity and egg diameter data were compared by one-way variance analysis. Data with time series were analysed by General Linear Model (GLM) (multivariate) and Tukey's test of multiple comparison. The minimum level of significance was set at P = 0.05. All statistical data was expressed as mean ± SE. Before analyzing the data, normality was cheeked through the Probability-Probability (P-P) Plots test and Bartlett's test was used to test for homogeneity of variances.

3. RESULTS

3.1. Effects of photoperiod (12L:12D and LL) on egg development and growth performances (up to 90 days post hatch, DPH) in juvenile catfish

No significant differences between the 12:12 (control) and LL treatments were observed for weight and length (Fig. 1A, B) except at 30 DPH where LL fish were significantly larger, resulting in a significantly lower condition factor (Fig. 2B) at the same stage. Between 60-90 DPH, significant increases in weight and length were observed in both treatments. No significant differences in cumulative mortality were recorded during this period of time between treatments (33-38 % of the initial population, Fig. 2B).

3.2 Effects of photoperiodic regimes on growth and first reproductive cycle in adult catfish

3.2.1 Growth parameters

- Weight

In female catfish, no significant differences between treatments were seen from 120 to 240 DPH at which point 12:12 fish were significantly heavier than fish under LL (Fig. 3A). This difference remained until the end of the trial with fish under 12:12 being

approximately 25% larger than LL fish at 330 DPH. At 270 DPH, female fish under 12-LL also became significantly smaller than control (12:12) fish, but this difference was recovered by 300 DPH. Interestingly, all treatments were significantly different from the LL treatments (except the 12-LL) at 300 DPH. In males, as for females, fish under LL were significantly smaller than fish under 12:12 and 12-LL becoming significant from 270 DPH with this difference remaining until the end of the trial (Fig. 2B). Male fish weight under LL-12 was significantly higher than fish under LL and 6:18 at 240 DPH. Fish under LL became significantly smaller than fish exposed to all other treatments at 270 DPH. At the end of the trial, both LL and 12-LL treatments were of significant lower weight than all other treatments.

Weight gain between 120-330 DPH showed LL<12-LL<6:6<12:12/6:18/LL-12, where the highest and lowest gain was found in the 06:18 (193 ± 6.1g) and LL (96 ± 8g) treatments respectively (Table 3).

- Length

Treatment effects were less pronounced for length. The main differences in female fish length were observed from 210 DPH when LL fish become smaller than fish under 6:18 (Figure 4A). This difference remained until the end of the trial. Fish under LL became significantly smaller than control 12:12 fish from 240 DPH onwards. At the end of the trial (330 DPH), LL fish were different from fish exposed to 12:12, 6:18 and LL-12. In males, first significant differences appeared at 240 DPH when LL-12 fish reached significantly greater length than LL and 06:18 fish (Figure 3B). Then, at 270 DPH, fish under 6:6 became significantly shorter than 12:12, 6:18 and LL-12 fish, but that decline was recovered at the next point at 300 DPH where the 12-LL was shorter compared with 06:18 and LL-12 treatments. At the end of the trial, LL-12 fish appeared to be the longest

although at this point they were significantly different to all treatments except the 6:6 fish and the 12-LL fish were the shortest.

- Condition factor (K)

In females, K only showed significant differences between treatments at 120 and 150 DPH with LL-12 being significantly different from all other treatments of 150 DPH (Fig. 5A). Significant increase in K was observed in all treatments from 120 DPH (0.6-0.68) to 150 DPH (0.70-0.78). Therefore K remained steady in all treatments (0.72-0.83). In males, significant differences were also only observed at the two first time points (Fig. 5B). Most treatments did not vary significantly between 120 and 150 DPH except 6:18 which increased (0.62 to 0.77). Most treatments increased up to 180 DPH and then remained steady until 240 DPH, from when they all showed a tendency to decrease (0.6 to 0.65). No significant differences between treatments in specific growth rate (SGR) were observed between 120-330 DPH. However, fish under the LL treatment appeared to have a lower SGR (0.53 ± 0.1) as compared to 6:18 (0.87 ± 0.08) (Table 3).

- Food consumption and feed conversion efficiency (FCE)

Food intake by catfish under the LL treatment was significantly lower than in all other treatments from 150 DPH onwards ranging from 10.7 to 26 g/fish/month throughout the experiment (Fig. 6). Overall in the other treatments, there was an increase in food consumption between 120 and 180 DPH (maximum of 41.8 g/fish/month in catfish exposed to 6:6 treatment at 180 DPH) followed by a reduction between 180 and 270 DPH (minimum of 20.5 g/fish/month in fish exposed to 12:12) and an increase again up to 330 DPH (ranging from 30 to 41.7 g/fish/month among treatments). Other main differences between treatments became apparent from 270 DPH when catfish under 6:6 and LL-12

displayed significantly higher food consumption than fish under 12:12. Also catfish feed consumption under 12-LL was significantly lower than under LL-12, 6:6 and higher than LL. These differences remained at 300 DPH. At the end of the trial, LL exhibited significantly lower feed intake than all other treatments and 6:6 displayed the highest feed intake (significant difference with all other treatments). This was confirmed by the total food consumed between 120-330 DPH ranking 6:6 > LL-12/6:18/ 12:12/12-LL > LL (Table 3). The feed conversion efficiency (FCE) was significantly lower in LL (58.37 %), 12-LL (54.58 %) and 6:6 (56.51 %) as compared to 12:12 (80.68 %) and 6:18 (87.37 %) (Table 3).

3.2.2 Cumulative mortality

Mortality of catfish from 150-330 DPH was overall lower than during the previous period from 60 to 120 DPH (Fig. 7). The first significant differences between treatments were observed at 150 DPH, where 12:12 and LL treatments recorded the highest mortality in contrast with the lowest mortality found in 6:18, 12-LL and LL-12 treatments. All treatments showed an increased mortality from 150 to 210 DPH. Subsequently mortalities stabilized in all treatments until the end of the trial. At that time, the 12:12 and LL treatments showed the highest cumulative mortality (11.3%), followed by the 6:6 treatment (9.2%) and the lowest groups were 6:18, 12-LL and LL-12 treatments (circa 6%). The cause of mortality could not be determined due to the strong cannibalistic behaviour observed in African catfish.

3.2.3 Sexual maturation

- Gonadosomatic index (GSI)

GSI in female catfish increased from 150 DPH (<5%) to 360 DPH (12 to 20%) in all treatments (Fig. 8A). Large variations were observed within treatments probably due to heterogeneity of size at each sampling point. No significant differences were observed up to 240 DPH when fish under 12:12 and LL showed significantly higher GSI (circa 10%) than fish under 6:6 and 6:18 (circa 5%). At 270 DPH, the treatments split into two groups, with both 12:12 and LL-12 treatment showing significantly higher GSI (circa 12% average) than all other groups (circa 7-8%) except 12-LL. No other differences were observed throughout the duration of the trial with a trend towards higher GSI in LL and 6:6 treatments. Furthermore, GSI profiles between treatments appear to differ between treatments with both 12:12 and LL-12 peaking at 270 DPH and remaining thereafter more or less steady whereas all other treatments (LL, 6:6. 6:18 and 12-LL) peaked at 360 DPH.

GSI in males showed different profiles than in females with levels remaining below 1% until 300 DPH (Fig. 8B). No significant differences were observed during this period although fish under 12-LL appeared to display slightly higher GSI at 180 DPH as well as fish under 12:12, 6:6 and LL-12 (circa 0.8 %) at 240 DPH in comparison to LL, 12-LL and 6:18. Similarly, male catfish under LL and 6:6 appear to have increased GSI at 300 DPH as compared to all other treatment but no significant differences could be seen due to high intra-population variations. The only significant differences between treatments were observed at 330 DPH where GSI in fish exposed to 12:12 (P<0.026) and LL (P<0.015) was significantly lower than in fish under 6:6, 6:18 and 12-LL. Furthermore, fish under 12-LL had significantly higher (P<0.023) GSI than fish under 6:6 and 6:18. Overall, GSI values appear to peak at 300 DPH in LL and 6:6 fish (1.3-1.4%) and 330 DPH for 12-LL

(1.9 %), LL-12 (1.4%) and 6:18 (1.1%). However, GSI in fish exposed to the control 12:12 photoperiod remain relatively steady (0.7-0.8%) from 180 to 360 DPH.

- Steroids

Plasma steroid levels of testosterone (T) and estradiol-17β (E2) in females were above the sensitivity threshold throughout the trial and the photoperiod regimes appeared to influence patterns of steroid release (Fig. 9). Plasma testosterone levels peaked in all treatments at 330-360 DPH with the main increase between 300-330 DPH (from 4 to 14 ng/ml) in 12:12, LL, 6:6 and 12-LL treatments (Fig. 9). However, levels remained lower in both 6:18 and LL-12 treatments (peak of approximately 7 ng/ml). Significant differences were observed between LL and all other treatments (except 12:12) at 240 DPH and 12:12 vs all other treatments (except LL) at 270 DPH (Fig.10A). As for estradiol, levels were much lower than for T, ranging from 1 to 8 ng/ml (Fig. 9, 10B). From 150-210 DPH, plasma E_2 concentrations were basal and no significant different between treatments and sampling points were found (Fig 10B). During this period, female GSI already increased significantly in most treatments (early vitellogenesis, Fig. 9). At 240 DPH, significantly increased plasma E2 levels were observed in 12:12 and LL treatments (Fig 10B). At 360 DPH, 06:18 was significantly lower than 12:12, LL and LL-12. The first peak of plasma E_2 was attained at 240 DPH for 12:12 (4.4 ng/ml) and LL (4.6 ng/ml) treatments and remained constant until 300 DPH whereas in the 6:6, 6:18 and 12-LL, plasma E_2 peaked at 330 DPH and remained constant thereafter (Fig. 9 and 10B).

In males, T levels were detected in the plasma from the start of the trial (150 DPH). In all treatments plasma levels peaked towards the end of the trial (330-360DPH) with mean values ranging from 2.5 to 29.2 ng/ml depending on the treatments (Fig. 11). Although fish under the LL treatment showed a steady elevation in T levels throughout the

trial, all other treatments displayed rapid increases at some point during the trial; between 300-330 DPH for most treatments (except 6:6 and 12-LL) where the increase was more pronounced from 270 DPH (Fig. 11, 12). No significant differences in plasma T levels between treatments at each sampling point were observed (Fig. 12).

- Gonadal development

Gonadal staging for both sexes was assessed from 150-360 DPH and same differences were observed between treatments. From 150 to 180 DPH, all females sacrificed from all treatments were at a pre-vitellogenic stage (S1) (Fig. 13). Signs of early vitellogenic stage (S2) first appeared at 210 DPH in all treatments with, however, different percentages between treatments (from 30% in 6:6 to 80% in LL-12). At 210 DPH, the first female at a late vitellogenic stage (S3) was found in the LL-12 treatment. Thereafter, at 240 DPH, except for 6:6 and 6:18 treatments, females in all other treatments reached late vitellogenesis (S3) with the highest occurrence in the LL treatment (60%). In the 6:6 and 6:18 treatments, females were still in stage 1 (40-50%) and 2 (50-60%). At 270 DPH, some females sacrificed in all treatments reached the mature stage (S4, 20-66% of females sampled depending on treatment) except in treatment 6:18 where females were still in stage 2 and 3. First sign of atresia was observed at 300 DPH in most treatments (except again in 6:6 and 6:18 in which atresia was only observed from 330 DPH).

In males, most individuals sampled at 150 DPH were in stage 1 and 2 (>70%) except in LL treatment where stage 3 males were already found (Fig. 14). At the next sampling point (180 DPH), all treatments showed stage 2 and 3 males except 6:18 in which males were at a less advanced stage of development (stage 1 and 2). First sign of ripening (S4) and ripe (S5) stages were observed, respectively, in treatments 12:12 and LL at 210 DPH and LL treatment at 270 DPH. By 300 DPH, stage 5 males were still only found in

treatments 12:12 (50%) and LL (80%). From 330 DPH, males in all treatment were at stage 4 or 5 with 60-70% in stage 5 at 360 DPH.

3.3 Effects of photoperiodic regimes on broodstock fecundity, egg diameter and catfish larvae hatching rate.

Six fish from 12:12, LL, 06:18 and 06:06 treatments were stripped for hatching rate, eggs diameter and relative fecundity. The relative fecundity ranged from 88 to 105 eggs/g of BW with no significant differences between treatments (Table 4) (P>0.05). Egg diameter of stripped eggs from fish exposed to LL was significantly larger than eggs from fish reared under 12:12, 6:18 and 6:6. Finally, no significant differences were found in hatching rate between treatments although values ranged from 35% in eggs from 6:6 broodstock to 44.4% in eggs from 6:18 fish (Table 4).

4. DISCUSION

Photoperiod manipulations are commonly used in the culture of temperate fish species with significant beneficial effects such as enhancement of growth performances, suppression of early maturation and spawning manipulation, allowing year round production in several annual and temperate species. Results from the present study showed that photoperiod had an impact on food consumed and growth performances but no major effects on sexual maturation in African catfish.

4.1 Effect of photoperiod on growth and survival of African catfish

Previous studies performed on the effect of photoperiod on growth performances of *C. gariepinus* all concluded that growth can be affected by daylength with fish performing better under short-day photoperiods (Britz and Pienaar, 1992; Almazan, 2004; Appelbaum

and Mcgeer, 1998; Appelbaum and Kamler, 2000; Adewolu, 2008). Similar results were reported in other catfish species like Silver catfish, *Rhamdia quelen* (Piaia *et al*., 1999). In the present study we studied the effects of photoperiod at two key stages, first during the initial 90 DPH (juvenile) and then during the adult stage (up to first maturation, 360 DPH). The growth performances during the initial period from hatching to 90 DPH did not differ significantly between the 12:12 and LL treatments except for the length and K at 30 DPH. These differences between our results and previous studies may be attributed to the different experimental designs or the genetic variation (Kinghorn, 1983; Thodesen *et al*., 2001). Mortality was very high (approximately 40%), with no significant differences between treatments at the initial period. Such high mortality at the early stages in the species has already been reported (Hung *et al*., 2002; Subagja *et al*., 1999). Acute cannibalism was reported in most catfish species during the larval stage. Hecht and Appelbaum (1987) demonstrated that cannibalism in *C. gariepinus* contributed the most to larval mortality. This may be related to the feed quality and the digestibility or to the primary development of the digestive system at first feeding.

However, during the later phase (from 120 to 330 DPH), clear effects were observed between populations exposed to the different light treatments, confirming previous reports, with especially a significantly lower growth in the LL treatment in comparison to the 12:12 treatment. Interestingly, fish reared under 12:12 until 120 DPH and then LL also show significantly reduced growth compared to fish under all other treatments at 330 DPH, such differences appearing from 300 DPH. This could suggest that photoperiod effects are life stage specific as no significant differences between 12:12 and LL could be seen prior to 240 DPH for length and 270 DPH for weight as already suggested for striped knifejaw, *Oplegnathus fasciatus* (Biswas *et al*., 2008). Furthermore, fish exposed to the opposite photoperiodic treatment (LL-12) was of similar size to control fish (12:12) suggesting that

early exposure to LL did not compromise their growth potential later on during the experiment. However, when it comes to short day photoperiod (6:18), results are not as clear and fish did not grow significantly better under this regime than 12:12 control or even 6:6 treatments although they were significantly longer than fish reared under LL. Condition factor data in this study showed no significant differences except at 120-150 DPH with 6:18 and LL-12 having lower K at 120 DPH. These results contrast with those of Almazan *et al*., (2005) who recommended use of a short light period during a 24h cycle (L6:18D) to achieve best growth rate in *C. gariepinus*. However contrasting findings were reported in channel catfish where no significant differences in growth were observed between control photoperiod (12D:12L) and a range of other photoperiodic treatments tested (from continuous darkness to continuous light including intermediary treatments with daylength of 6, 12 or 18 hrs) (Stickney & Andrews, 1971).

Enhanced growth rate can usually be explained by either increased food consumption or better feed conversion efficiency. In the present study, reduced food consumption was observed in fish reared under LL as compared to most of the other treatments (12:12, LL-12 and 12-LL) from 150 DPH till the end of the trial. This lower growth of catfish exposed to continuous photoperiod was accompanied not only by poor food intake but also by significantly lower FCE compared with 12:12 and 6:18 treatments. The lower food consumption may be due to nocturnal behaviour of catfish that are more active under long or continuous photoperiod, or be related to hormonal stimulation of appetite under a long or continuous photoperiod.

Similar findings were reported by Kilambi *el al*. (1970) in the channel catfish with higher food consumption in fish reared under a 10hrs daylength than 14 hrs. These authors thus concluded that there is a direct relationship between daylength and feed intake. Food consumption in the other treatments was similar although there was a trend for increased

feeding in fish exposed to the 6:6 treatment (significantly different to all other treatments at 330 DPH, especially to 12:12 treatment). However, surprisingly, these fish did not grow better than fish exposed to the 12:12 treatment. This may be explained by lower feed conversion efficiency (FCE) for the 6:6 treatment due to stress induced by repetitive day/night switches which could reduce the digestive and absorptive performance (Biswas, *et al*, 2008). This remains to be confirmed.

Many studies have been performed to test the effects of photoperiod on growth in a range of commercially important teleosts usually displaying diurnal activity. In Atlantic salmon (*Salmo solar*) and rainbow trout (*Onchorhynchs mykiss*), enhanced growth was observed when fish were exposed to extended daylength (Saunders *et al*., 1985; Berg *et al*., 1992; Taylor *et al*., 2005, 2006, 2009). Similar results were reported in Atlantic cod *Gadus morhua* (Folkvord and Ottera, 1993). However, in Artic charr (*Salvelinus alpinus*), another salmonid species, fish were shown to feed and grow well even in complete darkness (Jorgensen and Jobling, 1989). Furthermore, Atlantic halibut, *Hippoglossus hippoglossus*, growth appeared to be similar in fish reared under ambient photoperiod and LL (Hallaraker *et al*., 1995) although growth enhancement in juveniles exposed to LL has been reported (Jonassen *et al*., 2000; Simensen *et al*., 2000). Fuchs (1978) found no significant effect of photoperiod manipulation on the growth of sole, *Solea solea*, whereas an extended photoperiod was subsequently reported to have growth-enhancing effects (Fonds, 1979). Finally, Biswas and Takeuchi (2003) suggested that short photocycle (6L:6D) could be used to enhance the growth rate of Nile tilapia *Oreochromis niloticus.* Overall, photoperiod mediated growth effects in fish are clearly species specific depending on the daily activity rhythms (diurnal/nocturnal) and probably the light sensitivity which has been shown to differ greatly between species (Migaud *et al*., 2008) However, there are also large differences observed between studies performed in a given species which are likely to be

the result of different experimental conditions (feeding strategy, stocking densities, temperature profiles, tank rearing facilities and others) and the use of different stocks or strains. Importantly, in a species like African catfish, these factors can induce acute or even chronic stress responses, can increase aggressive behaviour and ultimately influence growth performances (Almazan *et al*., 2005). Lighting conditions are probably one of the most difficult environmental factors to control and recreate between studies as it is characterised by daylength, light intensities, spectral content and daily variations (e.g. dawn and dusk) (Boeuf et le Bail, 1999). Differences in light conditions could therefore explain differential results in fish performances and behaviour reported in light trials. Of interest, Han *et al.* (2005) showed reduced growth in Chinese longsnout catfish, *Leiocassis longirostris,* (4.8 ± 0.01 g) when exposed to low (5 lux) or high (443 lux) light intensities, where the specific growth rate (SGR) and feed conversion efficiency (FCE) were higher on the medium light density (74 lux). However, some species can grow better at low light intensity, such as striped bass larvae at 1 lux (Chesney, 1989), juvenile halibut at 1–10 lux (Hole and Pittman, 1995), juvenile haddock at 30 lx (Trippel and Neil, 2003). On the other hand, some species were reported to show improved growth at very intense light levels, sea bass larvae at 1400–3500 lux (Barahona–Fernandes, 1979), Atlantic cod larvae at 2400 lx (Puvanendran and Brown, 2002), and black porgy juvenile at 3000 lux (Kiyono and Hirano, 1981). It seems that the effect of light intensity on growth and survival are species-specific (Puvanendran and Brown, 2002). On the other hand, study of the growth performance in striped knifejaw, *Oplegnathus fasciatus* (body weight 100– 300 g) reared under four photoperiods (6L:6D, 12L:12D, 16L:8D and 24L:0D), showed a significant different of photoperiod with higher weight gain, SGR, and FCE in fish under 12L:12D than fish exposed to 6L:6D, 16L:8D and LL treatments (Biswas, *et al*., 2008).

4.2 Effect of photoperiod on mortality

Mortalities were high for the first three months post hatching for all treatments then gradually decreased. High cannibalism has been reported in *C. gariepinus* at the larvae and early juvenile stages (Hecht and Appelbaum, 1988). Due to cannibalism, mortalities can be observed from the age of 8 days post-fertilization or 4 days after the onset of external feeding (Appelbaum and Kamler, 2000), and can start to decrease 47 days after the onset of external feeding. Unfortunately, due to the method of mortality assessment, cannibalism could not be determined in the current trial. Two types of predator- prey relationship have been described: prey being caught tail first and swallowed up to the head, which is subsequently bitten off, (cannibalism type I), changing to swallowing of prey head first and whole (cannibalism type II) (Haylor, 1992). Due to the fact that tanks were checked three times a day during feeding, missing fish, corresponding to fish eaten by siblings in the tanks, during the study period were ≤ 1.5% and ≤ 0.5% for all treatments from 30-120 and 120 -330DPH, respectively. Previous studies have shown that cannibalism can contribute up to 70–83% of the total mortality accumulated during the first 46–50 days of *C. gariepinus* rearing (Hecht and Appelbaum, 1987; Appelbaum and Van Damme, 1988). However, several studies showed that cannibalistic behaviour is intensified by increasing size heterogeneity at these stages (Hseu, 2002; Smith and Reay, 1991). Size variation is caused by genetic differences which dictate individual growth rate (Celestin *at el*., 2008). Size variation is also a primary cause of agonistic behaviour, which in turn can have the same end effect as cannibalism. In addition, catfish is a carnivorous species which may prefer to feed on conspecifics rather than artificial feed thus the importance of satiation feeding. During the larvae and early juvenile stages, fish that are reluctant to accept the feed can become weaker which can be the target for larger cannibalistic fish (Baras and Almeida, 2001).

In the present study, from 150 DPH throughout the duration of the trial the highest cumulative mortality was observed in 12:12 and LL (7.3-10.5), against the lowest cumulative mortality for 06:18, 12-LL and LL-12 (4.4-6.5 %.) Appelbaum and Kamler (2000) suggested that survival of *C. gariepinus* juveniles was found to be suppressed by light. In contrast, Britz and Pienaar (1992) did not detect any differences in survival rate of *C. gariepinus* larvae reared in continuous dark or light and authors concluded that stress, aggression and cannibalism are reduced under dark conditions. From 150 DPH the mortality was stabilized until the end of the trial.

4.3 Effects of photoperiod on puberty

In the present study, no major effects of photoperiod were observed on puberty and gonadal development as in all treatments, fish matured and reached the later stages of gametogenesis from 270-300 DPH (stage 4, mature) for females and 270-330 DPH for males (stage 5, ripe). However, although none of the photoperiod treatments significantly suppressed maturation, the timing of key gametogenesis stages appeared to be influenced by photoperiod. Despite those effects, in both sexes significant differences in GSI were only found between treatments at 240 and 270 DPH in females and 330 in males. Interestingly in the present study, males under LL treatment at a ripe stage (5) were observed from 270 DPH. In contrast, males under 6:6, 6:18 and 12-LL treatments were delayed and reaching stage 5 from 330 DPH. Delay in females could also be seen in treatments 6:6 and 6:18 in which first sign of atresia were only detected at respectively 330 and 360 DPH as compared to all the other treatments (300 DPH).

From previous studies performed in Indian catfish, it has been reported that both photoperiod and temperature affect gonadal activity, but apparently, temperature is the more important factor (Vasal and Sundararaj, 1976; Sundararaj and Vasal, 1976). In

Chinese catfish *Clarias fuscus*, Young (1988) reported that females which developed mature ovaries were induced successfully under constant or long photoperiod at 25°C but at 30°C, regardless of the photoperiod, all female ovaries were fully matured. Moreover, maturation was delayed at 20 and 25°C under short photoperiod. Similar results were reported in Indian catfish (Vasal and Sundararaj, 1976). The delay in reaching the final maturational stages in these treatments may be due to the depressed levels of T and E_2 suppressed until 300 DPH. In females, E_2 plays a dominant role during vitellogenesis (Kagawa *et al*, 1982; Ijiri *et al*., 1995). In contrast, T is mainly involved in spermatogenesis and especially in spermatogonia multiplication and spermatocyte differentiation. In the present study, significant differences between treatments were only observed at 240, 270 (for both steroids) and 360 DPH (only for estradiol). Overall, plasma levels of both T (in both sexes) and E_2 (only in females) increased during the period of study to reach peak levels at 330 DPH. This is in agreement with histological observations. Interestingly, levels of E_2 and T at 240 DPH appeared to be reduced in the 06:06 in which ovarian development (S2) was shown to be delayed compared with the 12:12 treatment that achieved the late vitellogenic oocyte stage (S3). In males, 11-ketotestosterone (11-KT) is considered to be the main androgenic hormone and therefore the key steroid analysed in reproductive studies (Borg, 1994). However, due to technical problems with the 11-KT assay, no data could be provided in the present research programme. Future studies should investigate this hormone in catfish to explain potential photoperiodic effects on male spermatogenesis.

However, similar studies revealed that long photoperiod stimulated early puberty and maturation. Singh (1998) found that exposure of catfish *C. batrachus* to long photperiod (14L:10D) during the early preparatory phase can stimulate early development of the testis by activating the pituitary-gonadal axis. Guraya *et al.* (1976) found the ovarian

development in catfish, *Mystus tengara* was greater in fish treated with long photoperiod (14L:10D) than in controls (12L:12D), and the prespawning period was reduced considerably in fish treated with long photoperiod. Furthermore, during spawning and post-spawning periods, the ovaries are refractory, since long photoperiod is ineffective in maintaining gravid ovaries beyond the spawning period and ovarian regression during post the spawning period occurs regardless of the nature of the photoperiod treatment. On the other hand, Sundararaj and Sehgal (1970) pointed out that both long (14hr photoperiod / day) and gradually increasing photoperiod (11 or 12 to 14hr/day) are equally effective in accelerating ovarian recrudescence during the preparatory period in catfish (*H. fossillis*).

In tropical species such as European sea bass, gilthead seabream and Atlantic cod, long or continuous photoperiods have been shown to delay or even fully suppress sexual maturation and spawning, and enhance growth (Karlsen *et al*., 1999; Kissil *et al*., 2001; Davie *et al*., 2007b; Davie *et al*, 2008). In contrast, short days (6L:18D) can delay spawning of rainbow trout (Bromage *et al*., 1984). In salmonids, it has been reported that ovarian development and early vitellogenesis can be induced by a long or increasing daylength, whereas short or decreasing daylength would control the maturation and ovulation in those fish (Whitehead and Bromage, 1980).

4.4 Effect of photoperiod on reproductive performances and egg quality

No significant differences between treatments were found in hatching rate although a trend towards a lower mean hatching rate was apparent in the 6:6 treatment (35±7%) as compared to the 12:12, LL and 06:18 treatments (41±13, 43±6 and 44±7%, respectively). In a previous study performed in two catfish species (*C. gariepinus* and *C. anguillaris*), no effects of photoperiod (constant darkness/12L:12D) were found on the percentage of hatching in both species. However, illumination had a considerable influence on the

development and hatching time, where the eggs in the two species exposed to continuous light, hatched earlier (14-24hr) than those exposed to constant darkness (24-32hr) in both species (Inyang and Hetiarchchi, 1995). In another study on *C. gariepinus* and Asian catfish, *C. macrocephalus,* authors have shown that a short period of light (L6:18D) can significantly increase hatching rate and exposure of eggs to continuous light can advance or speed up the hatching process (Mino *et al*., 2008). In the current study, no significant difference in relative fecundity was found between treatments, whereas under the LL treatment, a higher mean fecundity was apparent (105.58±11 eggs/g of body weight) as compared to the 12:12, 6:18 and 6:6 treatments (88.34±6, 94.57±14 and 99.93±9 eggs/g of body weight, respectively). In contrast, egg diameter from fish maintained under LL was significantly larger (1.61±0.01mm) than from fish exposed to 12:12, 6:18 and 6:6 (1.58±0.01, 1.59±0.01 and 1.57±0.01 mm, respectively).

In Chinese catfish *Clarias fuscus,* Young (1988) showed that temperature had the strongest effect on ovarian maturation in terms of oocyte growth and fecundity. Interestingly in the present study the smaller egg diameter was observed in egg batches from fish exposed to the 6:6 treatment as compared to the LL treatment. The significantly smaller egg diameter in fish exposed to the 6L:6D photoperiod may be due to the lower E2 levels, which in turn reduce vitellogenin production.

4.5 Conclusions

The present results confirmed previous findings reported in other catfish species on the effects of photoperiod on growth of African catfish. Indeed, constant light reduced growth performances probably due to the levels of stress experienced by fish under this treatment (Almazan, 2004). However, photoperiod has no strong impact on *C. gariepinus* gonadal activity. Fish from all treatments reached the final stages of gametogenesis and no

major differences in hatching rate, relative fecundity and egg size were observed. Results suggested that constant light may slightly advance maturation in *C. gariepinus* (up to two months) and short daylength (6:6 and 6:18) could delay it (1-2 months). It must also be acknowledged that no continuous darkness treatment (DD) could be tested in the current study due to UK Home Office animal experimentation regulations. Indeed, long term exposure of fish to DD could compromise fish welfare. However, future studies should address the effects of such treatments in catfish given the strong nocturnal behaviour observed in the species. Many questions with regards to the effects of light on catfish physiology remained unanswered. If constant light negatively affected the growth performances, what are the effect of light intensity and wavelength on stress levels? If photoperiod has no strong impact on *C. gariepinus* gonadal activity, would using different light intensity and wavelength accelerate or delay gametogenesis?

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Table 1. Classification scheme used to identify the stage of oogenesis in female catfish showing stages S1, S2, S3 and S4. Adapted from Coward and Bromage (1998).

Stage	Definition	Appearance
S1	Pre-vitellogenic	Nucleus containing chromatin strands. Developing follicular layer. Vesicle at near edge of oocyte. Stains dark pink reducing to pale pink as less basophilic.
S2	Early stages of vitellogenesis	Small yolk granules starting at periphery. Vesicles seen throughout oocyte. Follicular layer can be seen to be more developed.
S3	Late stages of vitellogenesis	Yolk granules become larger yolk globules and empty vacuoles throughout oocyte. Very developed follicular wall. Nucleus central.
S4	Mature	Same as S3 but vesicle migration can be seen.

Table 2. Classification scheme used to identify stage of spermatogenesis in male catfish showing S1, S2, S3, S4, S5. Adapted from Babiker and Ibrahim (1979).

Stage	Definition	Appearance
S1	Immature	Mostly spermatogonia with some spermatocytes
S2	Maturing	Clusters of spermatocytes and a few spermatids
S3	Mature	Spermatogonia, spermatocytes, spermatids all present and few spermatozoa in middle
S4	Ripening	All stages present with abundant spermatozoa
S5	Ripe	Sperm ducts distended with spermatozoa and seminal fluid.

Table 3. Summary of overall (120-330 DPH) weight gain, food consumed, FCE and SGR Values are expressed as mean ± SEM Superscripts indicate significant differences between treatments.

Treatments	Initial weight (g)	Final weight (g)	weight gain (g)	Food consumed g/fish	FCE %	SGR %
12:12	50±2.8	238±13	188±16a	233.03±33.7a	80.68a	0.75
LL	46±2.7	146±9	96±17 b	163.72±37.3b	58.37b	0.63
06:06	45±2	210±12	165±12c	298.47±56.6c	56.51b	0.74
06:18	37±1.9	230±13	193±19a	247.5±24.6a	87.37a	0.87
12-LL	46±2.5	190±10	144±7d	264.47±1.4a	54.58b	0.68
LL-12	38±1.9	222±13	184±13a	265.05±1.6a	69.53ab	0.84

Table 4. Egg diameter (mm), relative fecundity (egg/g of body weight) and hatching rate (%) assessed in eggs stripped from first time female catfish spawner reared under different photoperiodic regimes (12:12, LL, 6:18, 6:6). Values are expressed as mean ± SE (n=6 females/treatment). Superscripts indicate significant differences between treatments.

Treatments	Eggs diameter (mm)	Relative fecundity (number of eggs/1g body weight)	Hatching rate (%)
12:12	1.58±0.01 [a]	88.34±6	40.87±13
LL	1.61±0.01 [b]	105.58±11	43.10±6
6:18	1.59±0.01 [a]	94.57±14	44.39±7
6:6	1.57±0.01 [a]	99.93±9	35±7

FIGURE LEGENDS

Figure 1. Weight (a) and length (b) of *C. gariepinus* reared under different photoperiodic regimes (12:12, LL) from hatching to 90 days post hatch. Values are exposed as mean ± SE (n=10-20 fish /sampling / treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 2. Condition factor (a) and cumulative mortality (b) of *C. gariepinus* under different light regimes (12:12, LL) from hatching -90 and 60-120 DPH respectively. Values are exposed as mean percentage ± SE (n=10-20 fish /sampling / treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the

Figure 3. Weight of females (a) and males (b) of *C. gariepinus* under different light regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 120-330 DPH. Values are exposed as mean ± SE (n=20/sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 4. Length of females (a) and males (b) of *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 120-330 DPH. Values are exposed as mean ± SE (n=20/sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 5. Condition factor of females (a) and males (b) of *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 120-330 DPH. Values are exposed as mean ± SE (n=20 /sampling / treatment). Superscripts indicate

significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 6. Food consumption of *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 120-330 DPH. Values of food consumption are exposed as mean ± SE (n=2 tanks / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 7. Cumulative mortality of *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 120-330 DPH. Values are exposed as mean percentage for cumulative mortality (n=2 tanks / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 8. Gonadosomatic index (GSI) of females (a) and males (b) *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 150-360 DPH. Values are exposed as mean ± SE (n=4-6 fish / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 9. Related of testosterone, steroidal and of Gonadosomatic index (GSI) on females *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 150-360 DPH. Values are exposed as mean ± SE (n=4-6 fish / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 10. Plasma levels of T (A) and E2 (B) on females *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 150-360 DPH. Values are exposed as mean ± SE (n=4-6fish / sampling/treatment). Superscripts indicate

significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 11. Related of testosterone, steroidal and of Gonadosomatic index (GSI) on males *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 150-360 DPH. Values are exposed as mean ± SE (n=4-6 fish / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

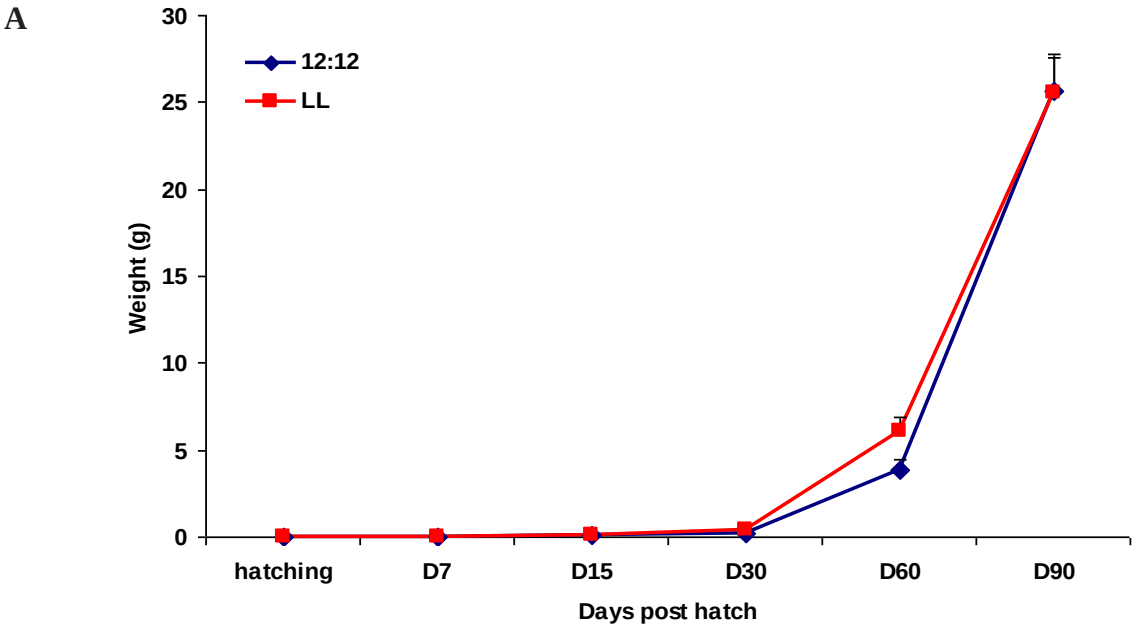
Figure 12. Plasma levels of T) on males *C. gariepinus* under different photoperiodic regimes (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) from 150-360 DPH. Values are exposed as mean ± SE (n=4-6 fish / sampling/treatment). Superscripts indicate significant differences ($p<0.05$) between treatment at each sampling point and they are stacked in the same order as the legend.

Figure 13. Stage of development in female *C. gariepinus* sampled from each photoperiodic treatment (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) throughout the trial (150-360 DPH). Stages of development are explained in Table 1. Numbers above bars indicate the number of female sampled at each time point.

Figure 14. Stage of development in male *C. gariepinus* sampled from each photoperiodic treatment (12:12, LL, 12-LL, LL-12, 6:6 and 6:18) throughout the trial (150-360 DPH). Stages of development are explained in Table 2. Numbers above bars indicate the number of male sampled at each time point.

.

Figure 1


A
30
25
20
15
10
5
0
Weight (g)
12:12
LL
hatching
D7
D15
D30
D60
D90
Days post hatch

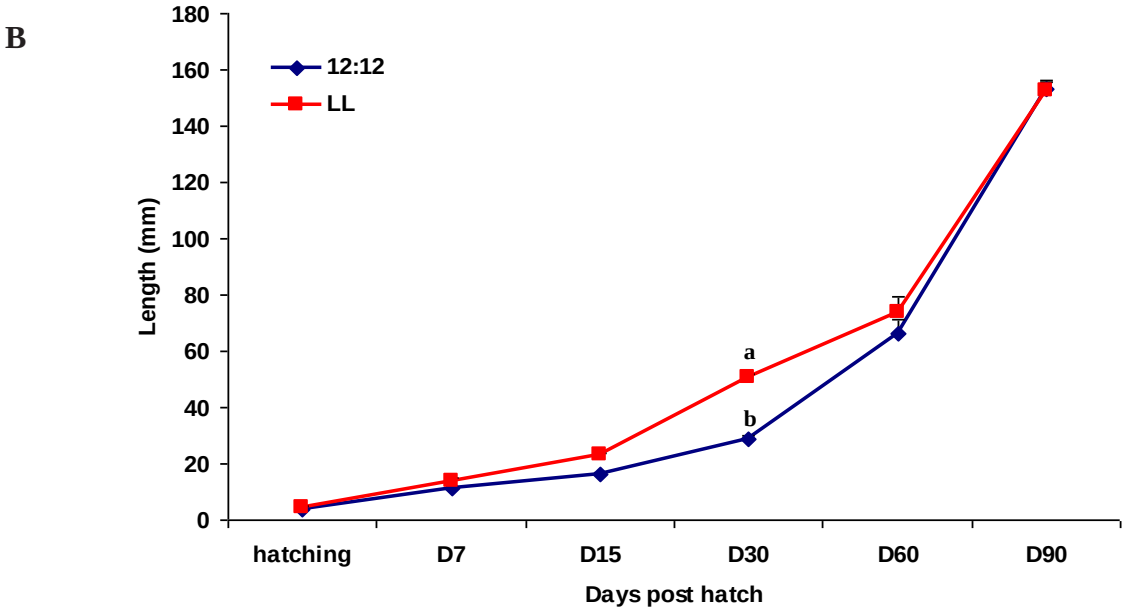
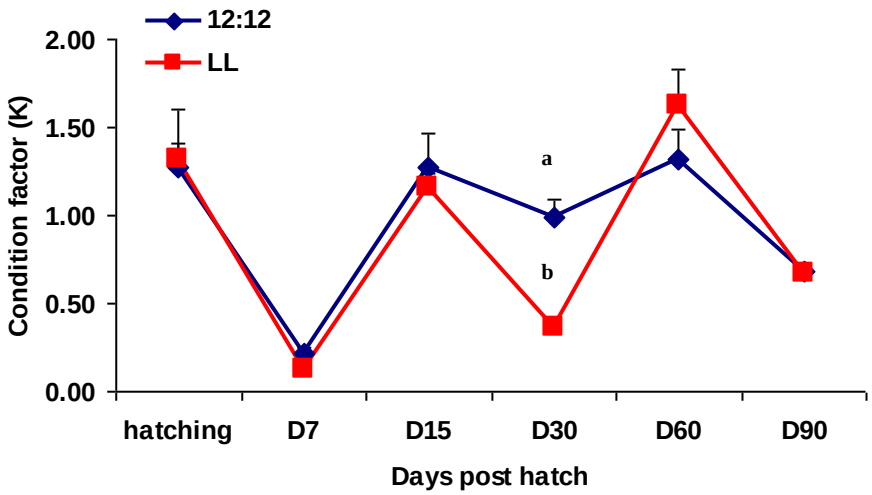

B
180
160
140
120
100
80
60
40
20
0
Length (mm)
12:12
LL
a
b
hatching
D7
D15
D30
D60
D90
Days post hatch

Figure 2

A


12:12
LL
2.00
1.50
1.00
0.50
0.00
Condition factor (K)
a
b
hatching
D7
D15
D30
D60
D90
Days post hatch

B

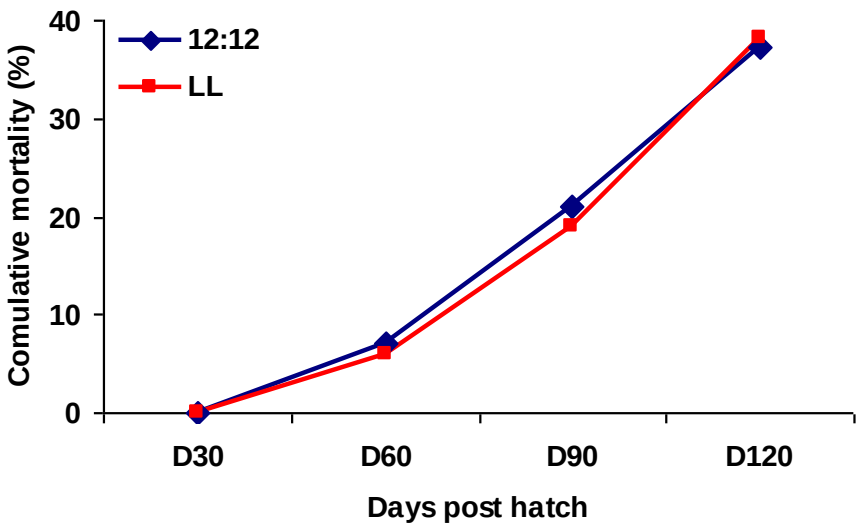
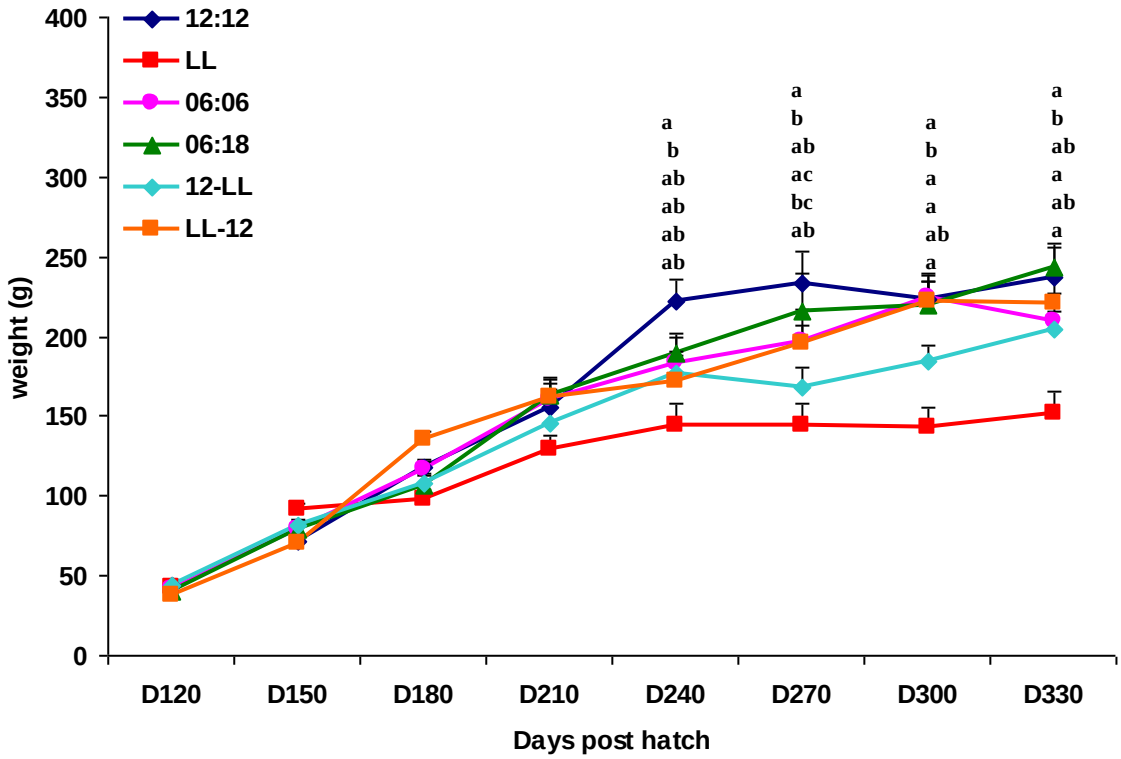

40
30
20
10
0
Comulative mortality (%)
12:12
LL
D30
D60
D90
D120
Days post hatch

Figure 3

A


12:12
LL
06:06
06:18
12-LL
LL-12
weight (g)
400
350
300
250
200
150
100
50
0
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch

B

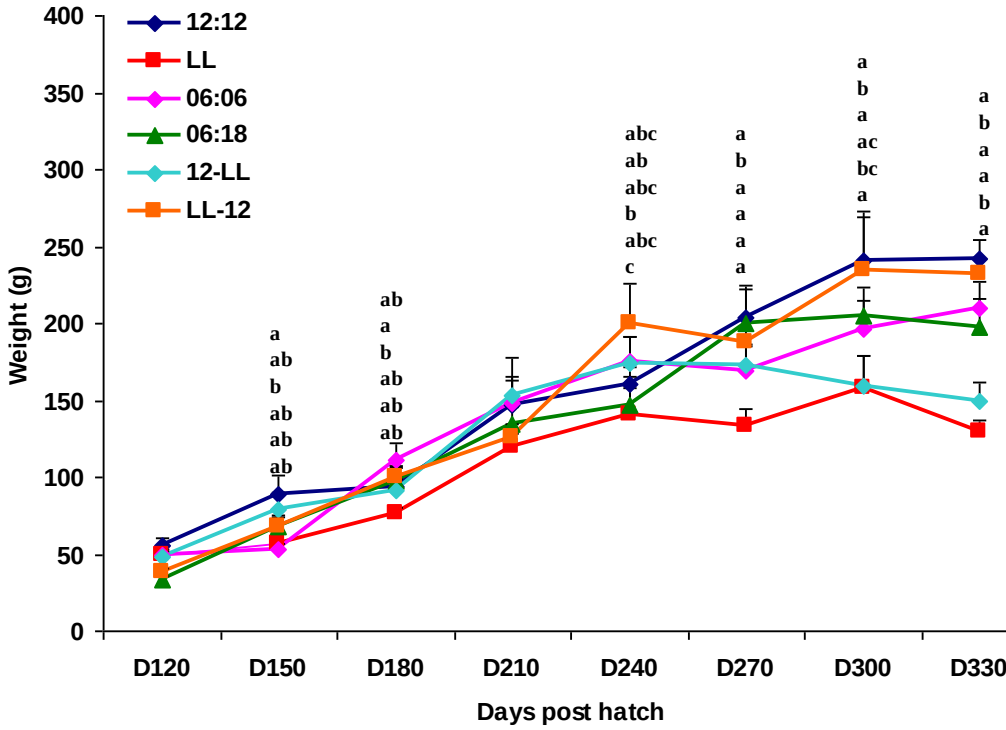
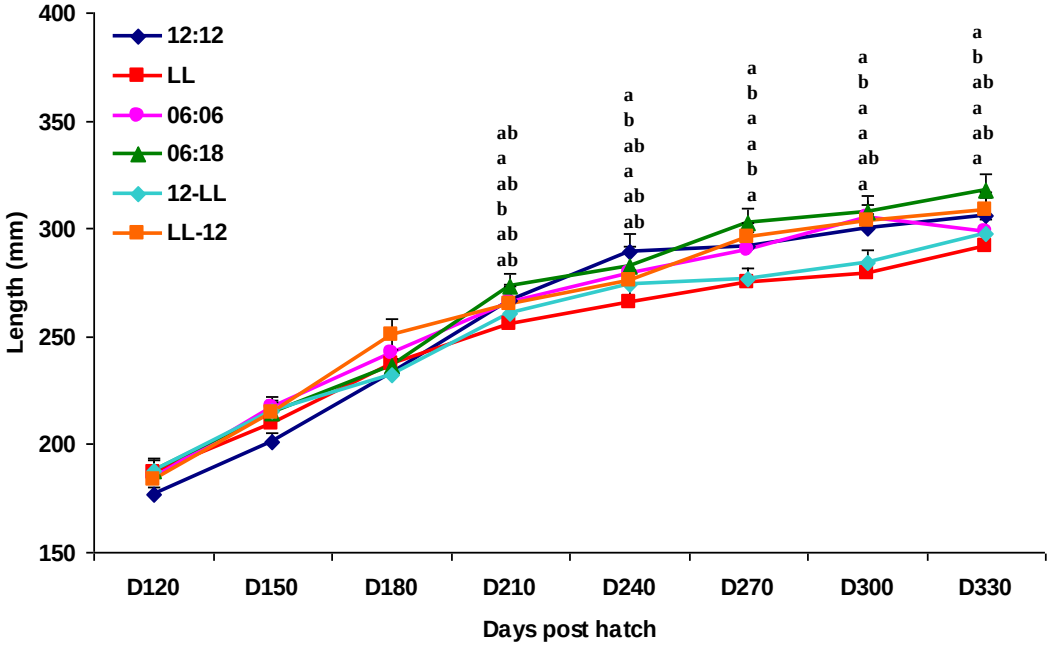

12:12
LL
06:06
06:18
12-LL
LL-12
Weight (g)
400
350
300
250
200
150
100
50
0
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch

Figure 4

A


12:12
LL
06:06
06:18
12-LL
LL-12
Length (mm)
400
350
300
250
200
150
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch

B

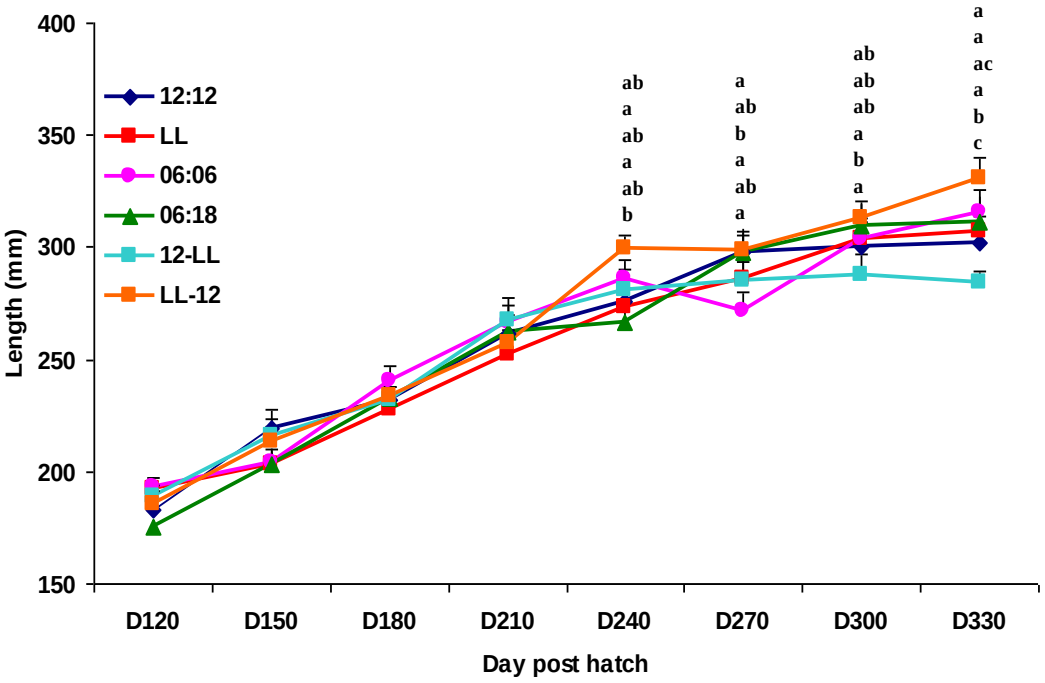
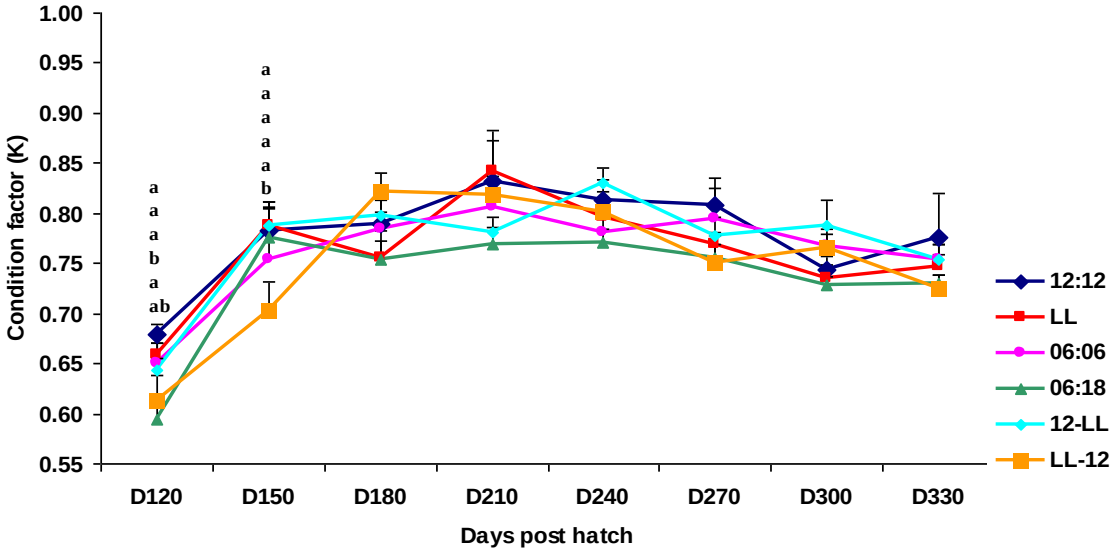

12:12
LL
06:06
06:18
12-LL
LL-12
Length (mm)
400
350
300
250
200
150
D120
D150
D180
D210
D240
D270
D300
D330
Day post hatch

Figure 5

A


1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
Condition factor (K)
a a a b a ab
a a a a a b
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch
12:12
LL
06:06
06:18
12-LL
LL-12

B

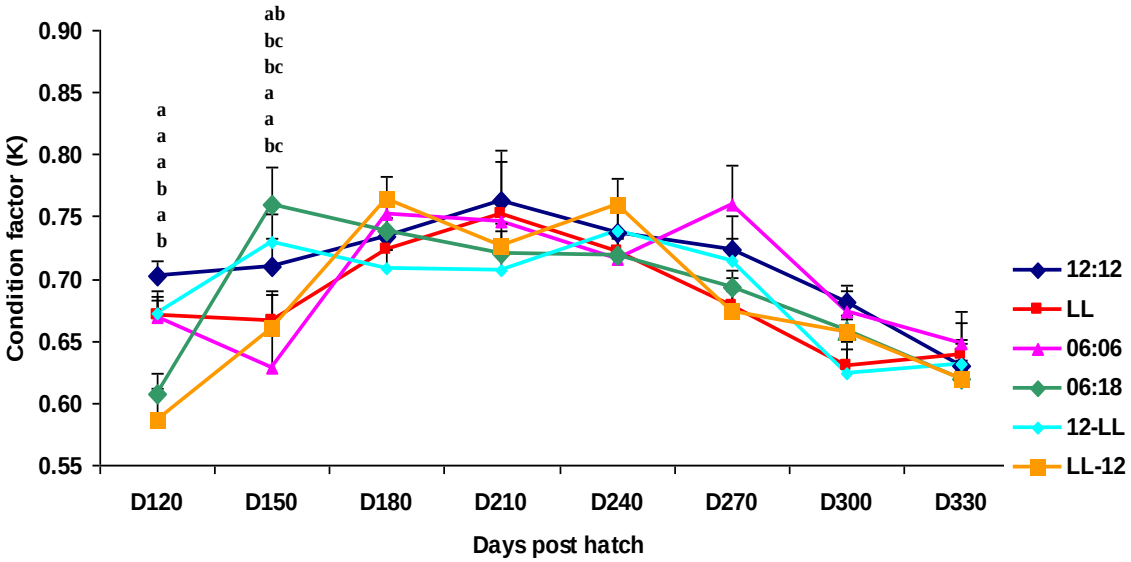

0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
Condition factor (K)
a a a b a b
ab bc bc a a bc
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch
12:12
LL
06:06
06:18
12-LL
LL-12

Figure 6

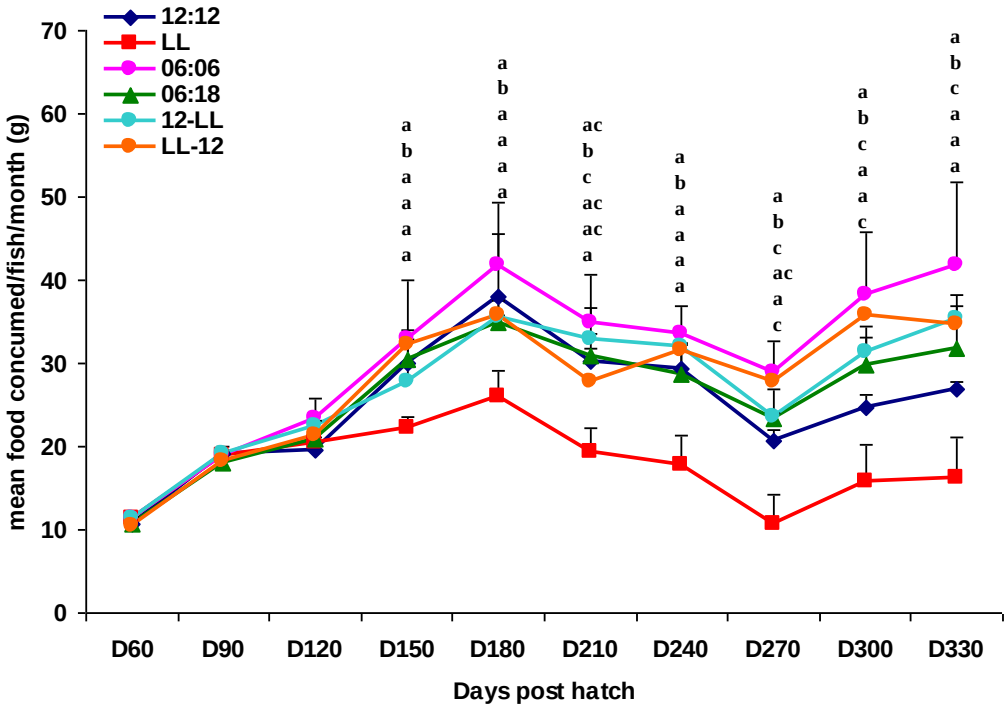

12:12
LL
06:06
06:18
12-LL
LL-12
mean food concumed/fish/month (g)
0
10
20
30
40
50
60
70
D60
D90
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch

Figure 7

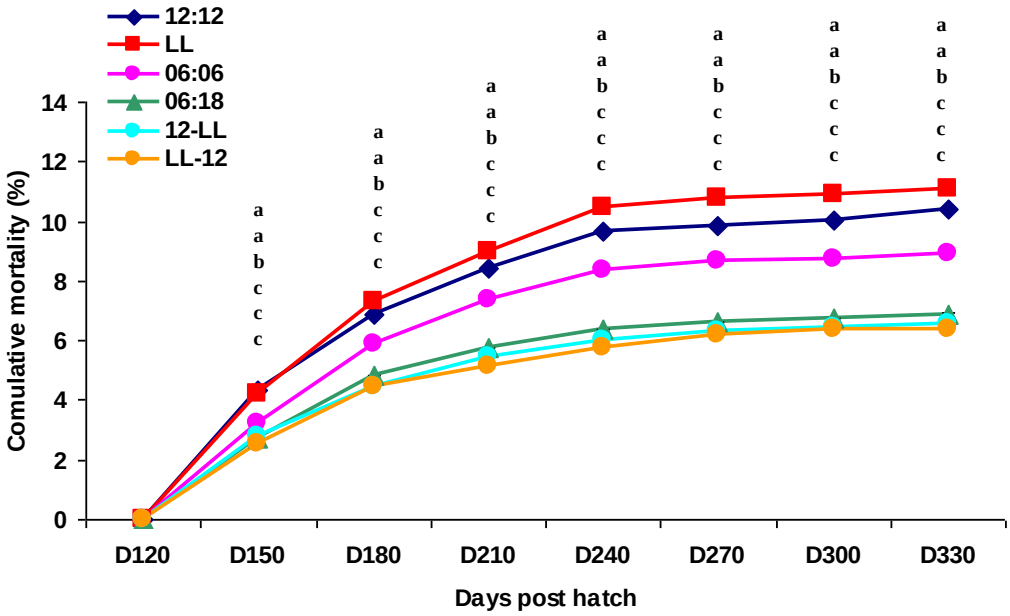

12:12
LL
06:06
06:18
12-LL
LL-12
Comulative mortality (%)
0
2
4
6
8
10
12
14
D120
D150
D180
D210
D240
D270
D300
D330
Days post hatch
a a b c c c
a a b c c c
a a b c c c
a a b c c c
a a b c c c
a a b c c c

Figure 8

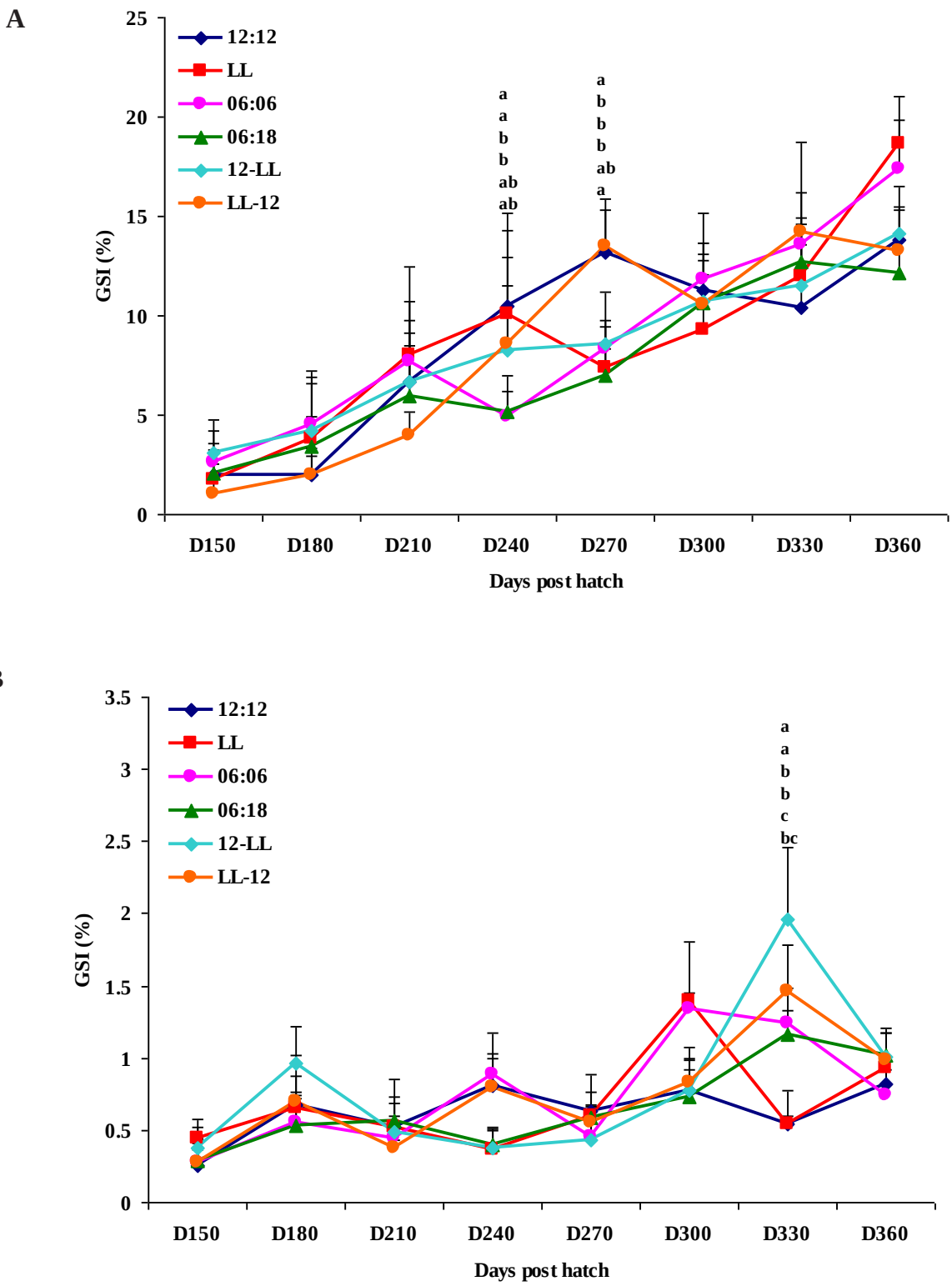
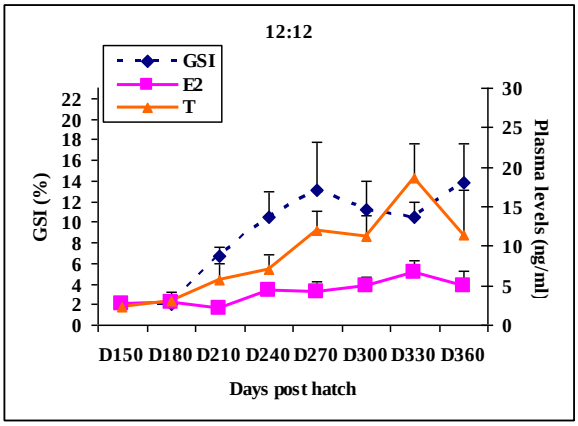
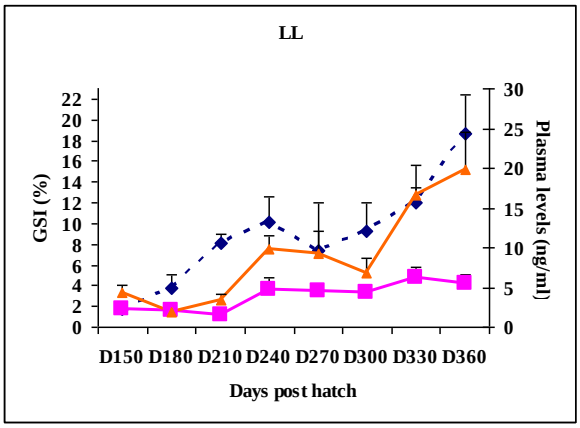
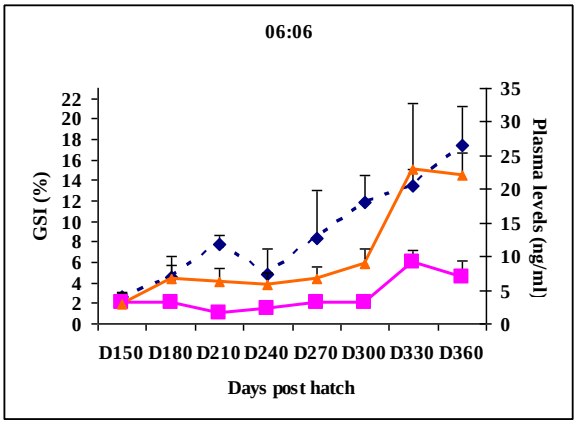
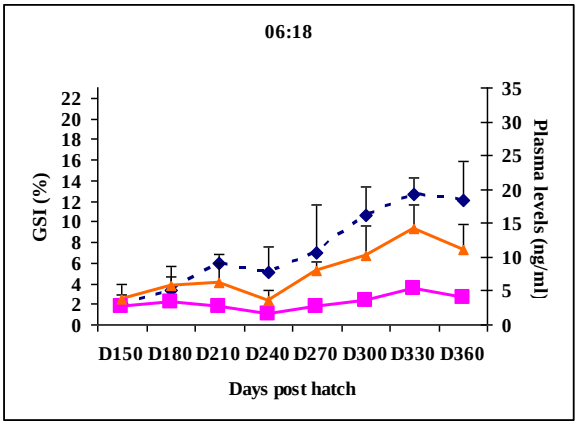

A
12:12
LL
06:06
06:18
12-LL
LL-12
GSI (%)
25
20
15
10
5
0
a a b b ab ab
a b b b ab a
D150
D180
D210
D240
D270
D300
D330
D360
Days post hatch
B
12:12
LL
06:06
06:18
12-LL
LL-12
GSI (%)
3.5
3
2.5
2
1.5
1
0.5
0
a a b b c bc
D150
D180
D210
D240
D270
D300
D330
D360
Days post hatch

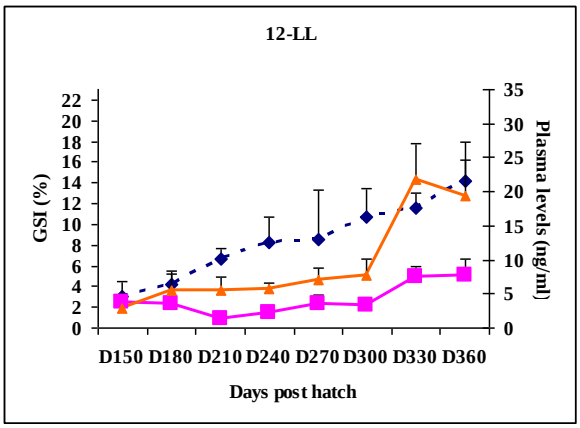
Figure 9


12:12
GSI
E2
T
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


LL
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


06:06
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


06:18
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


12-LL
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch

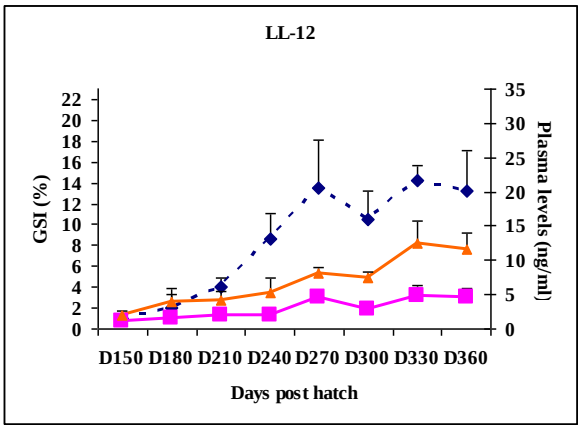
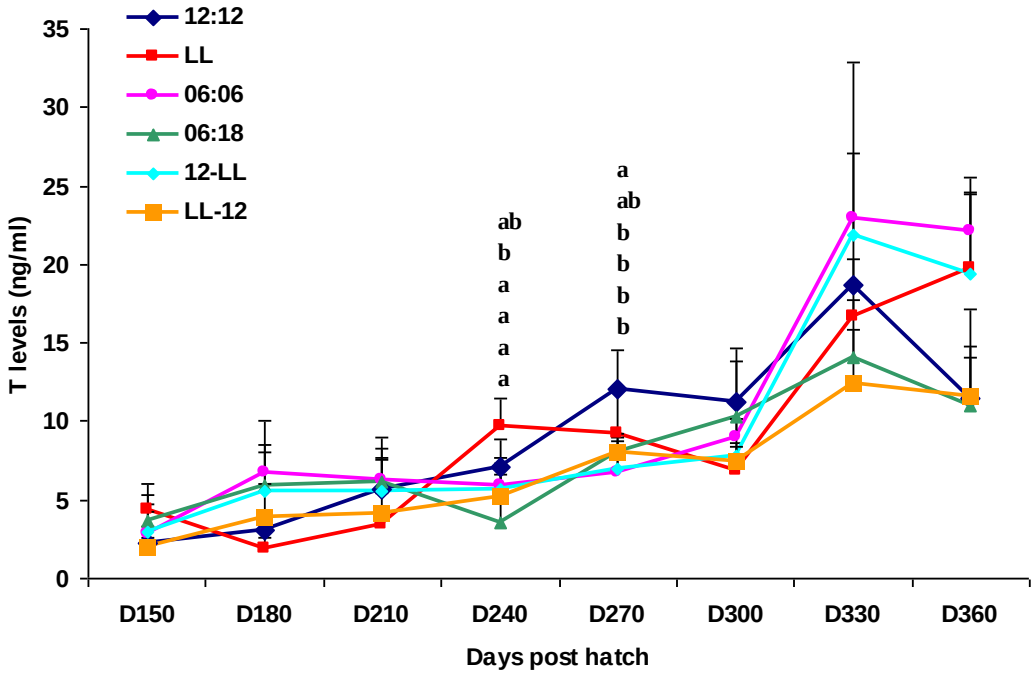

LL-12
GSI (%)
Plasma levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch

Figure 10

A


12:12
LL
06:06
06:18
12-LL
LL-12
T levels (ng/ml)
0
5
10
15
20
25
30
35
ab
b
a
a
a
a
b
b
b
b
a
D150
D180
D210
D240
D270
D300
D330
D360
Days post hatch

B

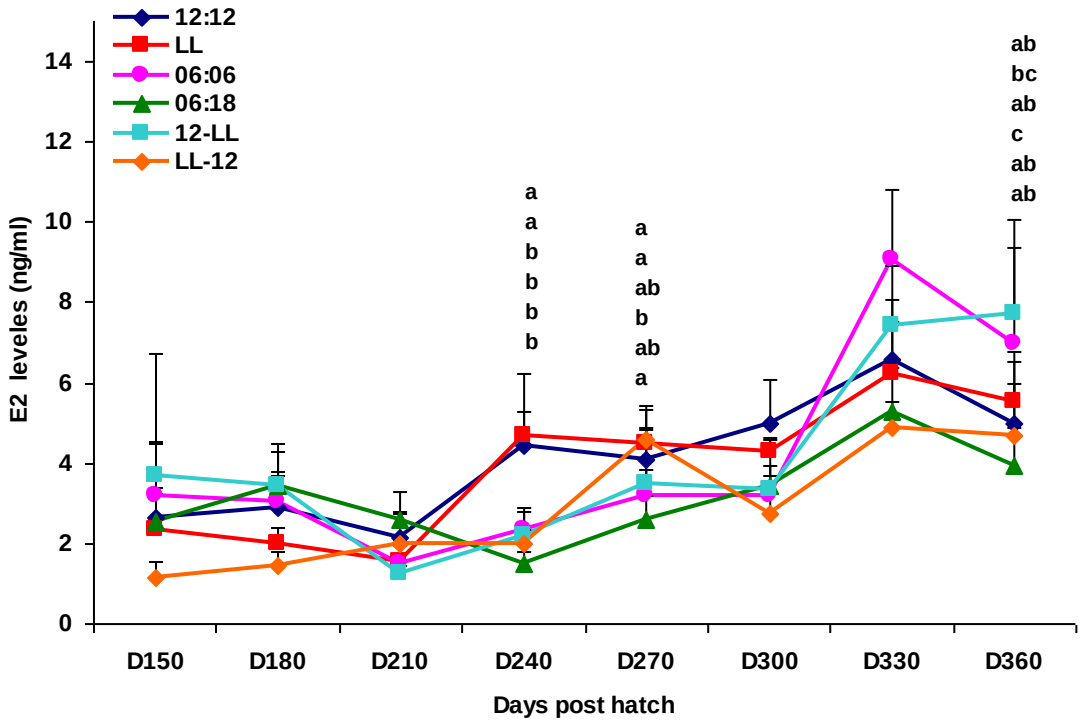
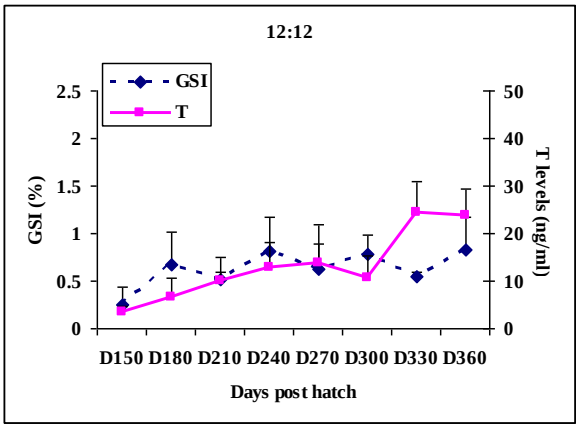
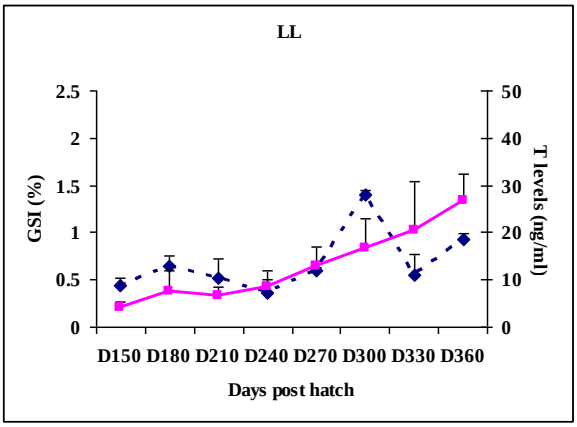
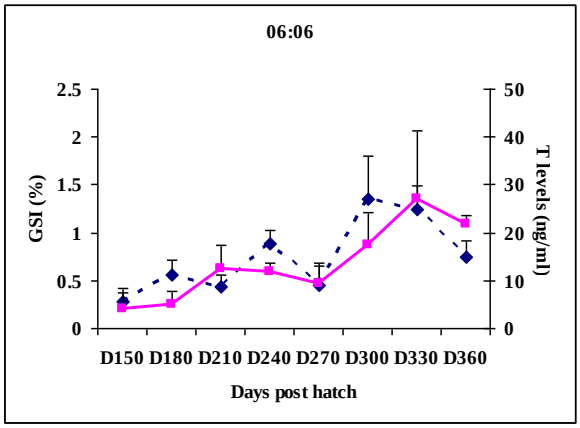
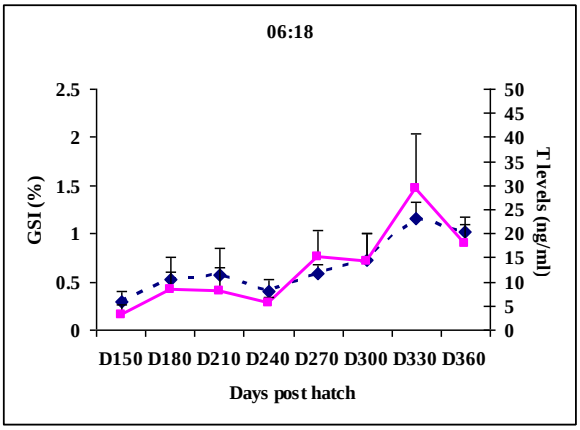

12:12
LL
06:06
06:18
12-LL
LL-12
E2 leveles (ng/ml)
0
2
4
6
8
10
12
14
a
a
b
b
b
b
a
a
ab
b
ab
a
ab
bc
ab
c
ab
ab
D150
D180
D210
D240
D270
D300
D330
D360
Days post hatch

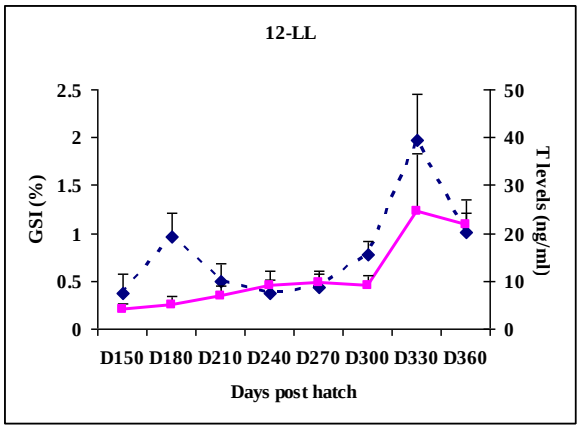
Figure 11


12:12
GSI
T
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


LL
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


06:06
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


06:18
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch


12-LL
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch

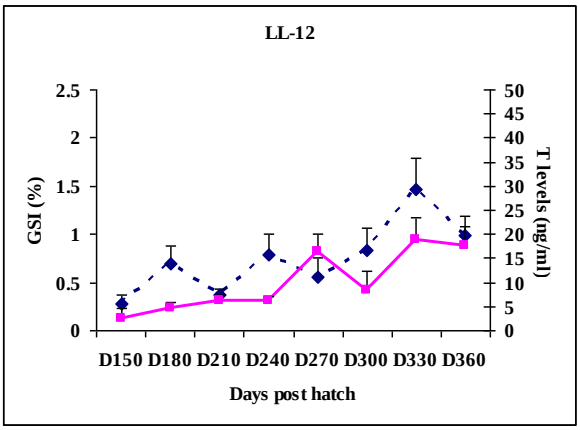

LL-12
GSI (%)
T levels (ng/ml)
D150 D180 D210 D240 D270 D300 D330 D360
Days post hatch

Figure 12

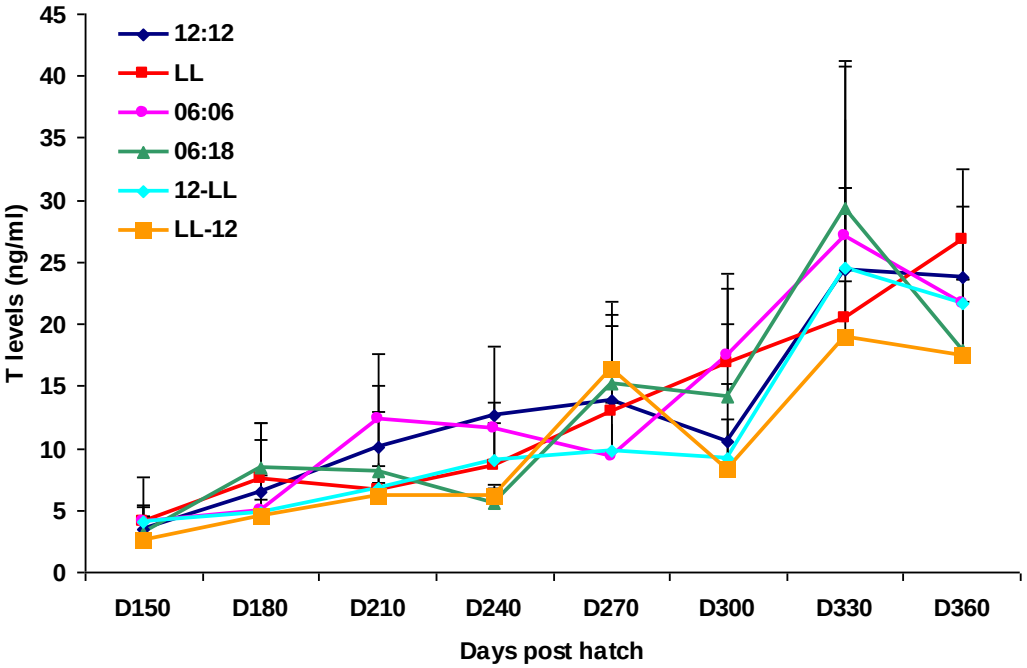

12:12
LL
06:06
06:18
12-LL
LL-12
T levels (ng/ml)
0
5
10
15
20
25
30
35
40
45
D150
D180
D210
D240
D270
D300
D330
D360
Days post hatch

Figure 13

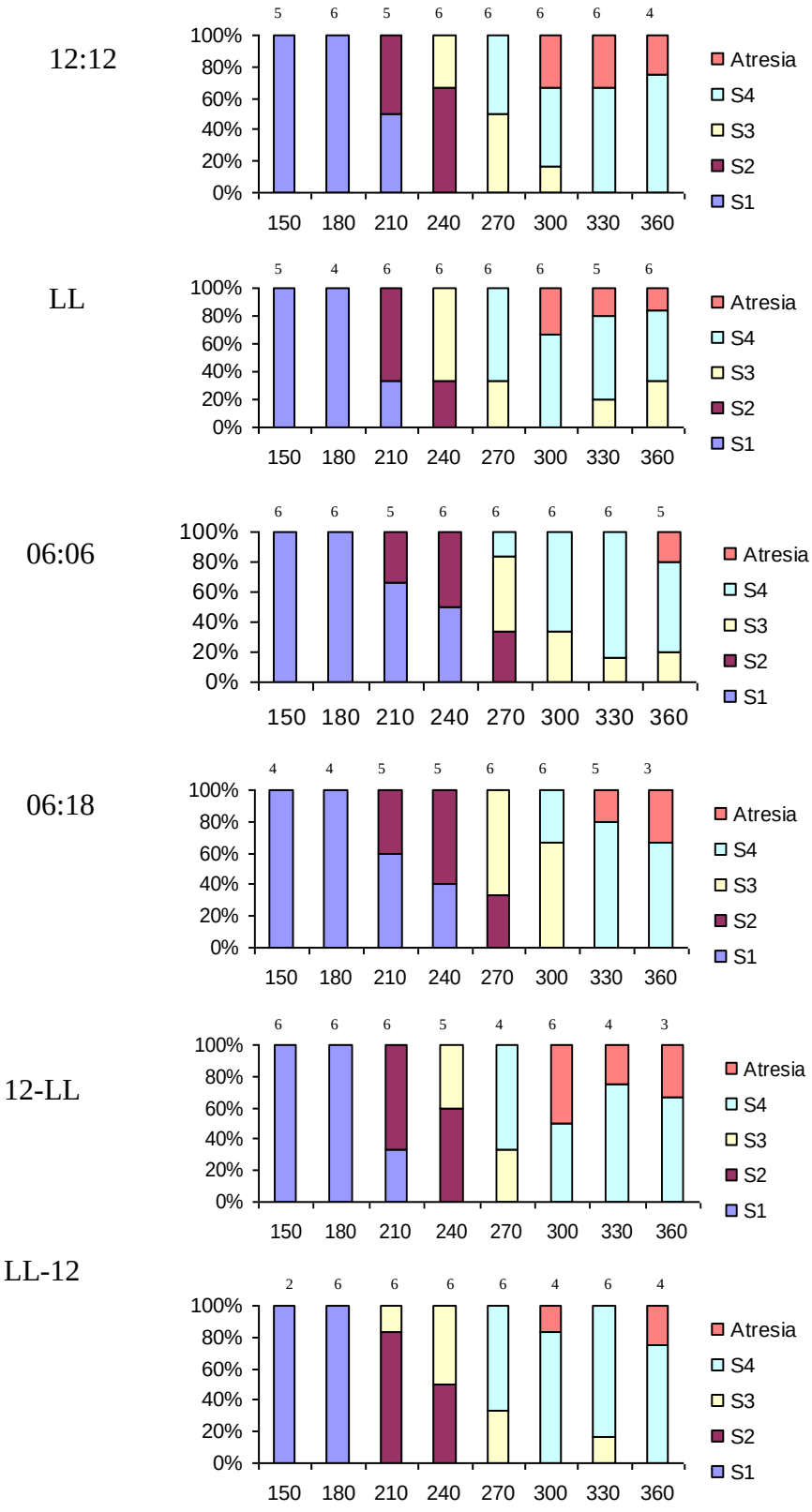
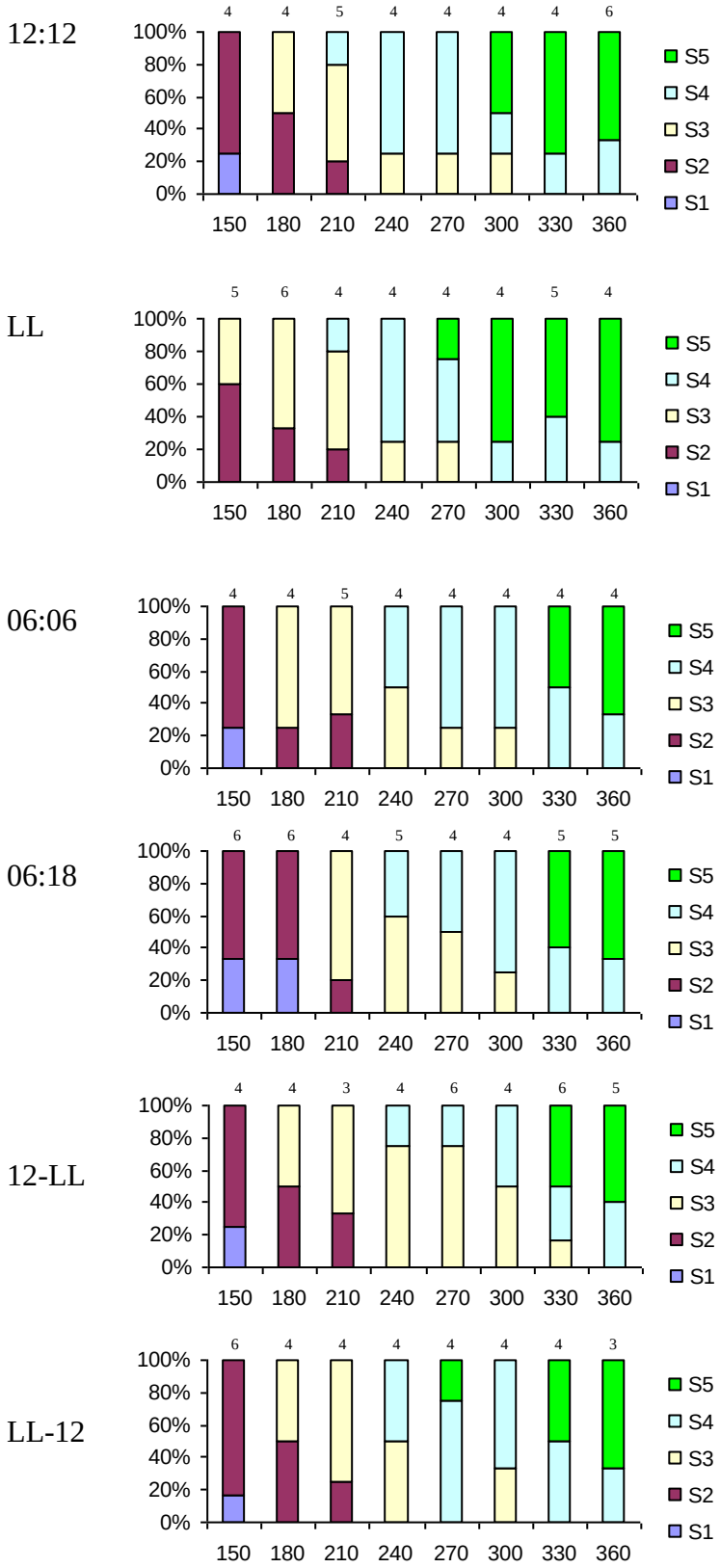

12:12
5 6 5 6 6 6 6 4
LL
5 4 6 6 6 6 5 6
06:06
6 6 5 6 6 6 6 5
06:18
4 4 5 5 6 6 5 3
12-LL
6 6 6 5 4 6 4 3
LL-12
2 6 6 6 6 4 6 4
100%
80%
60%
40%
20%
0%
150 180 210 240 270 300 330 360
Atresia
S4
S3
S2
S1
Days post hatch

Figure 14


12:12
4 4 5 4 4 4 4 6
LL
5 6 4 4 4 4 5 4
06:06
4 4 5 4 4 4 4 4
06:18
6 6 4 5 4 4 5 5
12-LL
4 4 3 4 6 4 6 5
LL-12
6 4 4 4 4 4 4 3
100%
80%
60%
40%
20%
0%
150 180 210 240 270 300 330 360
S5
S4
S3
S2
S1
Days post hatch

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Virtually all organisms exhibit daily rhythms in physiology and behaviour that are framed not only by external environmental stimuli, such as the daily light/dark cycle, but also by endogenous circadian clocks. Photoperiod is an important factor controlling the seasonal physiological cycles (such as reproduction and locomotor rhythms) of many fish species including sub-tropical and tropical species (Bromage *et al*., 2001; Randall *et al*., 2001; Gines *et al.*, 2003). Research into the photoperiodic control of physiology (principally growth and reproduction) has mainly focused on temperate species such as trout, salmon, sea bass etc., mainly because of the natural variation of photoperiod observed in these regions, while little is known concerning tropical species such as African catfish. Due to the variety of environments inhabited by fish, from temperate to tropical or freshwater to deep seawater, and the resulting high divergence demonstrated in fish physiology regarding biological rhythms in terms of feeding behaviour and locomotor activity (diurnal *vs.* nocturnal) and reproductive strategies (seasonal *vs.* continuous spawner), it is difficult to generalise results found in one fish species to all teleosts. The aim of this study was therefore to better understand circadian biology and the entrainment of two key physiological functions (growth and reproduction) by photic cues in a tropical species. African catfish, which inhabits a range of environments throughout the African continent, is becoming an increasingly important commercial species for aquaculture due to its rapid growth, high tolerance to poor water quality and high stoking densities. It is also believed that the species could also be a very interesting scientific model for chronobiology and environmental studies due mainly to its nocturnal activity rhythm and overall different physiology and morphology.

Many studies have focused on characterising the function of the pineal organ in fishes (e.g. Falcon, 1999; Max and Menaker, 1992; Gern *et al*., 1992; Molina *et al*., 1996;

Cahill, 1996). However, research into circadian biology to study the pineal gland as part of an entire system/network within the lower vertebrates has been sparse by comparison with that in mammalian and invertebrate models.

To our knowledge, African catfish has not been previously used in chronobiological studies. Results from the first chapter of this PhD project were very interesting as the photic regulation of the melatonin produced by the pineal gland clearly appeared to be very different from all other teleosts studied to date. Indeed, results suggested that melatonin production exclusively depends on light perceived by the eyes. Therefore, ophthalmectomised fish were unable to synthesize and release melatonin into the blood circulation during the dark phase.

Such a decentralized organization with specialisation of the eyes for light perception and pineal for melatonin production, similar in a way to Mammals (Underwood *et al*., 1990; Yamazaki *et al*., 1999) was reported for the first time in teleosts. The pineal gland would therefore serve as a enslaved secretory gland. These findings were supported by *in vitro* results that showed African catfish pineal glands in isolation were not able to normally produce melatonin at night, as usually seen in most other fish species studied so far (Max and Menaker, 1992; Randal *et al*., 1995; Molina *et al*., 1996). Similar results were obtained in the Nile tilapia and Mozambique tilapia., *Oreochromis mossambicus* (Nikaido *et al*., 2009). These results generate a number of interesting questions for future studies such as why the circadian organisation has been shaped differently in these species during evolution? How light perceived through non-visual photoreceptors in the retina is transmitted to the central nervous system (or pineal gland directly) ?. The lack of responsiveness of the pineal gland to light could be explained by the observed reduced light transmittance through the skull and therefore low light irradiance of the pineal gland in the African catfish as compared to other species.

A study performed on catfish species (*Clarias batrachus, Heteropneustes fossilis, Mystus vittatus, M. seenghala*, and *M. cavassius)* found two morphologically different types of pineal window: an opaque-looking pineal window in *C. batrachus* and *H. fossilis* and a translucent type of pineal window in *M. vittatus, M. seenghala*, and *M. cavassius* (Srivastava, 2003). In the present study, light penetration through the pineal window was lowest in catfish (1%) compared to a range of temperate and tropical species with the cranial bones acting as a strong filter against short wavelengths. These results together with the overall poor light transmission in the environment inhabited by the species could explain that in the absence of strong enough stimulation, the pineal gland might have lost its direct light perception ability. This could have occurred through a shift in the type of photoreceptor present in the pineal gland of the African catfish. Three types of cells producing melatonin have been described: true or modified photoreceptors and pinealocytes (Falcon, 2007). In the lamprey, for example, these different types have been shown to co-exist in the pineal (Ekstrom and Meissl, 2003). The presence or absence of these receptor types will shape the organisation of the circadian light axis and the need for extra-pineal light perception (e.g. retina and/or deep brain) (Ekstrom and Meissl, 2003). Future studies should therefore determine the nature of the pineal photoreceptors in the African catfish to confirm this interesting hypothesis.

Importantly, the current results supply further evidence suggesting that the mechanisms involved in the light perception and transduction through the central circadian axis would have radically changed in teleosts species probably reflecting the environment in which they have evolved. To date, only two kinds of circadian organization have been proposed *i.e.* salmon *vs.* other teleosts. It is presently suggested that a third organization could be at work in teleosts based on the photic control of melatonin production by the eyes and pineal gland.

The present research then focused on better characterizing diel melatonin production and endogenous entrainment through exposure to continuous photic regimes (continuous light, LL or darkness, DD) (**paper II**). A number of reproductive and growth performance studies have shown that tropical teleosts such as Nile tilapia and African catfish can be responsive to photoperiodic changes (Campos-Mendoza *et al.* 2004; Almazan-Rueda *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006). It is known that melatonin synthesised by the pineal gland and the eye, plays an important role as a photoneuroendocrine transducer in vertebrates. These tissues can convert the daily light-dark information into a daily rhythm of melatonin. Therefore the effects of photoperiod on the circadian endogenous melatonin rhythm system of the catfish were investigated.

The present results firstly showed a similar diel plasma melatonin profile in African catfish to that previously reported in most vertebrate species studied to date (Reiter 1988; Cassone 1990; Mayer *et al.* 1997; Pavlidis *et al.* 1999; Hardeland *et al.* 2006; Migaud *et al.* 2006). This is in accordance with the role of melatonin as a '*zeitgeber*' in entraining the physiology of the animal to seasonal daylength changes in their environment (Menaker *et al*., 1997; Falcon *et al*. 2006). Interestingly, results also showed that melatonin production or suppression can anticipate the change from night to day with basal melatonin levels observed 45 mins prior to the switching on of the light.

These observations clearly suggest the involvement of a clock-controlled system of melatonin secretion which is capable of anticipating the next photophase period. In particular the melatonin can be regulated by arylalkylamine-N-acetyltransferase (AANAT) or hydroxyindole-O-methyl-transferase (HIOMT) synthesis at the transcriptional and/or translational level, as previously suggested in other species (Appelbaum *et al*., 2005; Falcon *et al*., 2001; Klein *et al*., 1997).

In many vertebrates species including teleosts, constant photoperiod (i.e. LL/DD) has commonly been used to describe rhythmic melatonin production (Gern and Greenhouse 1988; Falcon *et al.* 1989; Kezuka *et al.* 1989; Okimoto and Stetson 1999b; Migaud *et al.* 2006; Takemura *et al.* 2006). When constant light (LL) was applied, day/night melatonin rhythms were abolished as expected, due to the constant photic inhibition of AANAT activity (e.g. one of the enzymes responsible for the conversion of serotonin into melatonin) as already reported in many other fish species (Falcon *et al.* 2007). An additional trial has been performed during the present research programme (appendix 1) aiming to test the light sensitivity of melatonin production by catfish *in vivo*. To do so, fish acclimated to a 12L:12D photoperiod were exposed to continuous light (LL) of three different light intensities (1, 20 and 60 lux) (LL). Results showed that African catfish is very sensitive to light with an intensity of 1 lux being enough to fully suppress the melatonin levels. Furthermore, interestingly in this study, when a light dark cycle was restored, melatonin levels were proportional to the prior light intensity exposure with significantly lower melatonin levels observed during the first dark period in fish that have been exposed to stronger light intensity. This would suggest that the pineal gland has somehow been desensitized and requires some time to produce melatonin normally.

However, when fish were exposed to constant darkness (DD), a strong endogenous melatonin rhythm (maintained for at least 4 days and 18 days in catfish and tilapia respectively) was found, demonstrating once again the presence of robust circadian oscillators in this species. Importantly, melatonin rhythmicity, assessed after few days of DD exposure, was still circadian (*i.e.*, cycling over approximately 24 h). It must, however, be acknowledged that this does not rule out potential phase-shift of the rhythms later on. This could not be assessed in the present study and should be studied further in the future. Taken together, the results presented in paper II raise interesting questions as to whether

these robust clock-controlled melatonin rhythms may eventually dampen and/ or free-run, or continue and what the mechanisms underlying these endogenous rhythms actually are.

The two first papers focusing on the circadian axis and endogenous melatonin rhythms in fish provided new insights into the potential entraining mechanisms of fish physiology by photic cues (daily and seasonal changes). Comparative studies between species might prove to be very useful to better understand how adaptations to specific environments during evolution have shaped the divergent systems found in teleosts. Future studies focusing on the pathways involved in light perception, melatonin synthesis and clock systems will be particularly relevant.

Following the knowledge gained on the light perception axis and circadian clock in catfish, the work then focused on the effects of light on catfish physiology (e.g. locomotor activity, growth and reproduction). The next aim of the present doctoral research was thus to investigate circadian behaviour of catfish exposed to different light regimes through the study of locomotor activity (**Paper III).** African catfish is a very interesting "model" due to its well reported nocturnal activity rhythmicity as compared to most other teleosts species. Locomotor activity is considered as a very useful tool to study and describe circadian mechanisms in both invertebrates and vertebrates (Iigo and Tabata, 1996; Eriksson, 1978; Reebs, 2002; Favreaua, 2009). Under light/dark cycles, fish show daily patterns of locomotor activity that can be classified into diurnal, nocturnal or crepuscular (Favreaua, 2009; Eriksson, 1978). However, in some teleosts species, the characterization of the daily pattern of activity is not straightforward as different individuals within the same species can show a great variability in their daily activity patterns resulting in a combination of activity patterns (Reebs, 2002; Favreaua, 2009).

Previous published data have shown variability in behavioural patterns obtained in other fish species. Indeed, when goldfish (*Carassius auratus*) were exposed to an LD

12:12 h cycle, the daily rhythm of locomotor activity also differed between individual fish: some were diurnal and others nocturnal (Sanchez-Vazquez *et al*., 1996). Furthermore, studies showed that sea bass (*Dicentrarchus labrax*) displaying diurnal feeding rhythms could be made nocturnal by restricting food availability to the night-time (Sanchez-Vazquez *et al*., 1995). In sharpsnout seabream (*Diplodus puntazzo*), spontaneous shifts from diurnal to nocturnal patterns of locomotor activity and *vice versa* were reported, as well as the existence of phase-independence between locomotor and feeding rhythms (Vera *et al*., 2006). In contrast, other teleosts species show clear daily rhythms of locomotor activity, such as the zebrafish and the tropical fish *Halichoeres chrysus*, which mostly confine their activity to the photophase under an LD cycle (Gerkema *et al*., 2000; Hurd *et al*., 1998), whereas the tench (*Tinca tinca*) shows a strictly nocturnal pattern, even under extremely short photoperiods (e.g., LD 22:2 h) (Herrero *et al*., 2003). Therefore, it has been generally accepted that activity patterns in fish show a strong plasticity (Ali, 1992; Madrid *et al*., 2001; Reebs, 2002). Freshwater teleosts have been proposed to have a more flexible circadian system than marine fish, probably due to the relative instability of their environment (Reebs, 2002).

The results obtained in this chapter provided interesting preliminary results which first confirmed the nocturnal activity of *C. gariepinus* as already shown in Japanese sea catfish *Plotosus lineatus* (Kasai, 2009), channel catfish, *Ictalurus punctatus*, (Goudie *et al*., 1983), sea catfish, *Arius felis* (Steele, 1984), Japanese catfish, *Silurus asotus* (Tabata *et al*., 1991), European catfish, *Silurus glanis* (Boujard *et al*., 1995), Brazilian cave catfish, *genus Taunayia* (Trajano & Menna-Barreto, 2000) and Indian catfish, *Clarias batrachus* (Ramteke, *et al*, 2009). Importantly, fish were also able to anticipate changes in their environment (day/night) as already seen in the previous chapter, this highlights once again the importance of internal clocks in this species.

In many locomotor activity studies performed in fish exposed to such conditions, fish lose external entrainment and their circadian rhythms can free-run with an approximate period (*t*) of 24 h in the case of circadian rhythms (Vera *et al*., 2009). Therefore to investigate the existence of endogenous control of behavioural rhythms in fish, constant environmental conditions are usually used, such as constant darkness (DD), constant illumination (LL), or ultradian pulses (very short LD photocycles) (Reebs, 2002). In the present study, clear circadian endogenous rhythms were observed under constant light (LL) or darkness (DD) during several days before losing rhythmicity. Indeed, under constant light or darkness, catfish were able of self-sustained activity rhythms during several days before losing rhythmicity. These free running rhythms were clearer in darkness than in light condition, and appeared to also depend on fish stocking densities. Some catfish showed free-running rhythms immediately after the removal of the LD 12:12 h cycle, whereas others were arrhythmic during the first few days under constant photic conditions and then showed free-running locomotor activity. Interestingly, the total activity levels varied depending on the stocking density with the highest activity seen in medium density treatments (5 fish/tank). These differences among fish reared individually or in groups could be explained by the existence of social interactions when animals become more active to defend their territory (Hecht *et al*., 1997; Hossain *et al*., 1998). Rearing fish individually resulted in some cases to arrhythmic activity pattern whereas group of fish showed more steady rhythmicity as already suggested in European catfish (Boujard, 1995). To our knowledge, these preliminary results are the first to be reported in African catfish and further studies are needed to better understand the effects of rearing environments on the species behaviour in relation to performance. Additional trials have been performed during this PhD project to investigate the effect of light intensities on catfish locomotor

activity, three different light regimes were applied (0.01, 10 and 50 lux) under the LD regime. Unfortunately the trial was not completed for technical problems.

Locomotor activity is definitely a very useful tool that could help to optimise rearing conditions in enclosed systems and explain variability in performances seen in different stocks in relation to photic environment but also water quality, temperature and a range of environmental and anthropomorphic perturbations that can be experienced by fish in artificial conditions.

Finally, the main target of this project was to test the effects of a range of photoperiodic manipulations on growth performance, sexual development and reproductive performance in African catfish reared from eggs to puberty (**Paper IV).** The present study investigated the effects of photoperiod at two key stages, first during the initial 90 DPH (juvenile) and then during the adult stage (up to first maturation, 360 DPH). Understanding of the effects of photoperiodic manipulations on reproduction has been used intensively in aquaculture to control spawning time and enable all year long supplies of seed (eggs/fry), control early maturation during on growing and as a result enhance growth, maintain good flesh quality and improve overall welfare status of farmed stock (Purchase *et al*., 2000, Bromage *et al*., 2001; Randall *et al*., 2001; Gines *et al.*, 2003). To date, such studies have been carried out mainly in the most commercially important species with salmonids serving as a reference in this field while little research has been done so far on sub-tropical or tropical teleosts (Bromage *et al*., 2001). Therefore the effects of photoperiodic manipulations on reproduction in these species are not well understood (Campos-Mendoza *et al*., 2004; Rad *et al*., 2006). It is important to note that even between closely related species (i.e. salmon vs. trout, cod vs. haddock), significant differences in photoperiodic effects have been observed (Bromage *et al*., 2001). However, it is of particular interest to compare the effects in very divergent species in terms of life cycle, environment and

phylogeny and tropical species have not been the object of many studies in this respect. Few previous studies have investigated the effects of photoperiod on African catfish performance although as these studies have mainly focused on given life stages without considering the whole life cycle and therefore potential effects of background rearing conditions, results are therefore difficult to compare. The aim of the present study was to consider the effects of photoperiodic manipulation on growth and reproduction throughout a production cycle. When fish were exposed during the early stages (up to 90 DPH) to a standard 12L:12D photoperiod or a continuous light regime (LL), no differences in growth performance or survival were observed.

These results did not confirm previous studies that reported better performance under short day photoperiods (Almazan, 2004; Appelbaum and Mcgeer, 1998). However, differences in experimental conditions especially lighting conditions characterised by daylength, light intensities, spectral content and daily variations (e.g. dawn and dusk) (Boeuf et le Bail, 1999), could explain such different findings. Moreover, mortality was high at the initial period which has already been reported in this species (Hung *et al*., 2002; Subagja *et al*., 1999). The cannibalism in *C. gariepinus* contributed the most to larval mortality as already observed in previous studies although this could not be assessed in the current study (Hecht and Appelbaum, 1987). This may be related to the feed quality and the digestibility or to the primary development of the digestive system at first feeding.

When photoperiod regimes were applied later during the life cycle (from 120 DPH), clear effects were observed with significantly lower growth observed in fish exposed to the LL treatment associated with lower food consumption and feed conversion efficiency in comparison to all the other treatments and especially with the 12:12 treatment. These results may be due to light induced stress suppressing appetite, increasing activity and social aggressive behaviour. Further studies are useful to confirm these hypotheses

Continuous darkness (DD) would be a very interesting and relevant treatment to test in African catfish due to its nocturnal activity. Unfortunately, due to UK Home Office regulation and technical difficulties (monitoring and sampling in such conditions), we could not realistically expose fish to DD for a long period of time as this could compromise their welfare. Future studies should definitely address these technical and welfare concerns and study the effects of such a treatment in catfish.

Interestingly, fish under the 6:6 regime showed higher food consumption in comparison to all other treatments, especially the 12:12 treatment. However, surprisingly, these fish did not grow better than fish exposed to the 12:12 treatment. This may be explained by lower feed conversion efficiency (FCE) in fish exposed to the 6:6 treatment due to the repetitive stress induced by the short photocycles that could result in higher activity and overall higher energy expenditure or potential reductions in digestive and absorptive performance as suggested by Biswas *et al*. (2008). Future studies on fish feeding activities when exposed to different light regimes will be useful to clarify the results obtained during the present programme of research. However, previous published data have shown large differences between species in the effects of photoperiod on growth. In Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*) for example, enhanced growth was observed when fish were exposed to extended daylength (Saunders *et al*., 1985; Berg *et al*., 1992; Taylor *et al*., 2005, 2006, 2009). However, short photocycle (6L:6D) could be used to enhance the growth rate of Nile tilapia *Oreochromis niloticus* (Biswas and Takeuchi, 2003). On the other hand, Artic charr (*Salvelinus alpinus*) feed and grow well even in complete darkness (Jorgensen and Jobling, 1989) whereas no significant growth differences were observed in Atlantic halibut (*Hippoglossus hippoglossus*) reared under ambient photoperiod or LL (Hallaraker *et al*., 1995). Overall, photoperiod mediated growth effects in fish are clearly species specific depending on the daily activity rhythms

(diurnal/nocturnal) and probably the light sensitivity which has been shown to differ greatly between species (Migaud *et al*., 2008).

In the present study, no major effects of photoperiod were observed on puberty, gonadal development, and egg quality with all fish recruited into puberty and developing gonads although differences in the timing of gametogenesis could be observed, especially a delay (circa 2 months) in females exposed to short daylength (6L:18D and 6L:6D). This is in agreement with findings obtained in carps and other cyprinids, catfish and other tropical and sub-tropical species suggesting that temperature plays a more important role in the control of reproduction than photoperiod (Bromage *et al*., 2001). Previous studies on Indian catfish, for example, showed that both photoperiod and temperature can affect gonadal activity, but temperature would be the predominant (proximate) cue with photoperiod playing a more permissive (ultimate) role (Vasal and Sundararaj, 1976; Sundararaj and Vasal, 1976). In general, it is accepted that high light intensities are required for growth optimization (Boeuf and Le Bail, 1999; Boeuf and Falcon, 2001) while shorter/longer photoperiods are needed to stimulate or alter maturation in fish (Amano *et al*., 2000; Bromage *et al*., 2001; Berrill et al., 2003) while, the light thresholds needed for reproduction (maturation) are not yet known (Bromage *et al*., 2001).

Nonetheless, temperature in the present trial was maintained constant throughout the experiment which raises the question of how reproduction was entrained and synchronised in the absence of a strong seasonal cue. Suggestions could include a size/condition threshold above which reproduction would proceed spontaneously even in the absence of the appropriate cues.

The work carried out during this PhD project clearly advanced our understanding of circadian rhythmicity, light perception and effects of photoperiod on physiology in a tropical species. Future studies are now required to further characterise the divergent

circadian system found in the African catfish and Nile tilapia and link it to evolutionary trends within vertebrates. Furthermore, studies carried out in this thesis have implications for the commercial production on catfish and could help to develop or refine husbandry regime to optimise the performances and welfare of farmed catfish.

CHAPTER 8

REFERENCES

8. REFERENCES

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APPENDIX

Appendix 1. Plasma melatonin profile of African catfish subjected to a) 12L:12D and LL regimes. Values shown are mean ± SEM (n= 6). Letters indicate significant (p<0.05) difference between sampling points. Open and filled boxes indicate photophase and scotophase respectively.