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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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CARLOS CRISTIAN MARTINEZ CHAVEZ B. SC. (HONS)

INSTITUTE OF AQUACULTURE, UNIVERSITY OF STIRLING

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of candidate: _____

Signature of supervisor: _____

Date: _____

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Abstract

Despite teleosts being the largest and most diverse group of vertebrates, fish models currently used to study photoperiodic effects on fish physiology have been limited to a few species, most of which are temperate seasonal breeders. The overall aim of this work was to expand our knowledge on circadian biology and environmental physiological effects in Nile tilapia (*Oreochromis niloticus niloticus*), a continuous breeding species of tropical-subtropical origin.

The circadian light axis of Nile tilapia is described with regards to melatonin production. Circadian melatonin profiles of fish under 12L:12D photoperiods were observed to be low at day and high at night, suggesting melatonin to be an entraining signal as observed in all other vertebrates. When constant light (LL) was used, such day and night fluctuations where abolished. However when fish where exposed to constant darkness (DD) a strong robust endogenous melatonin rhythm was found, suggesting the presence of circadian oscillators in this species. Importantly, this endogenous rhythm was observed to be maintained for at least three weeks under darkness and proved to be circadian in nature. Moreover, although the melatonin system was able to produce day and night melatonin rhythms when exposed to a different (6L:6D) photocycle, the oscillator appeared to not be entrainable to such a short photo cycle when exposed to DD, as melatonin levels remained high. When comparing the circadian organization of different teleost species including Nile tilapia, preliminary studies showed at least three divergent circadian light organizations in teleosts. Nile tilapia was characterised by a pineal gland far less sensitive than in other fish species as demonstrated through *in vitro* studies. Furthermore, pineal melatonin production was clearly dependent on the light perceived by the eyes as ophthalmectomy resulted in basal plasma melatonin levels during the dark period. These findings are the first to be reported in a teleost and could be comparable to the circadian light organization of higher vertebrates such as mammals.

The onset of puberty of Nile tilapia was studied with regards to the newly discovered Kiss1/GPR54 system. Such a system has recently been discovered in mammals and found to be the primary switch of the brain-pituitary-gonadal (BPG) axis. The results of this study not only suggest a link between the Kiss1/GPR54 system and the onset of

puberty in this tropical batch spawning teleost, that would be a highly conserved feature across vertebrates, but also that the transcriptional mechanisms regulating GPR54 expression could be directly or indirectly influenced by light.

Finally, a study was conducted on the effects of different intensities of continuous light (LL) on the growth and sexual development of Nile tilapia up to first maturation. The results showed a significant growth response of fish in all LL treatments compared to control fish. Importantly, this confirmed that LL enhances growth in this species and suggests that it is the light regime more than the intensity which is having an effect.

This work thus provides important basic knowledge of the light entrainment pathway and circadian melatonin rhythms in Nile tilapia. Of special importance is the discovery of a strong endogenous melatonin oscillator and a novel circadian organization in fish which would seem to be homologous to that observed in higher vertebrates. Moreover, this work provides evidence that the newly discovered Kiss1/GPR54 system has a similar role in fish as has been found in mammals and that such a system could be directly or indirectly regulated by light. If so, Nile tilapia and other fish species could become important models in the chronobiology and reproduction fields. Finally, this work not only increases our basic and applied knowledge of this species, but also broadens our understanding of the circadian light axis in teleosts and its mediatory effects on reproduction.

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1 General Introduction

1.1 The Nile tilapia *O. niloticus niloticus*

The Tilapiine tribe has been reclassified into three separate genera (Trewavas, 1983): *Tilapia* (substrate spawners), *Sarotherodon* (paternal or biparental mouthbrooders) and *Oreochromis* (maternal mouthbrooders) mainly based in their reproduction, feeding habits and biogeography (Rana, 1988; Macintosh and Little, 1995). However the common name 'tilapia' and/or 'Nile tilapia' is used for convenience throughout the text to describe *O. niloticus niloticus*.

Nearly all the tilapias suitable for culture belong to the *Oreochromis* genus, of which the Nile tilapia is the most important species due to its fast growth rate, adaptability to a wide range of culture conditions and high consumer acceptability. Over the past 30 years it has been transferred throughout the world to become the mainstay of tilapia farming in many different environments and at all levels, from subsistence to highly intensive production, reaching over 2.5 million tonnes by 2005 (Pullin and Lowe-MConnell, 1982; Macintosh and Little, 1995; Josupeit, 2007).

1.1.1 Culture problems

One of the characteristics of tilapias is the capacity to alter the age and size of first maturity in response to their environment. The environmental cues involved in the physiological switch from somatic growth to sexual maturation are unclear but several factors such as seasonality and nutritional status have been implicated (Rana, 1988; Lorenzen, 2000). Nile tilapia can sexually mature at 5 to 6 months old and breed when still at a very small size (Srisakultiew, 1993; Macintosh and Little, 1995). This presents a huge problem to farming mainly because energy is diverted from somatic growth to reproduction (Pullin and Lowe-Mconnell, 1982; Rana, 1988; Macintosh and Little,

1995) resulting in uncontrolled recruitment, stunting and energy losses. This balance between somatic growth and gonadal development increases in the favour of the latter as their environment becomes more limited and stressful (intensive culture in particular) (Pullin and Lowe-Mconnell, 1982).

1.1.2 Stock management strategies

Recent improvements in Nile tilapia broodstock management have focused on conditioning broodstock before spawning (regular replacement of females with rested conditioned fish to improve spawning synchrony) and on removing eggs for artificial incubation (Macintosh and Little, 1995). To solve the problem of differential growth, early maturation and uncontrolled spawning, different approaches have been taken through the years and some are still likely to be used depending on the farming strategy. Initially, this was managed through grading and harvesting the larger fish a number of times during the growing season, leaving the smaller ones to grow. The problem with this is that it generally involves selecting and using small size breeding fish every time the harvest is done, eventually reducing the average size of the population (stunting) (Pullin and Lowe-Mconnell, 1982). To overcome this problem, different authors suggested stock management systems which could separate the production and reproduction phases of the life cycle, coupled with genetic or hormonal manipulation of the cultured stock to eliminate the normal reproductive activity during the grow-out phase (Pullin and Lowe-Mconnell, 1982; Macintosh and Little, 1995). Nile tilapia have a marked sexual growth dimorphism with males growing faster than females most likely due to the energy requirements during gonadal development, however other possible sexual dimorphic mechanisms like compensatory growth could be at play (Barreto *et al.*, 2003). Monosex culture has been a way to solve the problem. Originally, monosex

was attained in three ways: manual sexing which has proven inefficient and labour costly, hybrid production (e.g. *O. mossambicus* x *O. hornorum*) which yield high percentages of male populations and sex reversal by hormone feeding at early stages (Pullin and Lowe-Mconnell, 1982; Devlin and Nagahama, 2002) which has recently had market acceptability problems and legal restrictions due to the use of exogenous hormones. However, recently it is more common to see the use of genetically selected tilapia broodstock (e.g. YY males) to produce all male populations. In short, the two greatest advantages of these techniques are the production of all male populations which have a greater somatic growth compared to females and by which uncontrolled spawning is restricted (Pullin and Lowe-Mconnell, 1982; Macintosh and Little, 1995). Importantly, recently new evidence has shown important and practical effects of photoperiod manipulations on the reproductive physiology of Nile tilapia which will be discussed in detail later. At present it seems that selective breeding and the use of genetically modified broodstock to produce all male populations are the best and most used techniques in hatcheries of mass production around the world.

1.1.3 Strains

There are a number of different colour strains in tilapia which can vary from wild (normal type), red, blotched to blonde, the latter not being actually used but has high market potential mainly because of its pigmentation characteristics (Scott *et al.*, 1987; McAndrew *et al.*, 1988; Hilsdorf *et al.*, 2002). The most commonly used is the red strain because of its high market acceptability and growth performances (McAndrew *et al.*, 1988). A number of red tilapia strains are known to exist world-wide. The most widely available is the Taiwanese red strain which is known to consist of a combination of at least four different species, the original red gene having come

from *O. mossambicus* being transferred by hybridization to various *O. niloticus* strains to improve growth characteristics of the stock (McAndrew *et al.*, 1988).

1.1.4 Life cycle

Tilapias engage in a communal breeding system based on a "lek" or "arena" which is guarded by a male and visited by receptive females. The males build and defend their territories, but there is no pair bond so polygamy is usual (Macintosh and Little, 1995). These mouth-brooding tilapia species have adapted the antipredator tactic of rearing eggs and fry in the relatively safety of the parental buccal chamber (Rana, 1988). In Nile tilapia once the mating and the fertilization has taken place, the female takes the eggs in its mouth and leaves the "lek" seeking calmer spaces. Tilapias produce a few hundred to a couple of thousand eggs in captivity (depending of age, size, reproductive status and genetic background). The female oral incubation starts immediately after fertilization and continues after fry hatch (~3 days) and begin to feed on their own (>10 days) and terminates approximately 30 days post fertilization when they physically cannot fit in the buccal chamber (Pullin and Lowe-Mconnell, 1982; Macintosh and Little, 1995). In the wild Nile tilapias are known to spawn year round but with a peak between December and June (Njiru *et al.*, 2006).

1.1.5 Embryonic development

Tilapia eggs, like those of other teleosts, are surrounded by a multilayered protective envelope, the chorion, which hardens as the eggs absorb water. Water hardened tilapia eggs are typically ellipsoid or ovoid in shape of around 1.65 x 2.00 mm to 2.60 x 3.15 and of ochre colour (Rana, 1988). The ontogeny and early development

of Nile tilapia has been described by several authors of which the most recent one (Morrison *et al.*, 2001) has summarized them all (Table 1).

Table 1.- Comparison of timing of some developmental features of Nile tilapia. Adapted from Morrison *et al*. (2001).

	Hours after fertilization (rearing temperature)					Stage					
Developmental features	Morrison *et al.*, 2001 (27-29^{0}C)	Galman, 1980 (26-27^{0}C)	Galman and Avtalion, 1989 (24-26^{0}C)	Rana, 1990 (28^{0}C)	Lingling and Qianru, 1981 (25-29^{0}C)	Shaw and Aronson, 1954	Nussbaum and Chervinsky, 1968	Galman, 1980	Galman and Avtalion, 1989	Rana, 1990	Lingling and Qianru, 1981
Zygote period											
Protoplasm bulges	1.5	1	0-1		0.5	1	1	2	1		1-2
Cleavage period											
2 cells	1.5-2	5	1-5	2-3	2	2	2	3	2	2	2-1
4-8 cells	2-3		2-3.5	4	2.5-3	3-4	3-4		3-4	3-4	2-2, 2-3
16 cells	4		4	5	4	5	5		5	5	2-4
32-64 cells	4-4.5		5.5	6	4.5-5	6	6		6	6	2-5, 2-6
Blastula period											
Morula or early blastula	5.5	7	5.5-10.5		5.5	7	7	4	6-8		2-7
Blastula	5.5-14	8	10.5-12	10	8	8	8	5	8-9	7	2-8
Gastrula period											
Early gastrula	22	11	24	10	11	9	9	6	10	8	2-9
30-40% epiboly, embryonic shield	22	15	24	14	28	10-11	10-11	7	10	9	3-2
Segmentation period											
Somite formation	26-30	30	40	30	37.5	12	12	9	11	10	4-2
Optic primordial	31	30	52	30	38	13	13	9	12	9	5-1
Brain segmentation	46	30	58		40	14	14	9	13		5-2
Pharyngula period											
Otic vesicles	76	48	65		40	15	15	11	14		5-2
Heart beat	50	60	72		52.5	15	15	12	15		5-4
Retinal pigment	76	72	65	72		18	19	13	14	11	
Hatching period											
Hatching	100-125	100	72	90-120	79	24	20	15	15	12	7
Early larval period											
Gill cover starts to form	124	150	72		127	20	21	17	15		prelarval
Mouth opens, jaw movements	147	156	98		127			22	18	17	“
Teeth	148		164							20	
Swimbladder inflates,	196	228	180		223						“
swimming										21	“
Active feeding	196	240	256		223					24	“

1.1.6 Growth and sexual maturation

In tilapia, sexual differentiation is influenced by stocking densities, Srisakultiew (1993) has shown that when reared at a density of 10-20 fry/L, almost 50% of the fry were sexually differentiated at 11 days post-hatching (DPH). The same study also showed that males grow faster than females and reach puberty and maturity faster than females, between 16 and 22 weeks for males and 22 weeks for females when reared at 27 ± 1^0C and fed commercial trout pellets *ad libitum*. Gonadal development in tilapia has been described by several other authors (Babiker and Ibrahim, 1978; Srisakultiew, 1993; Tacon *et al.*, 1996; Coward and Bromage, 1998) with similar results. In all these studies, the stage of oocyte development was visually determined through histological preparations and assessment of the leading oocytes, cohorts. Based on the findings, classifications of stages of development along both oogenesis and spermatogenesis were developed for Nile tilapia and serve as references. According to Coward and Bromage (1998) oogenesis is therefore divided in to seven stages as follow (Table 2): (1) chromatin nucleolar stage, (2) early perinucleolar stage, (3) late perinucleolar stage, (4) cortical alveolar stage, (5) vitellogenesis, (6) maturation and (7) germinal vesicle migration followed by ovulation and spawning. As for spermatogenesis, according to Babiker and Ibrahim (1978), there are also seven stages (Table 3): (1) Immature, (2) Maturing, (3) Mature, (4) Ripening, (5) Ripe, (6) Spawning and (7) Spent. Importantly, the whole ovogenesis and spermatogenesis can occur over a period of 3-4 months in comparison to temperate species in which it can take up to 8 months to 2 years (e.g. salmon and European seabass) (Bromage, 1995).

Table 2. Classification scheme used to identify the stages of oogenesis showing stages S1, S2, S3 and S4. Adapted from Coward and Bromage (1998).

Stage	Definition	Size Range (μm)	Appearance
S1	Pre-vitellogenic	7-345	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	224-658	Small yolk granules starting at periphery. Vesicles seen throughout oocyte. Follicular layer can be seen to be more developed.
S3	Late stages of vitellogenesis	428-1416	Yolk granules become larger yolk globules and empty vacuoles throughout oocyte. Very developed follicular wall. Nucleus central.
S4	Mature	428-1416	Same as S3 but vesicle migration can be seen.

Table 3. Classification scheme used to identify stages of spermatogenesis showing S1, S2, S3, S4 and S5. Adapted from Babiker and Ibrahim (1978).

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.

Fecundity in Nile tilapia has been found to be very variable and as in many other teleosts is affected by biotic (e.g. physiological including age, size, previous spawning history, genetic background) and abiotic (e.g. environmental including temperature, photoperiod, rainfall, quality of food) factors (Coward and Bromage, 2000; Ross, 2000). Typical fecundity in the wild has been found to be between 905 to 7619 oocytes for fish of 28 to 51 cm total length (Njiru *et al.*, 2006).

Nile tilapias are asynchronous spawners and they tend to breed throughout the year although in the wild they seem to reach a peak in the rainy season (Trewavas, 1983; Coward and Bromage, 2000). The inter-spawning interval (ISI) is also very variable (7 days to a month or more) even between siblings and as for fecundity it seems to be affected by many factors (biotic and abiotic factors previously mentioned) (Coward and Bromage, 2000). One of these is photoperiod, which has been found to

have an impact on ISI and will be discussed in further detail in 1.2.2 (Turner and Robinson, 2000; Campos-Mendoza *et al.*, 2004; El-Sayed, 2006).

1.2 Environmental control of physiology in teleosts

Biotic factors such as age, size, developmental stage (i.e. immature vs. mature) and nutritional status define the state of an organism at a given time and, depending on the species, will vary the degree to which environmental factors affect their physiology (Bromage *et al.*, 2001).

1.2.1 Biological rhythms

Temporal order is crucial for all living organisms as most of the main physiological and behavioural events are in tune with periodic fluctuations in the environment (Gerkema, 1992). Recent studies indicate that temporal synergisms of circadian rhythms have primary roles in neuroendocrine regulation of physiological and behavioural conditions (Meier, 1992). For this, living organisms use biological rhythms which can persist in un-entrained constant conditions in the laboratory, and are thus not simply caused by environmental cycles: they are endogenous, generated by the organism itself (Gerkema, 1992). This major advantage of rhythmic organization of physiology and behaviour concerns the coupling and synchronization of processes, a potential resulting from the 'physiological time' structure which in simple terms, restrict behavioural and physiological events from happening all at once (Gerkema, 1992; Foster and Kreitzman, 2005). They also confer many other advantages such as energy saving and avoidance of UV-mediated DNA damage as well as crucial synchronization with metabolic demands (Langmesser and Albrecht, 2006). Circadian rhythms are biological rhythms which evolved in adaptation to the rotation of the earth and moon in our solar system; they are synchronized with the alternation of day and night (photoperiod), with the tides, the lunar cycle, the annual seasons and feeding rhythms

(Gerkema, 1992; Boujard and Leatherland, 1992; Foster and Kreitzman, 2005). On the other hand non-circadian rhythms; ultradian (less than a day) and infradian (longer than a day) are also oscillating rhythms which at the cellular levels are related to biochemical and neurogenic oscillators, where feedback mechanisms are probably always involved and play an important role (Peters and Veeneklaas, 1992).

It is then logical to say that most of the physiological events (e.g. reproduction, growth, immune system, etc.) that are entrained and/or affected by environmental factors occur within both types of rhythms at some level (e.g. molecular, endocrine, etc.). So for example, while the timing of reproduction (as a whole) in seasonal species seems to be regulated by circannual rhythms over the year, the cascade of specific hormone events in the brain-pituitary-gonad axis is regulated by circadian and ultradian rhythms within each day.

1.2.2 Photic signals

One of the environmental factors that entrain biological rhythms in all vertebrates is light. Light is composed of a complex of external and ecological factors, including spectrum, intensity and photoperiod (Boeuf and Le Bail, 1999; Falcon *et al*. 2006). Importantly, photoperiod is a "noise free" signal as it remains constant over the years and reflects seasonality depending on the latitude. However, spectral content and intensity of the light is more complex as they also vary throughout the year but also during the day (e.g. dawn and dusk) and depends to a large extent to the local weather conditions as well as water absorbance properties. Light energy and wavelength are closely interlinked with shorter bandwidth wavelengths (i.e. blue-green light) having more energy than longer bandwidth (i.e. yellow-red light) which crucially affects the rate at which these are absorbed in water. Thus, light in the blue-green end of the visible

spectrum generally penetrates the water deeper (Lobban and Harrison, 1994). This would suggest that light quality is a highly variable component found in different water bodies and for which different species might be more or less sensitive. Although this notion is not new, studies on the effects of spectral quality and light intensity in teleosts have been limited until recently. Specifically, light intensity and its role in melatonin suppression have been well studied in several commercial and temperate fish species in order to explain some of its effects on reproduction and growth. The concept of light thresholds (intensity) has been deemed especially relevant in salmonids in which melatonin content in the blood was affected in a dose dependant manner by light (Bromage *et al.*, 2001; Porter *et al.*, 2001). In fact light intensity thresholds seem to be very important in the day/night perception of teleosts and are closely associated with spawning and migratory patterns as reviewed by Reebs (2002). Regarding spectral quality, recent studies have been focusing on testing spectral sensitivity of the pineal gland and retina in different fish species both *in vivo* and *in vitro* (Bayarri *et al.*, 2002; Migaud *et al.*, 2006; Ziv *et al.*, 2007; Migaud *et al.*, 2007b). These studies in general, provide evidence of different light sensitivities in teleosts although further studies as to the implications of this are clearly needed.

Although, there are a number of other important environmental factors displaying daily (circadian) and annual (circannual) variations which can also act as potential proximate cues (e.g. temperature, rainfall, food supplies, pheromones), it is photoperiod, or more specifically the seasonally changing pattern of day length, which is probably the most reliable and important signal for the cueing and timing of many physiological events, especially reproduction in most of the temperate fish species, temperature being also important (Zachmann *et al*. 1992; Bromage *et al.*, 2001; Besseau *et al.*, 2006). Many studies have been performed on a range of vertebrates and

temperate teleosts species, however, studies on tropical fish have only recently started and data is still scarce.

1.2.2.1 Seasonal and temperate species

Photoperiod manipulations are commonly used in the culture of temperate water 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 (Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, European seabass *Dicentrarchus labrax* and Atlantic cod *Gadus morhua*). Studies on salmon exposed to continuous light (LL) have enhanced growth in sea cages (Endal *et al.*, 2000) while exposure to winter photoperiods (short days) help to advance or synchronize maturation and smoltification (Berrill *et al.*, 2003). This is also true for male masu salmon (*Oncorhynchus masou*) in which testicular maturation is accelerated by a short photoperiod (8L:16D) and delayed by long photoperiods (16L:8D) (Amano *et al.*, 2000). In rainbow trout, the application of constant long days or continuous light from the winter solstice advanced spawning by up to 6 months depending on the duration of the window of exposure (Bromage *et al.*, 1984; Scott *et al.*, 1984). Further studies have shown that a shift from long to shorts photoperiods (18L:6D and then 6L:18D) can advance or delay maturation and spawning in trout (Davies *et al.*, 1992). The application of similar photoperiodic regimes from summer to autumn was also shown to enhance growth performances by up to 30% in juvenile trout (Taylor *et al.*, 2006). Finally, in Atlantic cod, continuous lighting from summer solstice resulted in a complete inhibition of maturation with a resulting significant growth effect (up to 1 Kg more at time of harvest) by saving energy normally used for gonadal growth (Davie *et al.*, 2007a; Davie *et al.*, 2007b). From these

few examples, it appears very clearly that photoperiod plays a major role in the entrainment and control of maturation and growth in temperate species which importantly are species-specific but also depend on the timing of physiological events (spawning in winter vs. spring in salmon and cod respectively) and the nature of the environment inhabited (mainly temperature variations).

Such significant effects of photoperiod indicate that these temperate seasonal fish species are very sensitive to light. Although the light thresholds needed for reproduction (maturation) are not known yet (Bromage *et al.*, 2001), in general it has been found 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). It is also important to mention that all the photoperiods tested to date have been designed around 24 hour L:D cycles (i.e 6L:18D and 18L:6D) and due to the annual reproductive cycle of the model species (e.g. salmonids) they have to run through a "relatively" long period of time to exert their effects.

1.2.2.2 The tropical batch spawner model

As previously mentioned, photoperiod research in teleosts has been focused on a few intensively produced and commercially important temperate species such as salmon, trout, European sea bass, halibut and more recently cod. However, photoperiodic studies in tropical and especially continuous batch spawner species has, until recently, been very limited. Seasonal temperate species are known to be greatly influenced by the varying day length to which they are exposed in higher latitudes and interesting questions arise as to whether the same mechanisms, effects and pathways are present in tropical and subtropical dwelling species where day length varies little or not

at all. Recent studies in Nile tilapia have shown that tropical species can also respond to light with regards to growth and reproductive performances which could ultimately be used as simple tool by the industry to promote growth and control reproduction in this species. Long and short photoperiods have been tested at different stages, showing in some cases better growth performance and higher reproductive performance in broodstock subjected to longer photoperiods compared to shorter ones (Ridha and Cruz, 2000; El-Sayed and Kawanna, 2004; Rad *et al.*, 2006). ISI were shown to be shorter under long photoperiods with higher fecundities (Campos-Mendoza *et al.*, 2004). Moreover it has recently been reported that further growth enhancement can be achieved when exposing fish to ultradian photoperiod cycles (e.g. 6L:6D) although the mechanisms by which this happens are still unknown (Biswas and Takeuchi, 2003; Biswas *et al.*, 2004; Biswas *et al.*, 2005a). More importantly, these short cycles might are also be capable of disrupting normal reproductive cycles by producing unviable eggs as suggested in a recent study (Biswas *et al.*, 2005a).

All these studies have been based on the observed effects (e.g. growth, fecundity, size, ISI) of different photoperiodic treatments and crucially no description of the circadian light axis and light perception in this species has been done, which could ultimately serve as a basis to design more informative studies and help us to understand the precise mechanisms by which these effects are regulated.

1.2.3 Temperature

Because fish are poikilothermic (thermal conformers) as their body temperature closely mirrors that of the environment, temperature is an important factor in the lives of teleosts and the range in which they thrive is thus species-specific (Ross, 2000). The first environmental manipulations in fish were performed in carps when it was observed

that temperature stimulated maturation (Bromage, 1995; Bromage *et al.*, 2001). There is now considerable evidence suggesting an important role for temperature in cyprinids, catfish and several other tropical and sub-tropical species, including tilapias, although it is likely that its effects are exerted directly on metabolic processes (e.g. cellular metabolism, gene expression and protein activity) (Bromage *et al.*, 2001; Pankhurst and Porter, 2003; Falcon *et al*. 2006). As such, it is still debated whether temperature is a proximate or a permissive factor. In any case, it is clear that it has greater effects in tropical species as opposed to temperate seasonal species where it usually acts more as a secondary cue (Bromage *et al.*, 2001; Pankhurst and Porter, 2003). In the specific case of Nile tilapia, temperatures above 20°C are required to stimulate reproduction, while high temperatures (30–35ºC) considerably reduced the reproductive performance (spawning activity, egg quality and hatching success) (Rana, 1988; Little and Hulata, 2000).

1.2.4 Feeding

As briefly mentioned earlier, the nutritional status of fish as in other vertebrates will depend on the quantity and quality of food available to them. However, because most organisms like fish do not feed constantly but exhibit a 'circadian-like' prandial pattern (Boujard and Leatherland, 1992), food may also serve as an entraining signal which has been the focus of many recent chronobiological studies. Similar to other factors discussed in this work, feed interaction especially regarding circadian and diel rhythms seem to be species-specific. For example it would seem that goldfish (*Carassius auratus*) is capable of adopting diurnal or nocturnal feeding activity patterns thus reacting directly to external factors such as food availability (Sanchez-Vazquez *et al.*, 1996; Aranda *et al.*, 2001; Reebs, 2002). More recently, food anticipatory activity in

this species has been observed, suggesting that feeding can entrain both behavioural (locomotor activity) and certain physiological (digestive related) patterns in this species (Vera *et al.*, 2007). In European seabass, light and feed entrainable oscillators seem to in part account for the dualistic feeding pattern in this species although other unknown factors cannot be ruled out (Sanchez-Vazquez *et al.*, 1995; Sanchez-Vasquez *et al.*, 1998; Aranda *et al.*, 1999a; Aranda *et al.*, 1999b; Azzaydi *et al.*, 2007). As such, studies in these and several other important aquaculture species such as salmonids, sharpsnout seabream (*Diplodus puntazzo*), barramundi (*Lates calcarifer*) and yellowtail (*Seriola quinqueradiata*) (Boujard and Leatherland, 1992; Barlow *et al.*, 1995; Azzaydi *et al.*, 2000; Kohbara *et al.*, 2000; Vera *et al.*, 2006; Noble *et al.*, 2007) provide increasing evidence of the importance and potential advantages of implementing this knowledge within the aquaculture industry.

1.2.5 Other factors

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 including tilapias are: salinity, water quality, pH, stress level, tides and rainfall (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/performance especially in batch spawner species like Nile tilapia. 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).

1.3 The circadian light axis: from light perception to melatonin production

The circadian light axis is comprised of all the different components (structures and pathways) by which light enters the organism and is transformed to a biological time signal (Foster and Hankins, 2002). Importantly, different specialized structures (e.g. pineal complex, retina, parietal eye, deep brain) and pathways have evolved across vertebrates, although the main basic components (e.g. the non-visual photoreceptor), remain (Menaker *et al.*, 1997; Foster and Hankins, 2002; Klein, 2004). Although still in its infancy, our knowledge of non-visual photoreception is improving at a great pace and already several types of photoreceptors have been described (i.e. cone and rod-like) and the mechanisms by which they are regulated have been studied (Falcon *et al.*, 1992; Cahill and Besharse, 1995; Rajendran *et al.*, 1996; Zordan *et al.*, 2001; Foster and Hankins, 2002; Decressac *et al.*, 2002; Vigh *et al.*, 2002; Fu *et al.*, 2005). However, the knowledge in this field is much more advanced in higher vertebrates and especially in mammals and is still scarce in fish.

1.3.1 Mammals: a centralized model

The most complex/evolved form of this axis is found amongst mammals where structures like the eye, brain (e.g. suprachiasmatic nucleus, SCN) and pineal complex form an integrated (non-visual) system where light/dark signalling is exclusively perceived through the eyes, the pineal gland not being photosensitive. However, some evidence also 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, 2003; Fu *et al.*, 2005). This centralised model of circadian organization is thus based on

the retina in the eyes which percieves the photic signals and transfer them 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, although it has been suggested that the secretion of glutamate at the RHT synapses might provide impaired transmission of light signals into the SCN (Holzberg and Albrecht, 2003). In response to the photic signals perceived by the eyes (e.g. light, darkness), the master clock in the SCN stimulates and entrains (by means of intracellular transcriptional/translational feedback loops) the appropriate mechanisms and peripheral oscillators that will ultimately regulate melatonin synthesis in the pineal (Jin *et al.*, 1999; Taghert, 2001; Foster and Hankins, 2002; Fukada and Okano, 2002; Schomerus and Korf, 2005). This model is thus characterised by a pineal gland which has lost its direct photosensitive capabilities, becoming a slave of the brain. It is possible that one of the causes of this evolutionary difference is the position of the pineal in higher vertebrates which has been engulfed by the expanded cerebral hemispheres and cerebellum, thus sitting in a position close to the centre of the brain. It is important to mention that the whole system is dependant on the different components to relay photic information to the rest of the organism (Aronson *et al.*, 1993). As such, ophthalmectomized animals and blind people (with absent or deficient non-visual photoreception) have impairment of the whole mechanism which conveys a biological signal of time in the organism (Zordan *et al.*, 2001). Nonetheless, when this happens, mammals seem to have multiple circadian oscillators throughout their body that can independently synchronize to different environmental stimuli (Zylka *et al.*, 1998; Herzog and Tosini, 2001; Foster and Hankins, 2002; Vansteensel *et al.*, 2007).

1.3.2 The teleost model

Although teleosts represent the most diverse group with the largest radiation in vertebrates (Kotrschal *et al.*, 1998), the circadian axis in fish has been previously described as being decentralized (as opposed to higher vertebrates). The reason for this generalization is that studied species to date have shown independent (directly photosensitive) light perception components (e.g. retina, pineal gland), whereas in mammals these form part of a centralized light entrainment organization dependant on the signalling pathways between them (Falcon *et al.*, 1989; Falcon *et al.*, 1992; Falcon, 1999; Falcon *et al.*, 2007). More importantly, although studied teleosts have the same physical components as the circadian axis in mammals (retina, RHT, SCN and pineal), the main feature that makes them so different to mammals is the lack of a 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; Holmqvist *et al*. 1992; Ekstrom, 1997; Vigh *et al.*, 2002) (Figure 1).

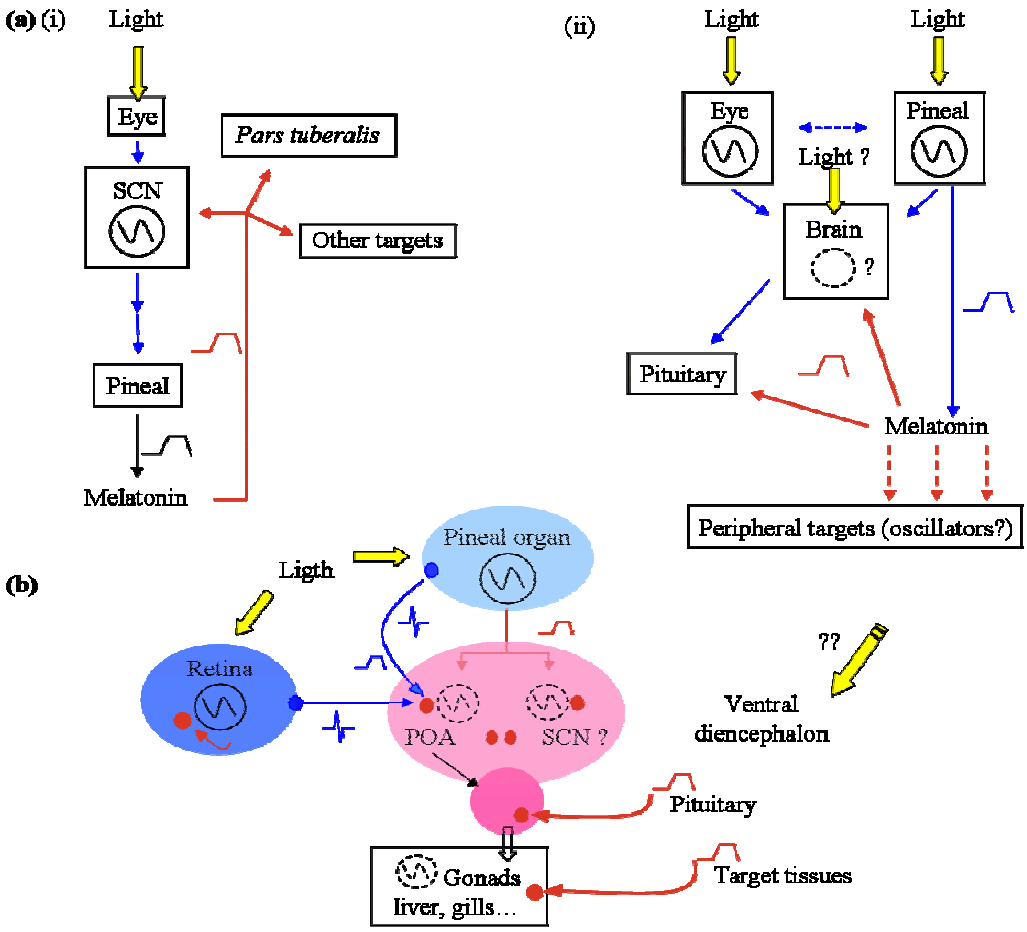

Figure 1.- Photoperiodic and circadian control of neuroendocrine functions.
(a) Fish versus mammals. In mammals (i) a linear flow leads to the rhythmic production of melatonin. Nonvisual information from the retina reaches the SCN of the hypothalamus through the retinohypothalamic tract (blue arrow). 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 other possible photosensitive and circadian structures in the ventral diencephalon (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).

1.3.2.1 The Pineal

The pineal complex in teleosts consists of a pineal and a parapineal organ. The latter consists in most teleosts of a small rounded body located in the left side of the brain above the epithalamus and in teleosts remains in a rudimentary form (Ekstrom, 1997; Vigh *et al*. 2002; Ekstrom, 2003). The pineal gland of fish is an evagination of the roof of the diencephalon which is located below a window in the skull (Ekstrom, 2003). It is a non-visual photosensitive organ that transduces light directly into a biological signal by producing melatonin only during the dark periods, which enters into circulation reaching the target tissues (Zachmann *et al*. 1992; Ekstrom, 1997; Boeuf and Le Bail, 1999; Bromage *et al.*, 2001; Falcon *et al*. 2006). Photoreceptor cells, neurons and ependymal interstitial cells compose the pineal epithelium and it is in these photoreceptors that melatonin is produced, released into the cerebrospinal fluid (CSF) and into blood circulation (Ekstrom, 1997; Falcon *et al*. 2006). Pinealectomy studies have confirmed the pineal as the main source of circulating melatonin at night although it has been suggested that melatonin produced in other tissues or organs such as the eyes could also enter the blood stream, although this has not yet been proven (Reiter, 1993; Porter *et al.*, 1996; Bubenik and Pang, 1997; Ekstrom, 1997; Ekstrom, 2003). In all fish studied to date, with the exception of salmonids, the photoreceptors in the pineal have self-sustained individual oscillators (clocks) which are capable of producing a circadian rhythmic production of melatonin *in vitro* under constant darkness conditions (DD) (Gern and Greenhouse, 1988; Falcon *et al.*, 1989; Zachmann *et al*. 1992; Zachmann *et al.*, 1992; Bolliet *et al.*, 1996; Cahill, 1996; Okimoto and Stetson, 1999; Falcon *et al.*, 2003; Iigo *et al.*, 2003b; Iigo *et al.*, 2004; Ron, 2004; Bayarri *et al.*, 2004a; Iigo *et al.*, 2007). The molecular components and mechanisms of these clocks have been recently well characterized in the zebrafish (*Danio rerio*) not only in the pineal but also in

several other tissues such as eye, liver, heart, brain and kidney, suggesting that the circadian light regulated clock mechanisms are well distributed throughout the organism (Cahill, 1996; Cahill, 1997; Kazimi and Cahill, 1999; Whitmore *et al.*, 1999; Cahill, 2002; Dekens *et al.*, 2003; Vallone *et al.*, 2005). Recently, the classic vision of pineal evolution through the gradual transformation of pinealocytes (a gradual regression of pinealocyte sensory capacity within a particular cell line) has been modified and now it is strongly believed that the evolution of the pineal receptor line (photoreceptor cells, rudimentary photoreceptor cells and non-photosensory pinealocytes) is due to 'changes in fate restriction within the neural lineage of the pineal field', in other words it is not the mature photoreceptors that evolve; but the developmental processes that shape them (Menaker *et al.*, 1997; Ekstrom, 2003; Falcon *et al*. 2006). This theory would explain the difference in the degree of importance of each photoreceptive component type and the difference in light circadian organizations found across vertebrates.

1.3.2.2 The Retina

Compared to higher vertebrates, the eyes of teleosts have also evolved and specialized under the pressure of the range of environments inhabited by fish (Kusmic and Gualtieri, 2000). They also have visual and non-visual photoreceptors capable of circadian entrainment and melatonin production (Cahill and Besharse, 1995; Falcon *et al.*, 2003; Foster and Bellingham, 2004). In most vertebrate species so far investigated, retinal melatonin is produced in parallel with that of the pineal (e.g. in darkness) (Reiter, 1993), however in some cases a reversed (phase shifted) pattern of melatonin production (e.g. high at day) has been found (Gern *et al.*, 1978; Yu *et al.*, 1981; Reiter *et al.*, 1983; Serino *et al.*, 1993; Iigo *et al.*, 1997; Besseau *et al.*, 2006). Moreover, the levels of melatonin found in the eye cups of these species are much higher than those

found in the blood circulation and it is thus believed that retinal melatonin has different roles than pineal melatonin and would be involved in homeostasis of the retina (see 1.4.1.1) (Cahill *et al.*, 1991; Grace *et al.*, 1991; Cahill and Besharse, 1995; Falcon *et al.*, 2003; Siu *et al.*, 2006).

1.3.2.3 The Brain

It has been suggested that the master clock in the SCN of higher vertebrates was derived from encephalic deep brain photoreceptors commonly found in vertebrates (Vigh *et al.*, 2002). Indeed, light has been shown to penetrate deep into the brain (Foster and Hankins, 2002; Vigh *et al.*, 2002), and deep brain photoreceptors have been identified in several vertebrate groups including teleosts such as catfish (*Silurus asotus*), European eel (*Anguilla anguilla*) and zebrafish (Foster *et al.*, 1994; Yoshikawa and Oishi, 1998; Yoshikawa *et al.*, 1999; Shand and Foster, 1999). After initial scepticism, it is now strongly believed that these encephalic photoreceptors have a role in non-visual light perception (e.g. irradiance) and entrainment (Menaker *et al.*, 1997; Yoshikawa and Oishi, 1998; Shand and Foster, 1999; Foster and Hankins, 2002). In teleosts, evidence of this has been shown in the ayu (*Plecoglossus altivelis altivelis*), where ophthalmectomized / pinealectomized animals responded to short photoperiods by undergoing gonadal development as opposed to animals held under long photoperiods (Masuda *et al.*, 2005). Similar results have been found in the catfish (*Heteropneustes fossilis*) (Garg, 1989), Mummichog (*Fundulus heteroclitus*) (Day and Taylor, 2005) and channel catfish (*Ictalurus punctatus*) (Davis *et al.*, 1986) although there are still doubts on the use of such substantial surgery techniques on the brain itself which could lead to other unwanted outcomes (Mayer *et al.*, 1997). Anyway, most of the evidence is indirect and further studies confirming the roles and action mechanisms

of these photoreceptors in the entrainment of circadian rhythms and reproductive physiology are still needed.

1.4 Melatonin: a role model molecule

Melatonin is present in the earliest life forms and is found in all organisms ranging from bacteria to humans (Conti *et al*. 2002; Tan *et al.*, 2003). As such, melatonin seems to have evolved to become a multipurpose molecule with different properties serving as a hormone, a tissue factor, an autacoid, a paracoid or an antioxidant vitamin (Reiter and Robinson, 1995; Beyer *et al.*, 1998; Conti *et al*. 2002; Tan *et al.*, 2003; Barrenetxe *et al.*, 2004). Furthermore, it would seem that melatonin is involved in many other physiological processes and pathways that still wait to be discovered. In this section we will focus only on one of the more reported roles of melatonin, which is a "zeitgeber" (time giver) in vertebrates.

1.4.1 Melatonin synthesis

The melatonin hormone has been one of the most investigated indole molecules that are synthesized in vertebrate pineal glands and recent data show that melatonin biosynthesis in teleosts follows the same pathway as in higher vertebrates (Falcon *et al.*, 1998). This pathway includes the uptake and conversion of tryptophan to 5-hydroxytryptamine (serotonin) through two enzymatic steps: hydroxylation of tryptophan by tryptophan hydroxilase (TPHO) which leads to the formation of 5-hydroxytryptophan which is then decarboxylated by means of the aromatic amino-acid decarboxylase to give serotonin. Serotonin itself can be N-acetylated by a specific enzyme arylalkylamine N-acetyltransferase (AANAT). The subsequent conversion of N-acetylserotonin to melatonin is catalysed by hydroxyindole-O-methyltransferase (HIOMT) (Zachmann *et al*. 1992; Gupta and Premabati, 2002) (Figure 2).

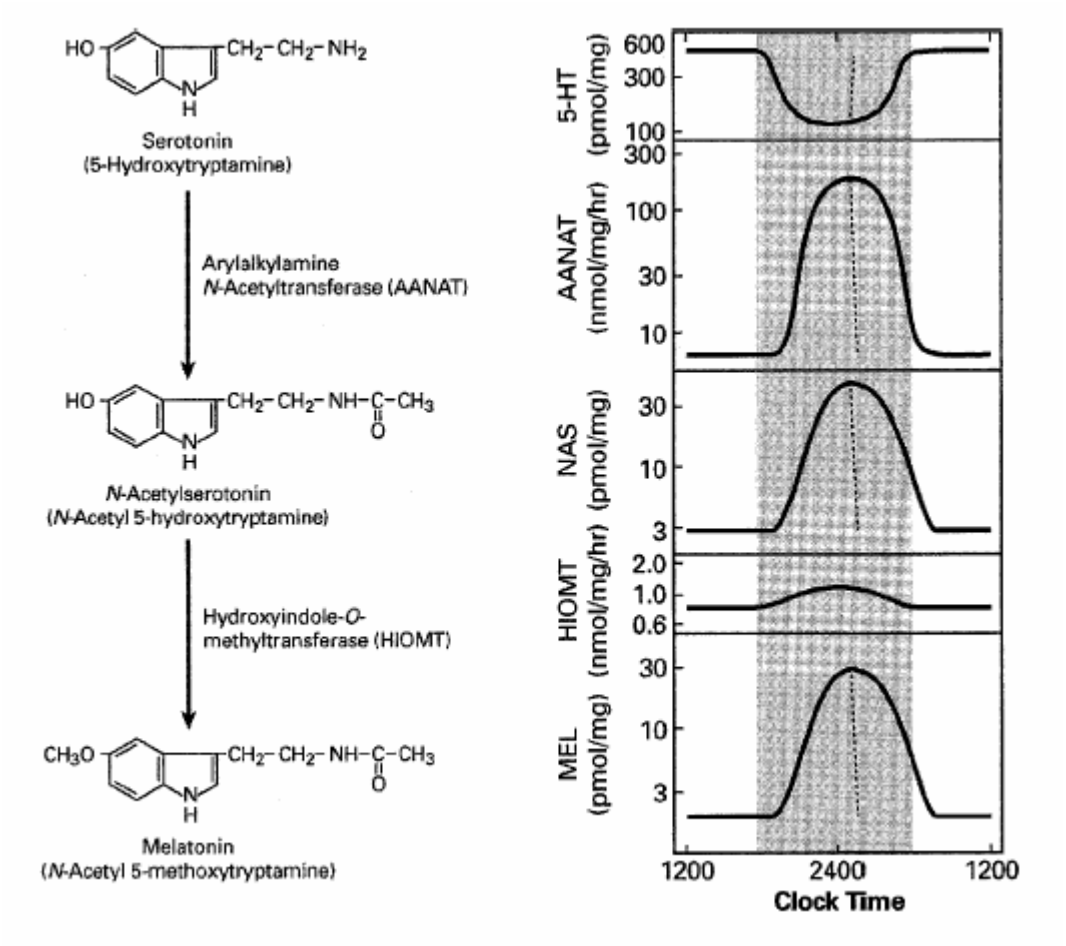

Figure 2.- Melatonin pathway is shown on the left; changes in pineal levels of the compounds and enzymes are shown on the right. Taken from Klein et al. (2002).

1.4.1.1 AANAT

As explained above, the enzyme arylalkylamine N-acetyltransferase (AANAT) is the first enzyme involved in the conversion of serotonin to melatonin. It is found in all vertebrates and is commonly known to be the melatonin rate-limiting enzyme (Klein *et al.*, 1997; Klein *et al.*, 2002). In mammals, birds and anurans, only one type has been found (AANAT), while in teleosts recent findings suggested the presence of at least 3 homologous genes differentially localized between the retina (AANAT-1a and 1b; homologous to non-teleost AANAT) and the pineal (AANAT-2) (Coon and Klein, 2006). The presence of the two different subtypes found in teleosts are attributed to

genome duplication events that happened early in teleost evolution (Coon *et al.*, 1999; Klein, 2004; Coon and Klein, 2006). The finding of differentially regulated AANAT genes should help us to better understand the shifted melatonin production observed in some lower vertebrates. Irrespective of the site, it would seem that AANAT, responsible for melatonin production at night across vertebrates, is regulated at the cellular level by cAMP and at the transcriptional levels by E-boxes and other promoter factors which restrict and regulate the production and activity of the enzyme during the photophase (Falcon *et al.*, 2001; Klein *et al.*, 2002; Iuvone *et al.*, 2005; Schomerus and Korf, 2005; Appelbaum and Gothilf, 2006; Humphries *et al.*, 2007). In the case of vertebrates that have a phase shifted profile (high at day) of melatonin production in the retina such as rainbow trout (Gern *et al.*, 1978; Benyassi *et al.*, 2000; Besseau *et al.*, 2006), seabass (Iigo *et al.*, 1997), gilthead seabream (*Sparus aurata*) (Zilberman-Peled *et al.*, 2004; Zilberman-Peled *et al.*, 2006), rats (Yu *et al.*, 1981; Reiter *et al.*, 1983) and frog (*Rana esculenta* and *Bufo viridis*) (Serino *et al.*, 1993), the precise regulatory mechanisms of AANAT and the specific gene subtypes present are not known yet. What is clear is that AANAT and indeed melatonin production in the retina is most likely to serve a very different physiological role in this organ (e.g. retinal detoxification) compared to that produced by the pineal gland and found in blood circulation (Klein, 2004; Coon and Klein, 2006; Klein, 2006). Importantly, new evidence in rats, showing that HIOMT and not AANAT is the rate limiting enzyme for melatonin production at night (Liu and Borjigin, 2005; Borjigin *et al*. 2007) has drawn new attention to the melatonin production pathway.

1.4.2 A biological signal of day length and its role in reproduction

Melatonin is produced by photoreceptors in the pineal and eyes of all studied vertebrate species (see 1.3.2.1 and 1.3.2.2). In the specific case of the pineal gland, melatonin is only produced at night, accurately reflecting the length of the day throughout the seasons and is thus described as a biological time keeping hormone or "zeitgeber" which entrains circadian (daily) and circannual (seasonal) rhythms in vertebrates (Menaker *et al.*, 1997; Falcon *et al*. 2006). As melatonin is a highly lipophilic molecule which easily crosses the cells of the membrane, the variations in melatonin content are likely to reflect the variations in pineal release (Falcon, 1999). In all vertebrates so far examined, including fish, levels of circulating melatonin are raised during the night and fall to basal during the day (Zachmann *et al*. 1992; Boeuf and Le Bail, 1999; Falcon, 1999; Bromage *et al.*, 2001; Falcon *et al*. 2006). As such melatonin would play a major role in synchronising many behavioural (locomotor, feeding, shoaling and migration activities) and physiological (growth, reproduction, immunity) processes across the animal kingdom. Although there is no doubt of the effects of photoperiod in fish reproduction (see 1.2.2.1), the specific pathways through which the light-dark cycle entrains and regulates reproductive processes in fish are still unknown (Mayer *et al.*, 1997; Falcon *et al.*, 2007).

Because melatonin has been suggested to be one of the factors that mediate the transduction of photoperiodic information to the brain-pituitary-gonadal axis (BGP axis) (Mayer *et al.*, 1997; Amano *et al.*, 2000) its role in fish has been actively investigated. This is normally done by studying correlations between melatonin levels, production and receptors and the physiological events of interest, or by experimental studies involving the removal of the source(s) of melatonin and/or administration of exogenous melatonin. Melatonin injections in most studies have either been without any significant

effects detected or have suppressed reproduction in fishes where long photoperiods stimulate breeding (Mayer *et al.*, 1997). Other studies using implants or administration via the water do not support the concept of melatonin being of major importance for photoperiodic effects in teleosts, probably because the levels artificially reached in the blood were much higher than physiological levels known in the species (Mayer *et al.*, 1997; Falcon *et al.*, 2007). *In vitro* studies have also been undertaken to test the effects of melatonin on gonadal and pituitary cells in the search for a direct link between melatonin and reproduction. In a few cases, melatonin has been shown to have some suppressing effect on *in vitro* gonad steroidogenesis (Mayer *et al.*, 1997), although once again tested using non-physiological concentrations. On the other hand, *in vitro* pituitary studies have recently proven some interaction between reproductive hormone synthesis and melatonin in trout pineals (Futter, 2002). The lack of consistency in the results and doubts regarding the techniques used (both *in vivo* and *in vitro*) it is safe to say that 'there is currently little evidence in support of a major role for melatonin in the control of reproduction in teleosts' (Ekstrom, 1997; Falcon *et al.*, 2007). Importantly the dopaminergic system which is known to inhibit maturation in vertebrates including teleosts (Dufour *et al.*, 1988; Melamed *et al.*, 1996; Levavi-Sivan *et al.*, 2004; Dufour *et al.*, 2005), has recently been successfully linked to the melatonin pathway and strong evidence of signalling between melatonin and this system has arisen in higher vertebrates (Hastings *et al.*, 1997; Kang *et al.*, 2007). Current efforts, linking both systems in fish might provide evidence of how melatonin may act on reproduction.

1.4.2.1 Melatonin receptors

Guanine nucleotide binding protein (G-protein) coupled receptors play a pivotal role in mediating the intracellular effects of numerous hormones, neurotransmitters and

autacoids (Lauffenburger and Linderman, 1993; Gubitz and Reppert, 1999). The use of melatonin radioligands (I-MEL) has led to the localization and characterization of a number of putative melatonin binding sites with well defined and distinct pharmacological profiles in a wide range of vertebrate species including teleosts (Davies *et al.*, 1994; Reppert *et al.*, 1996; Dubocovich *et al*. 1998; Priede *et al.*, 1999; Barrett *et al.*, 2003; Iigo *et al.*, 2003a; Amano *et al.*, 2003a; Amano *et al.*, 2003b). The most recent classification of melatonin receptor subtypes include the mel1a (Mt1 mammals), mel1b (Mt2 mammals) and mel1c by the International Union of Pharmacology (NC-IUPHAR). The mel1c has been found only in non-mammalian vertebrates (birds, amphibians and fish) (Sugden *et al.*, 1997; Vanecek, 1998; Dubocovich *et al*. 1998; Barrett *et al.*, 2003). All these receptors have an equilibrium dissociation constant (Kd) of less than 200 pM and exhibit pharmacological specificity (Reppert *et al.*, 1996; Reppert, 1997). In most vertebrates, high affinity melatonin receptors are present in the pars tubelaris (PT) of the pituitary in mammals and in the SCN amongst other sites (Vanecek, 1998; Priede *et al.*, 1999; Falcon *et al.*, 2003; Barrett *et al.*, 2003; Dardente *et al.*, 2003). In many birds, reptiles, amphibians and fishes, intense I-MEL binding was found in the retina and retinorecipient structures of the hypothalamus, thalamus and mesencephalon, in the thalamic and mesencephalic visual relay nuclei, in the visual integrative areas of the ecostriatum and in the lobus paraolfactorius (Vanecek, 1998).

In mammals, it has been suggested that the receptor MT1 subtype is responsible for the actions of melatonin in reproduction mainly because it is the only one present in the mammalian SCN and pituitary pars tubelaris which is the site of gonadotropin synthesis (Weaver *et al.*, 1996; Mayer *et al.*, 1997; Barrett *et al.*, 2003). Also, it has been found that in Siberian hamsters the Mel1b receptor does not encode a functional

receptor, nonetheless these animals exhibit seasonal reproductive and circadian responses to melatonin, suggesting that this type of receptor is not necessary for mediating reproduction, supporting the hypothesis that the MT1, which does encode a functional receptor in this species, mediates reproductive and circadian responses to melatonin in mammals (Weaver *et al.*, 1996). For further review see Falcon et al. (2007) and Pevet (2003).

So far there is little strong evidence for melatonin playing a physiological role in the photoperiodic control of reproduction in any animal group except mammals and even then it seems the action involves a complex neural circuit of inter-neurons as opposed to direct action to GnRH neurons (Malpaux *et al.*, 1998; Malpaux *et al.*, 2001; Simonneaux and Ribelayga, 2003; Pevet, 2003). It is clear that further experiments and comparative studies are needed to determine the physiological role of melatonin in non-mammalian vertebrates such as fish (Mayer *et al.*, 1997; Falcon *et al.*, 2007).

1.5 Endogenous rhythms and biological clocks

1.5.1 Endogenous rhythms in teleosts

Although a number of very useful models are routinely used in all fields of animal biology and physiology (rat, hamster and zebrafish), teleosts provide chronobiologists with a range of very interesting models due to the large diversity of environments inhabited and strategies adopted. For this reason studying the entrainment and output of endogenous rhythms in fish is becoming more important to better understand vertebrate circadian evolution. Amongst the most common studied rhythms in fish are feeding and locomotor activities. In goldfish, for example, it has been shown that dual phases of locomotor and activity rhythms (with circadian rhythmicity under DD) are present in these fish which are not restricted to a specific light period (e.g. light or darkness), suggesting that the locomotor activity in this species did not necessarily decide the feeding time (Sanchez-Vazquez *et al.*, 1996). Furthermore it has been observed that feeding-entrainable oscillators (FEO) exist in these species which can anticipate meal time to optimize food and nutrient acquisition (Vera *et al.*, 2007). A similar case was found in sharpsnout seabream where a phase shifted locomotor pattern (nocturnal) was observed in some fish (Vera *et al.*, 2006) showing the flexibility and plasticity of locomotor and feeding activity rhythms in some species. By contrast, locomotor activity preference during the light period was observed in the zebrafish as opposed to Senegal sole (*Solea senegalensis*) which displayed nocturnal activity (Bayarri *et al.*, 2004b). Zebrafish studies under constant photic conditions (LL and DD) have shown that the rhythms are circadian in nature and temperature compensated (Hurd *et al.*, 1998). Furthermore, recent work suggested that these locomotor rhythms

are also capable of entrainment by temperature cycles (Lopez-Olmeda *et al.*, 2006). Another study, in the primitive hagfish (*Eptatretus burgeri*), has pinpointed the preoptic nucleus (PON) as the source of endogenous rhythmic locomotor patterns in this species (Ooka-Souda *et al.*, 1993). Regarding reproduction, a study done in trout where fish, exposed to constant photoperiod and temperature for more than four years, showed trout to have an undamped free-running rhythm of spawning suggesting the existence of clock controlled (endogenous) mechanisms at work (Duston and Bromage, 1987). Clearly more studies are needed to locate such endogenous clocks, determine their presence in other species and their specific roles in reproduction or if the observed effects are due to the influence of a more complex series of clock networks and other unknown factors influencing fish physiology as a whole rather than specifically.

In brief, endogenous rhythms such as feeding and locomotor activities have helped dissect and locate common features of circadian function between vertebrates. Further studies in this field, especially in teleosts, could provide important knowledge of circadian rhythmicity. Moreover, such findings could prove crucial in the future sampling designs of fish studies and crucially could also be important to improve husbandry practices, for example feeding at regular intervals instead of randomly throughout the day could improve nutrient uptake and feed conversion rate in fish by anticipating these feeding regimes (Azzaydi *et al.*, 2007).

1.5.2 Clock rhythms

Although key components of the circadian system (photoreceptors involved in light reception, clock mechanisms that regulate the rhythms and neuroendocrine regulation of physiological functions) have been actively investigated and partially characterised in mammalian vertebrates (Simonneaux and Ribelayga, 2003), fish

circadian organization and clock controlled rhythms are still poorly understood. At the core of any circadian rhythm is a network of autonomous endogenous oscillators or biological clocks which in the case of mammals feed information to a master clock found in the SCN, synchronizing vertebrate physiology to the photic conditions (Foulkes *et al.*, 1997; Vigh *et al.*, 2002; Holzberg and Albrecht, 2003; Iuvone *et al.*, 2005). Understanding these endogenous rhythms in fish is still in its infancy. 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 (Herzog and Tosini, 2001; Zordan *et al.*, 2001; Fukada and Okano, 2002; Stehle *et al.*, 2003; Pevet, 2003; Iuvone *et al.*, 2005). In the last decade or so our understanding of the molecular bases of circadian clock mechanisms has substantially progressed. This has been shown to involve transcriptional and translational feedback loops involving a highly conserved set of "clock genes" across vertebrates (Iuvone *et al.*, 2005). In brief, these circadian feedback loops are characterized by positive (e.g. CLOCK, BMAL1 /MOP3) and negative components (e.g. Pers's, Cry's). Positive elements such as Clock and BMAL1 bind to E-box enhancers present in the promoter region of genes from the negative elements including period (Per 1 and 2) or cryptochrome (Cry 1 and 2) genes in the mammalian SCN and AANAT in photoreceptor cells where they promote gene expression activity. In turn proteins are synthesised which will ultimately block gene expression from latter positive elements (Dunlap, 1999; Chen and Baler, 2000; Albrecht, 2002; Iuvone *et al.*, 2005) (Figure 3).

This field is rapidly advancing and more clock-work components are frequently found broadening our view of the complexity of such molecular mechanisms. Also, important questions need to be answered at the integrative signalling level were specific

contributions and roles of central (e.g. SCN) and peripheral pacemakers (i.e. zebrafish cells) need to be answered (Whitmore *et al.*, 1999; Dekens *et al.*, 2003; Carr *et al.*, 2006; Vansteensel *et al.*, 2007; Kohsaka and Bass, 2007).

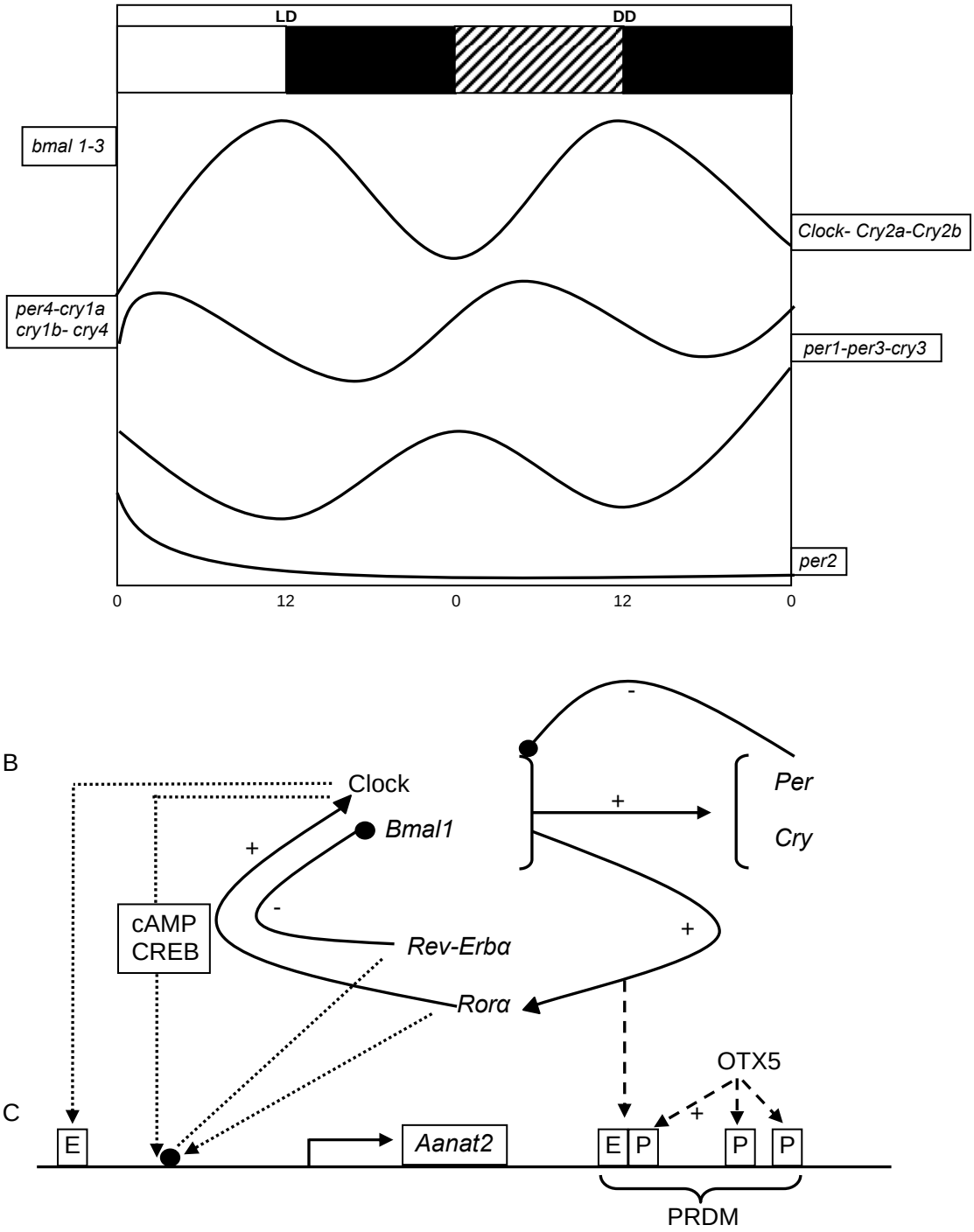

Figure 3.- Clock genes and AANAT2 gene regulation in fish.
(A) Phase relationship of clock gene variation in zebrafish under LD and DD. (B) The circadian feedback loop. (C) Induction of AANAT2 transcription by the clock machinery (interrupted lines) results from concomitant action of CLOCK/BMAL on one E-box (E) and of the photoreceptor specific homeogene (OTX5) on three photoreceptor-conserved elements (P) in the 3' unstranslated region of the AANAT2 gene, the photoreceptor restrictive downstream modulator (PRDM). Other possible pathways are indicated by dotted lines. Taken from Falcon et al. (2006).

1.6 The brain-pituitary-gonadal axis of teleosts

The Brain-pituitary-gonadal (BPG) axis is composed of three physiologically and hierarchically connected structures directly involved in the control of reproduction in vertebrates (Gore, 2002; Weltzien *et al.*, 2004). At all levels of this axis there are a number of key hormones being synthesized, released and acting on target tissues within the brain, the liver or the gonad which has been reviewed in fish by (Davies *et al.*, 1999; Weltzien *et al.*, 2004). In brief, gonadotropin-releasing hormone (GnRH) is produced by a specific group of GnRH neurons in the preoptic area (POA) of the brain which innervate directly to the pituitary, thus stimulating production of two gonadotropins; luteinizing hormone (LH) and follicle-stimulating hormone (FSH). As these hormones enter into circulation and reach the steroidogenic cell in the gonads (follicular cells in the oocytes and sertoli cells in the testes) they stimulate gonadal development (growth and final stages of maturation) by promoting the production of sex steroids and growth factors. Both sex steroids and growth factors are crucial for the regulation of reproduction, either directly in the gonad (paracrine, autocrine) or through positive and negative feedback mechanisms on the hypothalamus and pituitary (Yamamoto *et al.*, 1998; Gore, 2002; Yamamoto, 2003; Weltzien *et al.*, 2004) (Figure 4).

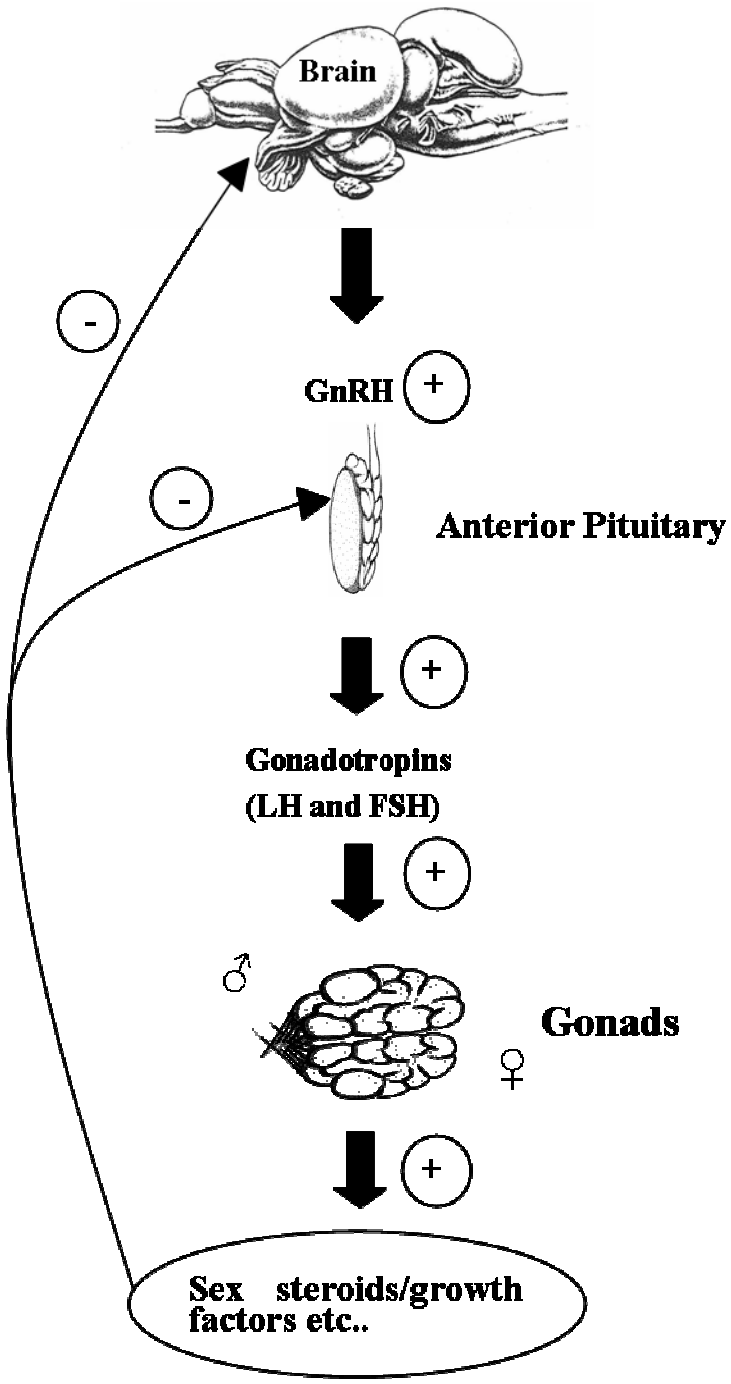

Figure 4.- Simplified diagram of the BPG axis.
The axons of GnRH neurons located in the hypothalamus project to the median eminence where they secrete the decapeptide into the portal vessels of the anterior pituitary. This induces the release of gonadotropins [leuteinizing hormone (LH) and follicle-stimulating hormone (FSH)] into the general circulation, which, in turn, stimulates gonadal functions and the production of sex steroids and inhibin. Sex steroids and inhibin exert negative feedback (-) at the level of the pituitary and hypothalamus. Positive (up regulatory) elements are marked by (+). Modified from Weltzien et al., (2004) and Cariboni et al., (2007).

1.6.1 GnRH

As in other vertebrates, in fish GnRH is a ten-amino acid peptide produced in certain nerve areas of the brain and its fundamental importance is supported by its structural conservation across species (Gore, 2002; Sherwood and Adams, 2005). Specifically, GnRH is expressed and produced very early in development in neurons that form and migrate from the olfactory region to form populations in three to four general regions: the preoptic area (POA), the midbrain and the terminal nerve as well as other areas in the forebrain (Yamamoto, 2003; Sherwood and Adams, 2005; Whitlock *et al.*, 2006; Cariboni *et al.*, 2007). To date, more than 20 forms of GnRH have been found in vertebrates (Figure 5). Fish share two forms of GnRH with other vertebrates; mammalian GnRH (GnRH-I) and chicken GnRH (GnRH-II) but in modern teleosts such as perciforms a third form (GnRH-III) is also found (Lethimonier *et al.*, 2004; Pawson and McNeilly, 2005; Sherwood and Adams, 2005). Again as for AANAT the diversity of GnRH forms found in teleosts is probably due to at least two genomic duplication events (Sherwood and Adams, 2005). Of the three GnRH forms (I, II and III) found in the brain of Nile tilapia and other perciforms, only GnRH-I was found in the POA of the hypothalamus and pituitary, where it is also the most abundant indicating that GnRH-I would be the hypophysiotropic form which plays a major role in gonadotropin secretion (Zohar *et al.*, 1995; Gothilf *et al.*, 1996; Parhar *et al.*, 1996a; Senthilkumaran *et al.*, 1999; Carolsfeld *et al.*, 2000; Hofmann, 2006). The specific role played by the other two forms of GnRH in teleosts is less clear although, based on the more variable patterns of expression in the brain, it has been suggested that GnRH-II acts through its specific receptor (rGnRH-II) as a neuromodulator, but also as a trigger of female sexual behaviour in higher vertebrates (Pawson *et al.*, 2003; Millar *et al.*, 2004; Kauffman and Rissman, 2004a; Kauffman and Rissman, 2004b; Kauffman *et al.*, 2005; Hofmann,

2006). Even less is known of the teleost-specific GnRH-III form, although evidence suggests it is likely to have a similar role in reproductive behavioural activity in male fish (Gore, 2002; Weltzien *et al.*, 2004; Hofmann, 2006) (Figure 5).

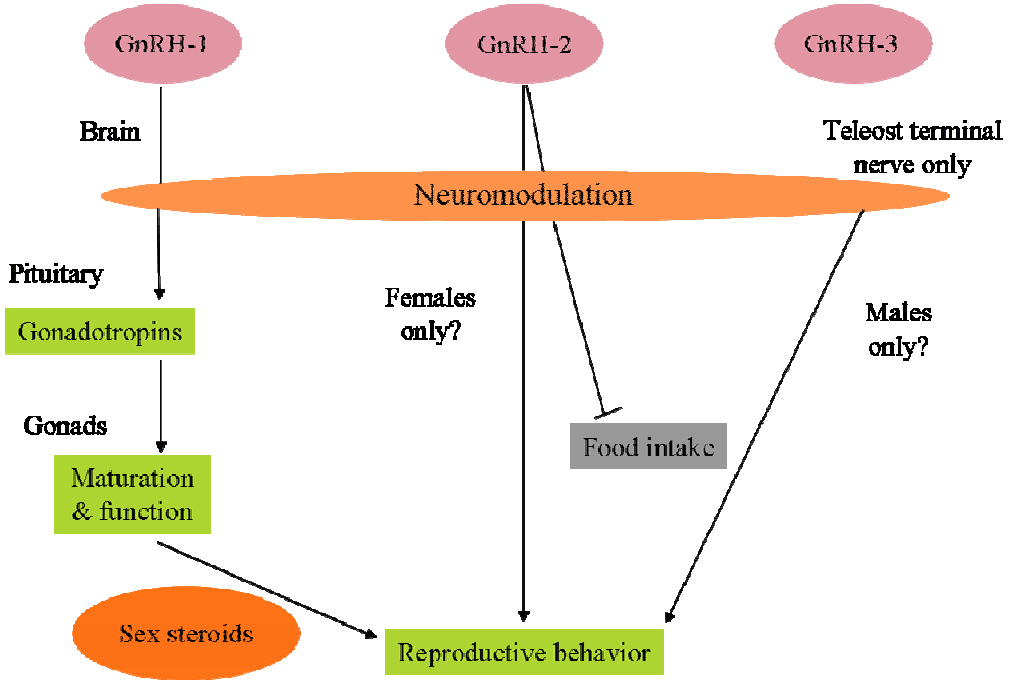

Figure 5.- Three GnRH subtypes influence reproductive behaviour in fish through hormonal and neuromodulatory pathways.
GnRH-I, which mainly controls gonadal maturation through gonadotropin release from the pituitary, probably also has neuromodulatory functions throughout the brain. Both GnRH-II and GnRH-III influence reproductive and probably other behaviours through neuromodulatory actions in the central nervous system. Taken from Hofmann (2006).

1.6.1.1 GnRH gene regulation

The regulation of GnRH gene expression through promoter regions has been studied in a number of animal models including tilapia (Kitahashi *et al.*, 2005; Sherwood and Adams, 2005). Indeed, GnRH mRNA expression studies in Nile tilapia brains have revealed GnRH types and site specificity suggesting different roles (Parhar *et al.*, 1996a; Parhar *et al.*, 1996b). In general, GnRH expression has been found to be regulated at a post-transcriptional level as GnRH mRNA expression has often not been correlated to transcribed product in early development (pre-pubertal). However when the organism enters puberty (attains sexual maturity/competence) a substantial surge in GnRH transcript levels and of its receptors (e.g. rGnRH-I) are commonly observed and used as means to study the mechanisms regulating gonadotropin release and onset of puberty in vertebrates in many species including fish (Gore, 2002; Nocillado *et al.*,

2007; Mohamed *et al.*, 2007). Importantly, as in other teleosts (Kah, 1986; Dufour *et al.*, 1988; Montero *et al.*, 1996; Dufour *et al.*, 2005), in Nile tilapia, dopamine has shown to down regulate the expression of GnRH receptors which ultimately affects gonadotropin stimulation (Safarian *et al*. 2001; Yaron *et al.*, 2003; Levavi-Sivan *et al.*, 2004; Levavi-Sivan and Avitan, 2005).

1.6.1.1.1 GnRH membrane receptors

The actions of GnRH subtypes in vertebrates including fish are mediated through binding to membrane-bound G-protein coupled receptors which through a series of cascade and stimulating events culminate in the mediatory effects of gonadotropin (e.g. LHβ and FSHβ) transcripts in vertebrates, including tilapia (Gur *et al.*, 2001; Yaron *et al.*, 2003; Levavi-Sivan and Avitan, 2005; Pawson and McNeilly, 2005; Chong *et al*. 2005). As it is logical to expect, several subtypes of GnRH receptors also exist. To date three distinct subtypes of GnRH receptors have been found in teleosts: rGnRH-I, rGnRH-II and rGnRH-III. They are in general specific to each one of the respective GnRH subtypes although cross-talk has been suggested to occur (Parhar *et al.*, 2005; Pawson and McNeilly, 2005; Soga *et al.*, 2005). Several other aspects of GnRH receptor expression and regulation in Nile tilapia have been actively studied, ranging from *in vitro/in vivo* mRNA expression, up/down regulation to social interaction (Melamed *et al.*, 1996; Safarian *et al*. 2001; Yaron *et al.*, 2003; Levavi-Sivan *et al.*, 2004; Levavi-Sivan and Avitan, 2005; Rispoli and Nett, 2005; Soga *et al.*, 2005; Au *et al.*, 2006) but also of special importance, work done using laser captured single cell profiling (gpcr) has significantly improved our understanding of regulatory mechanisms of such receptors in Nile tilapia (Parhar, 2003; Parhar *et al.*, 2003a; Parhar *et al.*, 2003b; Parhar *et al.*, 2005; Parhar, 2005).

1.6.2 Gonadotropins

The two main gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are stimulated by GnRHs and secreted by gonadotrope cells in the pituitary gland of vertebrates. LH and FSH consist of α and β subunits, the latter of which gives them specificity for receptor interactions (Yaron *et al.*, 2003). The actions of these hormones are as for the other previously described hormones (e.g. melatonin, GnRH), carried out through specific membrane receptors present in the cell membranes of the target tissues which, once activated, will relay information to the interior of the cell via the cyclic AMP second messenger system (Lauffenburger and Linderman, 1993; Swanson *et al.*, 2003; Yaron *et al.*, 2003); for further review see (Pawson and McNeilly, 2005). Both female and male gonads are the primary target for LH and FSH where they stimulate steroid (e.g. estrogens and 11-KT) by the steroidogenic cells. The potency of each hormone, the steroid being produced and profile will vary with the developmental stage of the gonad and the reproductive strategy of the species, but in general terms FSH is commonly found to be the main driver for incorporation of vitellogenin into the ovary (vitellogenesis) while LH is more important in the final stages of oocyte maturation in females and spermiation in males. In males, both hormones stimulate 11-ketotestosterone (11-KT) production during gametogenesis and a similar role as in females is observed for both gonadotropins (Swanson *et al.*, 2003; Yaron *et al.*, 2003; Weltzien *et al.*, 2004). Moreover, several studies have suggested an *in vivo/in vitro* effect of environmental cues on the regulation and secretion of gonadotropins which could act as neuromodulators of reproductive behaviours in fish (Vanecek, 1999; Parhar *et al.*, 2003b; Hellqvist *et al.*, 2004). Indeed, evidence of differential signalling pathways acting on GnRH subunits have been found

in tilapia, which can explain differential temporal regulation of fish gonadotropins (Melamed *et al.*, 1996; Gur *et al.*, 2001; Gur *et al.*, 2002; Millar *et al.*, 2007).

1.6.3 Sex steroids and feed back mechanisms

The gonads are the end point of the reproductive cascade and serve two main functions; germ cell development (spermatogenesis and oogenesis) and steroidogenesis/growth factor production. Spermatogenesis and oogenesis mark the beginning of puberty and subsequent reproductive cycles. Sex steroids, such as 11-KT in males and the estrogen 17β-estradiol (E_2) which is regulated by aromatase activity in females, have a crucial role in the stimulatory and regulatory effects of these processes, not only at the gonadal level (paracrine) but also as feedback to the brain and pituitary level where they are known to exert their effects by stimulating or inhibiting gonadotropin release (LH and FSH) in a typical endocrine fashion (Mateos *et al.*, 2002; Weltzien *et al.*, 2004; Pawson and McNeilly, 2005; Young *et al*. 2005) (Figure 4).

1.6.4 Kisspeptins and the GPR54 receptor: a new player in game

It is clear from the previous sections that our knowledge of the BPG axis in vertebrates and indeed fish has advanced at a great pace recently. Indeed, most of the endocrine pathways originating from "excited" GnRH neurons and the subsequent release of hormones controlling gametogenesis and final maturation in the gonads are fairly well understood. However, the factors and mechanisms in charge of triggering the GnRH cascade have until very recently been unknown. In fact, in July 2005, Science magazine listed as one of its 125 greatest unanswered scientific questions "What controls puberty?" In 2001, three teams of investigators working in tumour metastasis

discovered the natural ligand of the G-protein coupled receptor 54 (GPR54) (also called AXOR12) which they found to be highly expressed in non-metastatic tumour cells. The ligand originally termed metastin (because of its metastasis inhibitory properties) was discovered to be the product of the Kiss-1 gene (Muir *et al.*, 2001; Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). Then, in late 2003 two independent teams discovered that disabling mutations in GPR54 in mice and men were associated with failure to undergo puberty and presented immature sexual characteristics (hypogonadotropic hypogonadism) (de Roux *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007). Following these findings, the focus on the gene itself and the regulation of its ligand (now called kisspeptin) and its cognate receptor GPR54 were now the targets of many research groups particularly focused on reproduction which has led to very important discoveries in the last few years. It is now known that kisspeptin stimulates gonadotropin secretion by acting directly on GnRH neurons through GPR54 membrane receptors, which have in fact been found in Nile tilapia GnRH neurons (Parhar *et al.*, 2004; Messager *et al.*, 2005; Smith *et al.*, 2006; Kuohung and Kaiser, 2006). Moreover, expression of Kiss-1 mRNA in the brain is also regulated by sex steroid feedback in mammalian models (Popa *et al.*, 2005; Smith *et al.*, 2006). Also, GPR54 has been suggested to have a neuromodulatory role similar to some GnRH subtypes as it has recently has been found to be required for sexual differentiation of the brain and behaviour (Kauffman *et al.*, 2007). Although initial work was done exclusively on mammals, several groups took on the task to determine whether this Kisspeptin/GPR54 system was a conserved feature across vertebrates. The final outcome of this is still pending, but it would seem from the species of vertebrates so far studied (recently including a few teleosts) that this system is conserved and crucial for the activation of the BPG axis (e.g. puberty and sexual maturation). Moreover, because a loss of Kiss-1

or GPR54 signalling cannot be overcome by compensatory mechanisms (as is possible with other levels of the GnRH cascade) (d'Anglemont de Tassigny *et al.*, 2007; Roa *et al.*, 2008), it would seem that this system stands at the top of the known BPG axis in form of a physiological "bottleneck" where the slightest malfunction would consequently disable the rest of the GnRH cascade and BPG axis.

1.6.4.1 Kisspeptin in teleosts

Towards the beginning of this work, the only information about the kisspeptin/GPR54 system known in fish was the cloning and sequencing of the receptor in Nile tilapia which was found to be highly expressed in GnRH (types I and II) neurons of mature fish (Parhar *et al.*, 2004). Since then and just previous to the publication of our work, new evidence of GPR54 expression during puberty in 4 other teleost species became available in cobia (*Rachycentron canadum*) (Mohamed *et al.*, 2007), grey mullet (*Mugil cephalus*) (Nocillado *et al.*, 2007), fathead minnow (*Pimephales promelas*) (Filby *et al.*, 2008) and zebrafish (van Aerle *et al.*, 2008). These showed high homology of the GPRP54 amino acid sequence and strongly suggest that the Kiss1/GPR54 system would be a conserved feature across vertebrates.

2 Aims of thesis

As it is clear from the introduction, there are many environmental and endocrine pathways related to biological rhythms and reproduction fairly well known in higher vertebrates. However, clear gaps in our knowledge is lacking in teleosts especially regarding the *in vivo* entrainment by light and its mediatory effects on reproduction. Also unknown is the role of the Kisspeptin/GPR54 system in fish as research has only recently shed some light to its importance in mammals. The aim of this PhD project was therefore to study some aspects of these two interesting and highly important areas which are believed to be closely interlinked. This research was carried out in a tropical fish species, the Nile tilapia *O. n. niloticus*, which although being considered as a model species for many biological studies has surprisingly not been characterised in terms of environmental sensitivity and reproductive physiology and not used in the field of chronobiology. One of the main reasons for choosing Nile tilapia in this project was the different characteristics (e.g. reproductive strategy and its natural tropical habitat) compared to most of the other fish species studied to date.

The general objectives of this work were to a) describe the circadian light axis with regards to melatonin production and determine potential physiological effects when photoperiod was altered and b) to study the onset of puberty of Nile tilapia with regards to the kisspeptin/GPR54 system to determine potential links as found in higher vertebrates.

Specific objectives are listed below:

- To describe through *in vivo* and *in vitro* techniques, the circadian light axis of Nile tilapia regarding production and inhibition of circulating melatonin levels and presence of endogenous melatonin oscillators.

- Investigate the role of the eyes in the entrainment of circadian rhythms in Nile tilapia.

- Determine the effects of constant illumination and different light intensities on the physiology of growth and sexual development of reared Nile tilapia.

- Determine potential associations between onset of puberty and the surge of expression of reproduction related genes (e.g. rGnRH and GPR54) in Nile tilapia.

- Investigate potential links between the environmental relay of information (e.g. photoperiod) and the Kisspeptin/GPR54 system during the onset of puberty in Nile tilapia.

The results of this research and related findings from collaborating researchers are presented in the following five manuscripts. The manuscripts have either been published (I, III and IV) or ready to be submitted (II and V) in peer reviewed journals in the relevant areas.

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 provided all data related to Nile tilapia. Co-authors provided data from other species (rainbow trout, Atlantic salmon, European seabass, Atlantic cod and African catfish). Submitted manuscript was written in collaboration between all authors.

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Evidence for differential photic regulation of pineal melatonin synthesis in teleosts

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. First, 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.

H. Migaud, A. Davie, C. C. Martinez Chavez and S. Al-Khamees

Reproduction and Genetics Group, Institute of Aquaculture, University of Stirling, Stirling, UK

Key words: circadian axis, melatonin, ophthalmectomy, pineal gland, teleosts

Address reprint requests to Herve Migaud, Institute of Aquaculture, University of Stirling, FK9 4LA, Stirling, UK.
E-mail: herve.migaud@stir.ac.uk

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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 [1–3]. There is extensive literature describing the gross structure and examining the potential role performed by these individual components, in particular the pineal, in nonmammalian vertebrates; however, there is limited work considering the system as a whole and discussing its interaction [4]. 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 [5–7]. 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 [7–9]. 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 [4, 6, 10, 11]. Although there has been much work focused on melatonin as it is the main endocrine signal shown to be regulated by photoperiod [12–15], its role in regulation of physiological rhythms such as reproduction remains unclear in teleosts [4, 16].

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 [3, 17]. In mammals, previous studies have demonstrated through ophthalmectomy [18–22] that photoentrainment is exclusively mediated by retinal photoreceptors and as such pineal photoreceptors have lost their direct light sensory abilities in comparison with lower vertebrates, reducing their role solely to a melatonin secretory gland [6]. However, unlike mammals, in all teleosts species studied so far, to our knowledge, as in birds, ex vivo studies have shown that the pineal gland was directly photosensitive [12–15, 23–28]. Such reports came from studies mainly performed in temperate fish species but also two tropical species, the goldfish *Carassius auratus* [29] and zebrafish *Danio rerio* [30]. In summary two forms of circadian organization have been previously suggested in fish relating to melatonin secretion by the pineal gland [3, 4, 6, 9]: (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 generalize results that one finds in a given fish species to the whole teleost phylogenic class [9], 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 behaviour and locomotor activity (diurnal versus nocturnal) and reproductive strategies (iteropare versus continuous spawner), it is unlikely that one unique circadian organization (retina–pineal gland network) exist in fish. The objective of this study was, therefore, first to compare the effects of ophthalmectomy on melatonin production in a diverse range of teleosts species 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 6–18 hr photophase) except for tilapia and catfish which were held at constant temperature (27 ± 1°C) and photoperiod (12 L:12 D). All experiments were carried out in accordance with the Animal (Scientific Procedures) Act 1986, UK.

Experiment 1: in vivo ophthalmectomy

Fish were acclimated to a 12 L:12 D photoperiod for at least 2 wk before surgery. The fish were anaesthesized using a 1:20,000 concentration of 2-phenoxyethanol solution (0.2 mL/L; Sigma Poole, UK). 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., Hounslow, UK) and cicatrin antibiotic (The Wellcome Foundation Ltd., London, UK) 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 (1 mL/L; Sigma) and then blood sampled at day (12:00 hr) and night (04:00 hr) over two consecutive days (n = 4–8 depending on species and sampling). No sham operation could be

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 2005) NBFRF[a]	150 ± 19 g	86 ± 13 g	600 ± 151 g
Atlantic salmon	*Salmo salar*	Howietown Fisheries (March 2005) MERL[b]	114 ± 12 g	193 ± 37 g	1850 ± 250[d]
European sea bass	*Dicentrarchus labrax*	Llyn Aquaculture (spring 2003) MERL	660 ± 123 g	152 ± 22 g	609 ± 100 g[d]
Atlantic cod	*Gadus morhua*	MMF[c] (spring 2005) 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 g	216 ± 71 g	523 ± 150 g
African catfish	*Clarias gariepinus*	Tropical Facilities at IOA (spring 2005)	160 ± 27 g	420 ± 85 g	

NBFRF, Niall Bromage Freshwater Research Facility; MERL, Machrihanish Marine Environmental Research Laboratories
[a]Niall Bromage Freshwater Research Facilities, IoA.
[b]Machihanish Environmental Research Laboratories, IoA.
[c]Machrihanish Marine Farms (cod hatchery).
[d]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.

performed because of 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 acclimatized to a 12 L:12 D 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-wk 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 per hour and a fraction collector automatically collecting samples every hour after passing through the culture chambers [28]. The culture media (ref: R8755; Sigma) was supplemented with HEPES sodium salt (ref: H3784, 4.77 g/L; Sigma) as a pH regulator with the pH adjusted to 7.4 and penicillin–streptomycin (10 mg/L) and Fungizone (5 g/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 (1 mL/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, because of 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 4700°K Black Body radiator (4700 K CRI 99, 10° spread; Solux, Rochester, NY, USA) providing a light intensity of approximately 12 W/m^2 at the pineal level during the day (measured by a single channel light sensor; Skye instruments, Powys, UK). Only selected media samples were analysed (2–3/day–night periods depending on species) for melatonin levels corresponding to 4, 8 and 12 hr 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., Lutterworth, UK) in phosphate buffer and observed under ×100 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 [28]. All fish were killed by a lethal dose of anaesthesic 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 standardized distance (26 cm) 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 fibre optic cable and cosine corrector (EPP2000c; Stellarnet Inc., Oldsmar, FA, 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 to seven equal narrow bandwidths using bandpass interference filters (Melles Griot Photonics Component Group, Ely, UK) characterized by a Full Width Haft Maximum of 80 nm (centre wavelengths: 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61 nm). Differences in relative transmittance between filters were corrected by the use of neutral density filters in order to balance light intensity at 5 W/m^2, 1.6×10^{15}photons/s/cm^2. Readings were recorded in W/m^2 (400–740 nm) 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 3 pg/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 one-way analysis of variance (ANOVA) using a general linear model with treatment and time as tested factors (replicate nested within treatment). When comparing mean melatonin levels ex vivo (experiment 2, four to six pineal glands per species, two to three day–night periods, two to three samples analysed per period) and penetration of the light through the pineal window (experiment 3), statistical analyses were carried out by ANOVA followed by Tukeys multiple comparison test. Data are expressed as mean + S.E.M. No replicate effects were observed and as such data were pooled. All statistical tests were carried out with Minitab v14.1 (Minitan Ltd., Coventry, UK). The minimum level of significance was set at $P \leq 0.05$.

Results

No significant differences in melatonin profile and levels were observed in ophthalmectomized fish as compared with intact

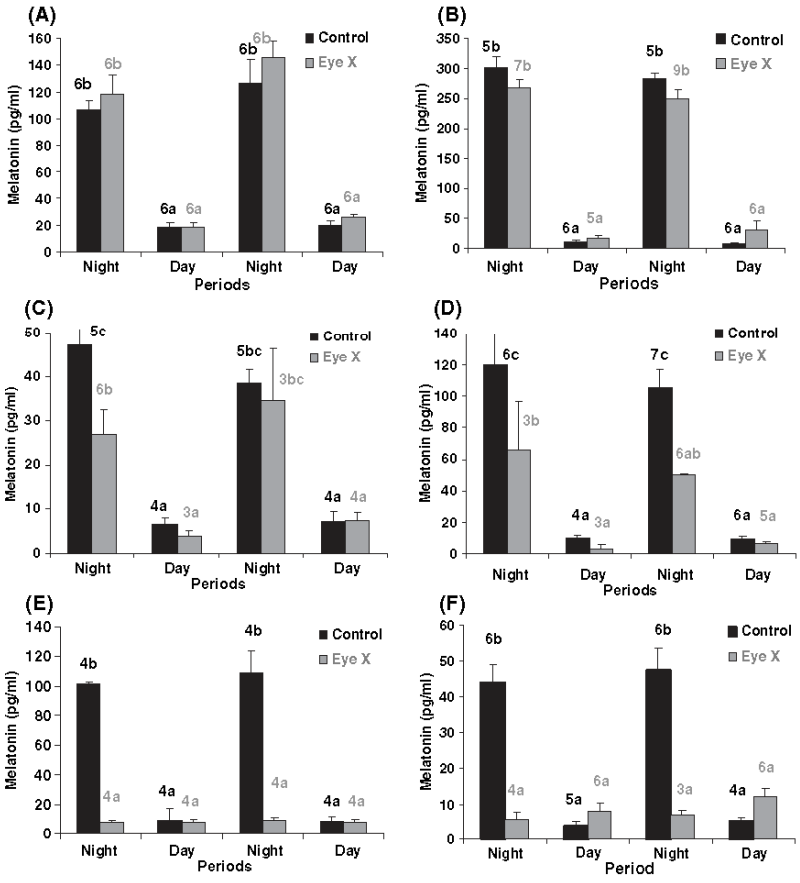

Fig. 1. Effect of ophthalmectomy (Eye X) on in vivo plasma melatonin levels in comparison with intact fish (control) in rainbow trout (A), Atlantic salmon (B), sea bass (C), Atlantic cod (D), Nile tilapia (E) and African catfish (F). Values are expressed as mean ± S.E.M. (three to seven individuals per sampling point). Superscripts denote significant differences (general linear model, $P < 0.05$) and numbers sampling size.

fish in both Atlantic salmon and rainbow trout (Fig. 1A,B). However, in ophthalmectomized sea bass and cod melatonin levels were significantly lower at night as compared with intact fish except in sea bass during the second night (Fig. 1C,D). With regard to Nile tilapia and African catfish, night plasma melatonin increase was suppressed in ophthalmectomized fish with levels remaining comparable with basal day levels (Fig. 1E,F). Relative to night levels in controls, plasma melatonin in ophthalmectomized 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 12 L:12 D cycle, rhythmic melatonin production were observed with low day levels (below 100 pg/mL in rainbow trout and Atlantic cod, and below 500 pg/mL in Atlantic salmon and sea bass) and high night-time levels (mean levels from 2500 to 3700 pg/mL in trout, salmon and cod and 1200 pg/mL in sea bass, Table 2). Melatonin synthesis and release from Nile tilapia pineal glands was very low at night (15.9 ± 2.8 pg/mL), however, a day–night rhythm was still observed although levels were below the assay sensitivity threshold (day levels of 0.6 ± 0.4 pg/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

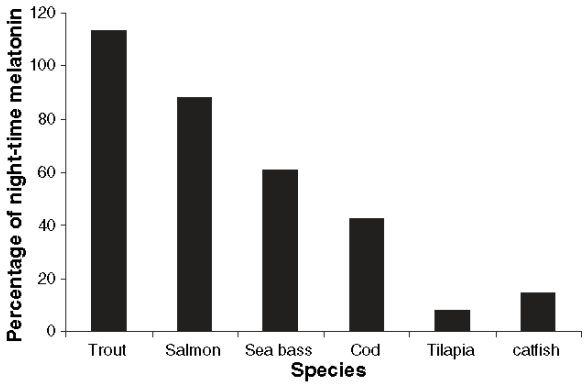

Fig. 2. Summary of the relative percentage of night-time melatonin levels in ophthalmectomized fish as compared with control fish in all species studied. Values are expressed as mean of n = 3–7 individuals over two night periods.

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.

Table 2. Comparisons between melatonin levels in the plasma and produced by pineal glands in culture

Species	Plasma (pg/mL)		Pineal culture (pg/mL/hr)		Relative melatonin production in plasma/pineal	
	Night-time	Daytime	Night-time	Daytime	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)	–	–	–	–

Levels are expressed as mean ± S.E.M. with n representing the number of animals sampled and number of pineal glands, respectively, for in vivo (plasma) and ex vivo (pineal culture) experiments.

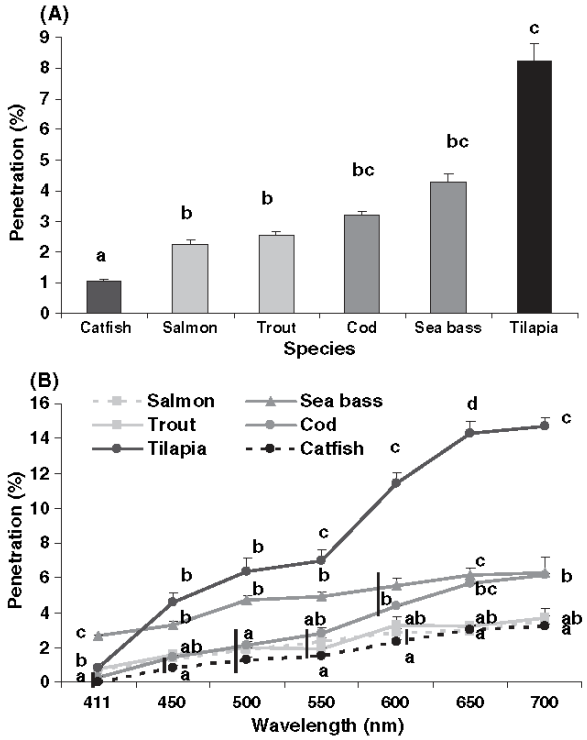

Fig. 3. Percentage of white artificial light (A, Solux bulb) and narrow bandwidth light at 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61 nm (centre wavelengths) (B, Solux bulb + bandpass interference filters) through rainbow trout (n = 4), Atlantic salmon (n = 7), sea bass (n = 6), Atlantic 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.

Light penetration through the pineal window in the six 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 > 550 nm.

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 compartmentalized [31] which contrasts with that of lower vertebrates and invertebrates that possess a network of independent oscillatory components [32]. Many studies have focused on characterizing the function of the pineal organ in fishes [e.g. 3, 12, 13, 25–30]. 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 versus other teleosts [6, 9]. 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 [33] 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 [4, 33]. 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 [18, 34–36] as opposed to higher vertebrates where retinal melatonin synthesis is enhanced in the scotophase as in the

pineal gland [7, 37]. 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 [9] while melatonin from the eyes could be involved in the paracrine protection and adaptation of the retina [34, 36, 38].

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 [39] as well as birds [40, 41] and amphibians [11]. 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 [41, 42]. In fish, studies have shown that three different types of pinealocytes (true and modified photoreceptors and pinealocytes) co-exist in the lamprey or pike [6], although it is thought that pinealocytes are the evolved form of the true pineal photoreceptors; in mammals only pinealocytes remain [6, 8]. 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 that 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 (20 pg/mL/hr) 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 [43] resulting in levels 20 times as high in the CSF as in blood [44]. 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 organization also relying on retinal photoreception has been suggested in a more primitive fish species, the hagfish, *Eptatretus burgeri* [45, 46]. 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, in 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 recognized that the effects of postsurgery 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 [17, 47] where in some cases [47], samples were taken 2-5 wk postsurgery as opposed to 48 hr in the present study. This could thus confirm that postsurgical 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 postsurgery 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 ≥650 nm (>14% versus <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 [45] that the inherent advantage of localized (decentralized) 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 centralized 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 [45]. 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

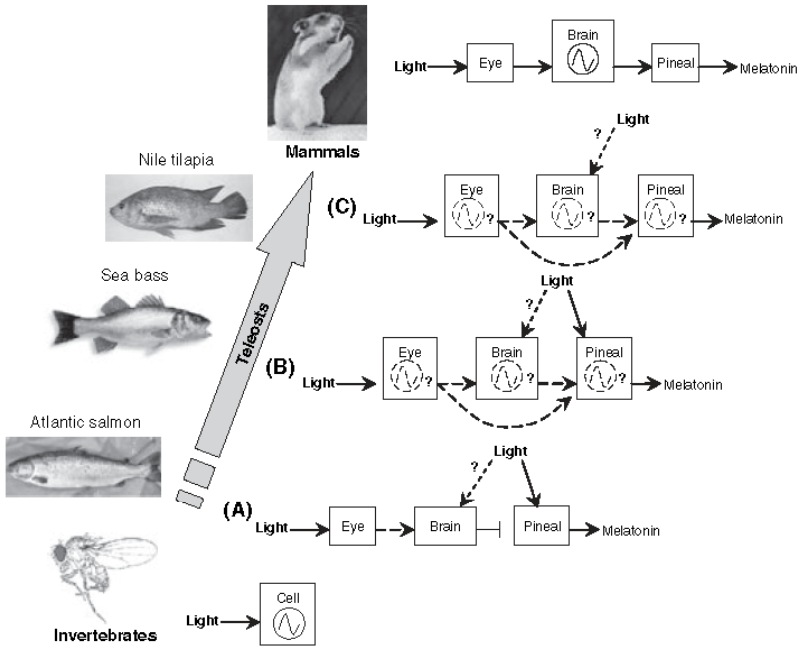

Fig. 4. Suggested evolution of the regulation of pineal melatonin synthesis by the circadian axis in teleosts. In addition to the two types of circadian organization already proposed in fish (A and B), a third type could exist where pineal light sensitivity would be dramatically reduced (C). The regulation 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).

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 [48], the differences in circadian organization 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 [45]. 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) [49], 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.

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References

1. Collin J-P, Voisin P, Falcon J et al. Pineal transducers in the course of evolution: molecular organization, rhythmic metabolic activity and role. Arch Histol Cytol 1989; **52**: 441–449.
2. Takahashi JS. Molecular neurobiology and genetics of circadian rhythms in mammals. A Rev Neurosci 1995; **18**: 531–553.
3. Falcon J. Cellular circadian clocks in the pineal. Prog Neurobiol 1999; **8**:121–162.

4. Ekstrom P, Meissl H. The pineal organ of teleost fishes. Rev Fish Biol Fish 1997; **7**:199–284.
5. Korf H-W, Schomerus C, Stehle JH. The pineal organ, its hormone melatonin and the photoneuroendocrine system. Adv Anat Embryol Cell Biol 1998; **146**:1–100.
6. Ekstrom P, Meissl H. Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors. Phil Trans R Soc Lond 2003; **B**:1679–1700.
7. Simonneaux V, Ribelayga C. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 2003; **55**:325–395.
8. Malpaux B, Migaud M, Tricoire H et al. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J Biol Rhythms 2001; **16**:336–347.
9. Falcon J, Besseau L, Sauzet S et al. Melatonin effects on the hypothalamo–pituitary axis in fish. Trends Endocrinol Metab 2007; **18**:81–88.
10. Steele C, Tosini G, Siopes T et al. Time keeping by the quail's eye: circadian regulation of melatonin production. Gen Comp Endocrinol 2006; **145**:232–236.
11. Wright ML, Francisco LL, Scott JL et al. Effects of bilateral and unilateral ophthalmectomy on plasma melatonin in rana tadpoles and froglets under various experimental conditions. Gen Comp Endocrinol 2006; **147**:158–166.
12. Max M, Menaker M. Regulation of melatonin production by light, darkness, and temperature in the trout pineal. J Comp Physiol A 1992; **170**:479–489.
13. Gern WA, Greenhouse SS, Nervina JM, et al. The rainbow trout pineal organ: an endocrine photometer. In: Rhythms in Fish. Ali MA, ed. Plenum, New York, 1992; pp. 199–218.
14. Alvarino JMR, Randall CF, Bromage NR. Effects of skeleton photoperiods on melatonin secretion in the rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult Fish Manag 1993; **24**:157–162.
15. Randall CF, Bromage NR, Thorpe JE et al. Melatonin rhythms in Atlantic salmon (*Salmo salar*) maintained under natural and out-of-phase photoperiods. Gen Comp Endocrinol 1995; **98**:73–86.
16. Bromage NR, Porter MJR, Randall CF. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 2001; **197**:63–98.
17. Mayer I, Bornestaf C, Borg B. Melatonin in non-mammalian vertebrates: physiological role in reproduction? Comp Biochem Physiol 1997; **118A**:515–531.
18. Underwood H, Barrett RK, Siopes T. Melatonin does not link the eyes to the rest of the circadian system in quail: a neural pathway is involved. J Biol Rhythms 1990; **5**:349–361.
19. Lockley SW, Skene DJ, Arendt J et al. Relationship between melatonin rhythms and visual loss in the blind. J Clin Endocrinol Metab 1997; **82**:3763–3770.
20. Foster RG. Shedding light on the biological clock. Neuron 1998; **20**:829–832.
21. Yoshikawa T, Oishi T. Extraretinal photoreception and circadian systems in nonmammalian vertebrates. Comp Biochem Physiol 1998; **119B**:65–72.
22. Yamazaki S, Goto M, Menaker M. No evidence for extraocular photoreceptors in the circadian system of the Syrian hamster. J Biol Rhythms 1999; **14**:197–201.
23. Falcon J, Brun Marmillon J, Claustrat B et al. Regulation of melatonin secretion in a photoreceptive pineal organ: an in vitro study in the pike. J Neurosci 1992; **9**:1943–1950.
24. Zachmann A, Falcón J, Knijff SCM. Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the white sucker (*Catostomus commersoni*) *in vitro*. Gen Comp Endocrinol 1992; **86**:26–33.
25. Molina-Borja M, Falcon J, Urquiola E et al. Production of melatonin by the gilthead sea bream pineal: an *in vivo* and *in vitro* study. Fish Physiol Biochem 1996; **15**:413–419.
26. Bolliet V, Ali MA, Lapointe FJ et al. Rhythmic melatonin secretion in different teleost species: an *ex vivo* study. J Comp Physiol 1996; **165**:677–683.
27. Yanez JH, Meiss H. Secretion of the metoxyindolamines melatonin, 5-metoxytriptophol, 5-metoxyindoleacetic acid, and 5-metoxytriptamine from trout pineal organs in superfusion culture: effect of light intensity. Gen Comp Endocrinol 1996; **101**:165–172.
28. Migaud H, Taylor JF, Taranger GL et al. Pineal gland sensitivity to light intensity in salmon (*Salmo salar*) and sea bass (*Dicentrarchus labrax*): an *in vivo* and *ex vivo* study. J Pineal Res 2006; **41**:42–52.
29. Iigo M, Kezuka H, Aida K et al. Circadian rhythms of melatonin secretion from superfused goldfish (*Carassius auratus*) pineal glands *in vitro*. Gen Comp Endocrinol 1991; **83**:152–158.
30. Cahill GM. Circadian regulation of melatonin production in cultured zebrafish pineal and retina. Brain Res 1996; **708**:177–181.
31. Morse D, Sassone-Corsi P. Time after time: inputs to and outputs from the mammalian circadian oscillators. Trends Neurosci 2002; **25**:632–637.
32. Tamai TK, Vardhanabhuti V, Arthur S et al. Flies and fish: Birds of a feather. J Neuroendocrinol 2003; **15**:344–349.
33. Kezuka H, Iigo M, Furukawa K et al. Effects of photoperiod, pinealectomy and ophthalmectomy on circulating melatonin rhythms in the goldfish, *Carassius auratus*. Zool Sci 1992; **9**:1047–1053.
34. Falcon J, Gothilf Y, Coon SL et al. Genetic, temporal and development differences between melatonin rhythm generating systems in the teleosts fish pineal organ and retina. J Neuroendocrinol 2003; **15**:378–382.
35. Garcia-Allegue R, Madrid JA, Sanchez-Vazquez FJ. Melatonin rhythms in European seabass plasma and eye: influence of seasonal photoperiod and temperature. J Pineal Res 2001; **31**:68–75.
36. Besseau L, Benyassi A, Moller M et al. Melatonin pathway: breaking the 'high-at night' rule in trout retina. Exp Eye Res 2006; **82**:620–627.
37. Cahill GM, Besharse JC. Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. J Neurosci 1991; **11**:2959–2971.
38. Iuvone PM, Tosini G, Pozdeyev N et al. Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. Prog Retina Eye Res 2005; **24**:433–456.
39. Oshima H, Yamada M, Goto K et al. Pineal and retinal melatonin is involved in the control of circadian locomotor activity and body temperature rhythms in the pigeon. J Comp Physiol A 1989; **166**:217–266.
40. Brandstatter R. Encoding time of day and time of year by the avian circadian system. J Neuroendocrinol 2003; **15**:398–404.

41. Jimenez AJ, Fernandez-Llebrez P, Perez-Figares JM. Central projections from the goldfish pineal organ traced by HRP-immuno-cytochemistry. Histol Histopathol 1995; **10**:847–852.
42. Yanez J, Anadon R. Neural connections of the pineal organ in the primitive bony fish *Acipenser baeri*: a carbocyanine dye tractactivity. J Comp Neurol 1998; **398**:151–161.
43. Tricoire H, Locatelli A, Chemineau P et al. Melatonin enters cerebrospinal fluid through the pineal recess. Endocrinology 2002; **143**:84–90.
44. Skinner DC, Malpaux B. High melatonin concentrations in third ventricular cerebrospinal fluid are not due to Galen vein blood recirculating through the choroid plexus. Endocrinology 1999; **140**:4399–4405.
45. Menaker M, Moreira LF, Tosini G. Evolution of circadian organization in vertebrates. Braz J Med Biol Res 1997; **30**:305–313.
46. Kabasawa H, Ooka-Souda S. Circadian rhythms in locomotor activity of the hagfish, *Eptatretus burgeri* (IV). The effect of eye ablation. Zool Sci 1989; **6**:135–139.
47. Bayarri MJ, Rol De Lama MA, Madrid A et al. Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in sea bass. Brain Res 2003; **969**:175–182.
48. Kusmic C, Gualtieri P. Morphology and spectral sensitivities of retinal and extraretinal photoreceptors in freshwater teleosts. Micron 2000; **31**:183–200.
49. Helfman GS, Collette BB, Facey DE. The Diversity of Fishes. Blackwell Science, Oxford, 1997.

4 Paper II: Confirmation that Nile tilapia eyes are required for night time plasma melatonin

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Confirmation that Nile tilapia eyes are required for night time plasma melatonin

Martinez-Chavez C.C. and Migaud H.

Institute of Aquaculture, University of Stirling, Stirling, UK. FK9 4LA

Abstract

The aim of this work was to confirm previous findings suggesting that the eyes are required for night-time melatonin production in Nile tilapia and further characterise this divergent circadian light axis. To do so, first, melatonin levels at day and night were measured in eyecups and plasma to determine patterns of melatonin production in each case. Secondly, the effects of total ophthalmectomy (as already published) and partial ophthalmectomy on the suppression of melatonin production were determined at night *in vivo*. *In vitro* pineal culture was also performed to determine pineal light/dark sensitivity in culture. Finally, to investigate whether such findings could be related to post-surgery stress rather than disruption of the light circadian axis, melatonin analyses were performed immediately (following 24 h) and 7 days after ophthalmectomy with cortisol levels assessed as an indicator of stress. Our results showed a reversed pattern of melatonin production in the eye cups of Nile tilapia compared to blood circulating levels, suggesting a different role played by melatonin in these two tissues. Then, total ophthalmectomy resulted in the suppression of night-time melatonin production confirming previously reported data in the species. Furthermore, interestingly, although pineals in culture were shown to be photosensitive, night-time melatonin levels were much lower than seen in other species (up to 20-30 pg ml^{-1}). Importantly, partial ophthalmectomy (left or right eye enucleation) did not affect melatonin production at night suggesting melatonin suppression at night in totally ophthalmectomized fish was not due to surgical stress. Finally, when performing sampling immediately or one week post-surgery, no difference in the melatonin profiles were observed. It is therefore unlikely that post-surgery stress would explain such suppression in melatonin production although all fish displayed high cortisol levels (115-168 $pgml^{-1}$) most probably due to social and handling stressors by which it was impossible to assess the

surgery-related stress. Taken together, these results bring further evidence of a new type of circadian light axis in a teleost species where as in mammals, eyes are required to sustain full amplitude melatonin rhythms.

Key words: Ophthalmectomy, Melatonin, *in vitro*, pineal, retina, cortisol, Nile tilapia

Introduction

Melatonin is at the core of the vertebrate circadian system; nonetheless it also fulfils several other paracrine roles many of which still need to be studied (Beyer *et al.*, 1998; Falcon *et al.*, 2003; Barrenetxe *et al.*, 2004; Iuvone *et al.*, 2005; Hardeland *et al.*, 2006). Typically, melatonin is produced at night in two main sites: the pineal and the retina. Melatonin produced by the pineal gland is released into the bloodstream and cerebrospinal fluid and would act as a "zeitgeber" by accurately representing the day-night phases (endocrine role) although substantial knowledge on the underlying mechanisms by which melatonin acts to entrain physiological events remains to be found especially in teleosts. At the retina level, melatonin has been suggested to play paracrine functions like protecting and detoxifying the retina (Falcon *et al.*, 2003; Klein, 2004; Iuvone *et al.*, 2005; Siu *et al.*, 2006). In a number of teleost species, melatonin rhythmic production by both tissues is in phase (low during day and high at night) but in some other vertebrates (including teleosts), melatonin production in retina can be reversed thus being higher during the day than at night (Gern *et al.*, 1978; Yu *et al.*, 1981; Reiter *et al.*, 1983; Serino *et al.*, 1993; Iigo *et al.*, 1997b; Besseau *et al.*, 2006). These differential patterns of secretion could be linked to differences within the melatonin cascade leading to the production of melatonin and more specifically arylalkylamine N-acetyltransferase (AANAT) which is the enzyme, found in all vertebrates, involved in the conversion of serotonin into melatonin (Klein *et al.*, 1997). In mammals, birds and anurans only one subtype is found (AANAT), however in teleosts recent findings have suggested the presence of at least 3 homologous genes differentially localized between the retina (AANAT-1a and 1b; homologous to non-teleost AANAT) and the pineal (AANAT-2) (Begay *et al.*, 1998; Falcon *et al.*, 2003; Tosini and Fukuhara, 2003; Iuvone *et al.*, 2005; Coon and Klein, 2006). Such

information in teleosts helps to better understand these reversed patterns of melatonin production and overall the evolution of the circadian organization.

The circadian light organization (light perception and the melatonin entrainment pathway) in teleosts has, until very recently, been shown to follow a decentralized organization as compared to that of mammals where light input is strictly fed through photoreceptors in the eyes, reaching the master clock in the SCN through a retinohypothalamic tract (RHT) which in turn directly controls melatonin production in the pineal (Reiter, 1993; Herzog and Tosini, 2001; Zordan *et al.*, 2001; Tamai *et al.*, 2003; Ekstrom, 2003). In teleosts however, no homologous circadian master clock has been found to date and independent photoreceptive organs (eyes, pineal, deep brain) and cells in culture have been found to produce robust circadian rhythms on their own (Falcon *et al.*, 1989; Kezuka *et al.*, 1989; Iigo *et al.*, 1991; Zachmann *et al.*, 1992; Bolliet *et al.*, 1996; Cahill, 1996; Okimoto and Stetson, 1999; Iigo *et al.*, 2003; Iigo *et al.*, 2004; Bayarri *et al.*, 2004; Migaud *et al.*, 2006). However, a recent comparative study performed in several teleost species including European sea bass (*D. labrax*), Atlantic cod (*G. morhua*), Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*) has suggested the existence of more than one circadian model of light perception in teleosts (Bayarri *et al.*, 2003; Migaud *et al.*, 2007). Such organizations are characterized by either a photosensitive pineal gland perceiving and producing melatonin (salmonids) or a partial (seabass and cod) and even total (Nile tilapia and African catfish) dependence on the eyes to normally produce full amplitude melatonin rhythms. These findings are undoubtedly of great interest to the chronobiology field and require further confirmation. The aims of this study were thus to further investigate the light perception mechanisms in the tropical batch spawner Nile tilapia. To do so, a series of trials were performed to first determine day/night melatonin rhythms in

eyecups and plasma. Secondly, the study aimed at replicating the ophthalmectomy trial and confirming the resulting suppression of melatonin production at night *in vivo,* as already reported as well as testing the effects of partial eye enucleation (one eye removed). *In vitro* pineal culture was also used to determine pineal light sensitivity in isolation as an indirect confirmation of the lack of photo-responsiveness of the Nile tilapia pineal gland. Thirdly, to investigate whether such findings could be related to post-surgery stress rather than disruption of the light circadian axis, melatonin analyses were performed immediately (following 24 h) and 7 days following surgery with cortisol levels assessed as an indicator of the level of post-surgery stress.

Material and methods

Experimental animals

Mixed sex red Nile tilapia's (220.38 ± 5.46 g) were obtained from the tropical aquarium facilities at the Institute of Aquaculture, University of Stirling. All fish were raised under 12L:12D conditions and were acclimated in the experimental tanks prior to the start of the study. The experimental tanks formed part of a closed water recirculation system maintained at 27 ± 1^{o}C as previously described (Campos-Mendoza *et al.*, 2004). Feeding occurred to satiation with commercial trout pellets (Standard Expanded, Skretting, Cheshire, UK). In all experiments lights were switched on at 08:00 h and turned off at 20:00 h. 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. 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. Experimental tanks were lit using standard 60 watts GLS

bulbs (CPC, Leeds, UK) providing a light intensity of approximately 0.75 W/m^2 at the water surface (measured by a single channel light sensor, Skye instruments, Powys, UK). Ophthalmectomized fish behaviour was monitored using infrared cameras and digital video recording equipment (Pakatak Ltd, Essex, UK). All trials were carried out according to international ethical standards (Touitou *et al.*, 2006). Night sampling was performed under a red dim light with the head of the sampled fish covered.

Experiment 1: Day and night melatonin levels in plasma and eye cups.

In order to determine daily melatonin fluctuations in plasma and eye cups of Nile tilapia, fish (n=5) were blood sampled and eye cups were removed in the middle of the day and night (i.e. 14:00 h and 02:00 h) under a 12L:12D photoperiod. Whole retinas were homogenized in 2ml of phosphate-buffered saline (PBS) (10 mM phosphate buffer containing 140 mM NaCl and 0.1% sodium azide, pH 7.5) with 1% albumin (fraction V; Sigma, St Louis, MO, USA) as previously described (Garcia-Allegue *et al.*, 2001) and 500µl of clear supernatant was analysed. Results are presented in Fig. 1.

Experiment 2: Effects of total or partial ophthalmectomy.

Following acclimation of fish to 12L:12D in the experimental tanks, a total of 48 fish were ophthalmectomised (either totally or partially). To do so, fish were anaesthesised and the ophthalmectomy consisted of cutting out the membrane around the eye, lifting the eye and sectioning the optic nerve. A drop of a 3/1 w/w mix of Orahesive powder (ConvaTec, Squibb & Sons Ltd., Hounslow, UK) and cicatrin antibiotic (The Wellcome Foundation Ltd., London, UK) were applied to the eye socket. No mortalities were observed following the surgery. An intact group of 16 fish was used as a control. The control fish were anesthetised and handled in the same manner as the fish receiving the

surgery except that no surgical procedure was performed. No sham operation could be performed because of limitations in fish number and restrictions placed by our local ethical review committee. The surgical procedure happened during the afternoon (17:00 h) and sampling (n=4 / treatment) commenced during the night of the same day for two complete cycles (Fig. 2).

Experiment 3: *In vitro* melatonin production by isolated pineal glands.

To reveal if the pineal organ of tilapia was photosensitive and capable of producing daily melatonin fluctuations on its own, pineal culture studies were carried out according to (Migaud *et al.*, 2007). In total, 6 pineals of Nile tilapia were dissected 2 hours before darkness (22:00 h) and placed individually in flow through chambers inside an *in vitro* cabinet (26 ^{0}C). Pineals were then exposed to a normal LD cycle and hourly samples were collected during two consecutive 12L:12D cycles. Two night (02:00 h and 06:00 h) and two day (14:00 h and 18:00 h) sample points were performed and melatonin analysed (Fig. 3).

Experiment 4: Effects of post-surgery stress on melatonin rhythms in ophthalmectomised *vs*. intact Nile tilapia.

This trial was designed to investigate whether ophtalmectomy post-surgery stress monitored through plasma cortisol levels could affect LD melatonin rhythms in Nile tilapia. To do so, total ophthalmectomy was repeated according to the protocol described above (Exp. 2) while another group of fish were left intact as a control. Sampling occurred at two different times post-surgery (in the following 24 hrs and a week later), where 4 fish were sampled at each time point during two dark and one

consecutive light periods (Fig. 4). Melatonin and cortisol analysis was performed as described below.

Melatonin assay

Homogenized eye cups and blood samples were centrifuged at 1200 G for 15 min at 4^0C (Jouan CT422) and plasma stored at -70 ^{0}C until analysed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). 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 $pgml^{-1}$. Aliquots of pooled rainbow trout plasma were used as quality controls (QCs) and the intra- and inter-specific assay coefficients of variation were 2.1-3.7 % and 5.5 %, respectively.

Cortisol assay

Cortisol was assayed by radioimmunoassay previously described by Ellis *et al*. (2004) and modified by North *et al*. (2006). Aliquots of pooled rainbow trout plasma were used as quality controls (QCs) and the intra-specific assay coefficients of variation was 4.2 %.

Statistical analysis

The data were analysed using MINITAB® Release 14.13 (Minitab Ltd., Coventry, UK). Data are expressed as mean ± SE values. When necessary data was transformed using the natural logarithm and all data conformed to normality and homogeneity of variance following Kolmogorov-Smirnov and Bartlett's tests. Melatonin levels were analysed using a General Linear Model (Zar, 1999) followed by Tukey's post hoc tests or

unpaired t test to identify where significant differences occurred. Significant differences were determined at $p \leq 0.05$.

Results

Experiment 1: Day and night melatonin levels in plasma and eye cups.

Significant day-night melatonin profiles were shown in both the eye cups and blood (Exp. 1, Fig. 1a and b). Mean melatonin levels in the eye cups were significantly higher (619.6 ± 17.2 pg/eye) during the day than during the night (140.8 ± 53.6 pg/eye), in contrast plasma levels which were significantly higher at night (49.7 ± 6.7 $pgml^{-1}$) compared to day (16.02 ± 1.9 $pgml^{-1}$).

Experiment 2: Effects of total or partial ophthalmectomy.

Ophthalmectomy resulted in the complete suppression of plasma melatonin synthesis and release at night (both night periods tested) with similar circulating blood levels to the day period (<20 $pgml^{-1}$) (Exp. 2, Fig. 2). However, partial (i.e. left or right) enucleation did not significantly affect plasma melatonin production at night, except during the first night period tested where right eye ophthalmectomy resulted in significantly lower plasma melatonin levels (67.45 ± 5.40 $pgml^{-1}$) than at night in control fish (95.26 ± 6.80 $pgml^{-1}$). However, right or left eye enucleated fish showed a tendency towards lower levels as compared to control fish at night during both night periods tested. A normal day-night plasma melatonin rhythm was observed in control fish. Video recordings showed a return to normal feeding and social dominant-submissive behaviour within 24 hrs post-surgery.

Experiment 3: *In vitro* melatonin production by isolated pineal glands.

In vitro results showed that Nile tilapia isolated pineal glands responded to day-night rhythm with higher melatonin levels (20-30 pg ml^{-1}) at night than during the day where levels were close or below the sensitivity threshold of the assay (<4 pg ml^{-1}, Fig. 3).

Experiment 4: Effects of post-surgery stress on melatonin rhythms in ophthalmectomised *vs*. intact Nile tilapia.

Ophthalmectomy performed in this experiment resulted in a suppression of night-time melatonin production as observed in experiment 2. No differences between the two times post-surgery were observed with levels remaining in all cases basal as during the day (<20 $pgml^{-1}$). Mean (day-night) cortisol levels of enucleated (124.1 ± 31.5 and 115.3 ± 28.8 $pgml^{-1}$) and control (169.0 ± 41.4 and 138.1 ± 41.0 $pgml^{-1}$) fish were found to be high at both sampling points respectively.

Discussion

Although the pineal gland is undoubtedly the main site of melatonin production in vertebrates, it has been suggested that melatonin can also be produced by other organs (Cahill *et al.*, 1991; Bubenik and Pang, 1997). Many reports have specifically shown rhythmic melatonin production in the retina of vertebrates including fish (Gern *et al.*, 1978; Iigo *et al.*, 1997a; Iigo *et al.*, 1997b). Furthermore, it has been suggested that melatonin produced by the retina could contribute to circulating plasma levels (Reiter, 1993; Tosini and Fukuhara, 2003), although no evidence of this has been given to date. As shown previously in different species of vertebrates (including some teleosts), in our study retinal melatonin production was higher during the day than during the night (>4 fold) whereas melatonin production in the blood was found to increase during the dark phase (>3 fold), providing the fish with an accurate biological reading of the length of

the day (endocrine role) (Coon *et al.*, 1999). Given that the amplitude in melatonin levels between both periods (day/night) in the eyes appeared to be much higher and reversed to those found in the plasma, we suggest that melatonin found in the eye cups of Nile tilapia is most likely having a different role than that melatonin produced by the pineal gland. Retinal melatonin could play a paracrine role by protecting the retina against harmful free radicals generated by light during the day as already suggested (Benyassi *et al.*, 2000; Simonneaux and Ribelayga, 2003; Klein, 2004; Besseau *et al.*, 2006). A similar reversed pattern of melatonin synthesis between the plasma and retina has also been reported in the European sea bass (Iigo *et al.*, 1997b; Bayarri *et al.*, 2002) and may be a common evolutionary characteristic amongst other perciforms. A similar phase shifted (180^0) pattern occurs in trout (*O. mykiss*) where at least two different melatonin synthesis cascades appear to exist. In trout, this is possible due to the presence of different precursor subtypes differentially expressed in the pineal (AANAT2) and the retina (AANAT1) (Falcon *et al.*, 2003; Besseau *et al.*, 2006). The recent discovery of more than one AANAT1 subtype (AANAT1a and b) in two species of puffer fish and medaka further complicates the picture in teleosts and also suggests multiple roles for these genes other than melatonin synthesis (Coon and Klein, 2006). Importantly, until the regulation of different patterns of melatonin synthesis in the eye and pineal of Nile tilapia are clearly understood direct comparison of melatonin levels produced at both sites is not straightforward. Furthermore, the out of phase production of melatonin, together with the higher melatonin levels found at night in retina (compared to plasma) do not rule out the possibility that retinal melatonin production contribute to circulating levels, which could in part explain basal circulating levels (<20 $pgml^{-1}$) in this and other species and thus will need to be addressed in the future.

Irrespective of the role of the retinal and pineal melatonin, the previously reported results on the role of the eyes in the control of the pineal melatonin production Migaud et al. 2007) are of great interest to the Chronobiology field as for the first time a "mammal-like" circadian light axis was suggested in teleosts without considering endogenous rhythmicity nor the existence or not of a SCN like structure in fish. These findings clearly required further investigations. In the current study, *in vivo* (exp. 2) and *in vitro* (exp. 3) experiments were performed to confirm the effects of ophthalmectomy on suppression of circulating melatonin levels and determine the effects of partial ophthalmectomy. It is first important to mention that no mortalities were observed following the surgery and that, through video recording, it was possible to demonstrate that fish normally fed within 24hrs following the surgery and even displayed normal social behaviour typical of this species (i.e. dominant display). The results of the ophthalmectomy showed that while control and partially ophthalmectomized fish continue to produce circulating melatonin in a normal circadian pattern (low at day and high at night except for right eye enucleated fish during the first dark period), plasma melatonin was completely suppressed at night in totally ophthalmectomized fish with levels remaining basal as during the day. These results confirmed that the eyes are needed for the pineal gland to normally produce and secrete melatonin into the blood circulation. Although not a novel finding by itself, it is felt important that such an important divergent circadian light axis was confirmed in fish. Importantly, a similar disruption of the light circadian axis by ophthalmectomy in mammals has provided proof of novel non-visual photoreceptors in the eyes which are fundamental for the circadian light axis and thus photoentrainment of endogenous rhythms through the RHT pathway (Herzog and Tosini, 2001; Zordan *et al.*, 2001).

In vitro results also confirmed that Nile tilapia pineal glands do not follow the general photo-responsiveness pattern observed in all teleosts studied so far. Indeed, numerous reports on a range of teleosts have shown that fish pineal glands are photosensitive and when placed in culture, produce daily melatonin rhythms with typical low at day and high at night levels and, with the exception of salmonids, are also capable to sustain these rhythms under constant darkness (Falcon *et al.*, 1989; Bolliet *et al.*, 1996; Ron, 2004; Migaud *et al.*, 2006; Iigo *et al.*, 2007). More importantly, night time *in vitro* melatonin levels in these species are typically at least ~10-30 fold higher than those found during the same period in plasma (Kezuka *et al.*, 1989; Zachmann *et al.*, 1992; Iigo *et al.*, 2004; Takemura *et al.*, 2006; Migaud *et al.*, 2007). However, in the current study, although isolated pineal glands appeared to be directly photosensitive by producing melatonin during the dark periods, their sensitivity to day-night rhythm appeared to be far less pronounced than previously observed in other fish species with very low levels produced at night (<30 pg ml^{-1} equivalent to plasma levels at night). As a comparison, levels >2-3 ng ml^{-1} have been reported in temperate species such as sea bass or Atlantic cod equivalent to ~25-30 fold levels measured in the blood (Bayarri *et al.*, 2004; Migaud *et al.*, 2006; Iigo *et al.*, 2007). These results would indicate that Nile tilapia pineal glands are somewhat different than in other fish species previously studied. It must, however, be acknowledged that although *in vitro* pineal culture is a standard procedure routinely used in our laboratory, further optimization of the technique in this species might still be needed. Taken together, these *in vivo* and *in vitro* results confirmed that photic stimulus from the eyes (and/or deep brain photoreceptors) seem to be required for the rhythmic pineal melatonin production as opposed to all other teleosts studied to date (Gern and Greenhouse, 1988; Falcon *et al.*, 1989; Zachmann *et al.*, 1991; Iigo *et al.*, 1991; Iigo *et al.*, 1998; Bayarri *et al.*, 2003; Iigo *et al.*, 2007;

Migaud *et al.*, 2007). However, the possibility that the melatonin suppression was due to post-surgery stress could not be overlooked. In the current study, no differences were observed when fish were sampled immediately (next 24hrs following surgery) or one week following ophthalmectomy, with the same night-time melatonin suppression. Although stress levels through the measurement of plasma cortisol were monitored, it clearly appears that sampling procedure was not appropriate as high levels were recorded in ophthalmectomized fish as well as intact control fish. Such high levels, were comparable to those found in stress related studies in Nile tilapia (Vijayan *et al.*, 1997; Barcellos *et al.*, 1999; Al-Khamees, 2000; Barreto and Volpato, 2007) and might therefore have been an artefact of sampling. Previous studies have shown that cortisol levels in tilapia (*O. mossambicus*) rose two-fold only 4 minutes after netting and handling (Ron *et al.*, 1995) and in Nile tilapia higher levels to those found in the current study were reached 5 minutes after handling (Al-Khamees, 2000). Indeed, when analysing the present data, a clear effect of sampling time between the four individual fish sampled at each time point was observed with low levels (up to 50 ng/ml) in the first fish sampled irrespective of the photoperiod and increasing levels in the following fish (data not shown). However other factors such as low stocking density (4 fish/tank) known to lead to dominance and aggressive behaviour (stressors) in Nile tilapia may have also contributed to the high levels observed in controls and enucleated fish (Barcellos *et al.*, 1999; North *et al.*, 2006; Barreto and Volpato, 2006; Overli *et al.*, 2007). Other possible stressors would include the waterborne cortisol released from ophthalmectomized fish in the recirculating system. Nevertheless, no difference was observed between controls and ophthalmectomized fish when only the first individuals sampled (at each period and treatment) are considered. This could suggest that suppression of night melatonin in ophthalmectomized fish would not be due to high

stress levels. We would argue then that cortisol is not the most useful parameter to monitor stress response on the light entrainment pathway as it would seem not to affect the capacity of fish to synthesize melatonin although interestingly it has been suggested to have a stimulatory effect in melatonin production in trout (Larson *et al.*, 2004).

The new evidence presented in this paper confirms the existence of a more centralized circadian organisation in a teleost similar to that of mammals. However, although comparable elements of the circadian axis of mammals (SCN, RHT) are present in teleosts, it is the lack of a central pace maker that sets them apart from teleosts (Tilgner *et al.*, 1990; Holmqvist *et al*. 1992). However, several other structures and components such as the preoptic nucleus, deep brain photoreceptors, melanopsin's, visual pathways and/or cerebrospinal fluid may act as similar circadian components in fish by mediating light information directly or indirectly (Wullimann and Meyer, 1990; Skinner and Malpaux, 1999; Nicholson, 1999; Tricoire *et al.*, 2002; Drivenes *et al.*, 2003).

This study strongly suggests the involvement of the eyes in the circadian light axis of Nile tilapia in the melatonin generating system which could also potentially involve the brain (through neural pathways such as the retinohypothalamic tract) as occurs in mammals (Herzog and Tosini, 2001; Lee *et al.*, 2003), although this remains to be confirmed through ultra structural, binding and gene expression studies. Further ophthalmectomy studies and their effect on circadian behaviour (i.e. feeding, locomotor activities) disruption should enable us to create a better picture of the complexity of this new system and the importance of these findings.

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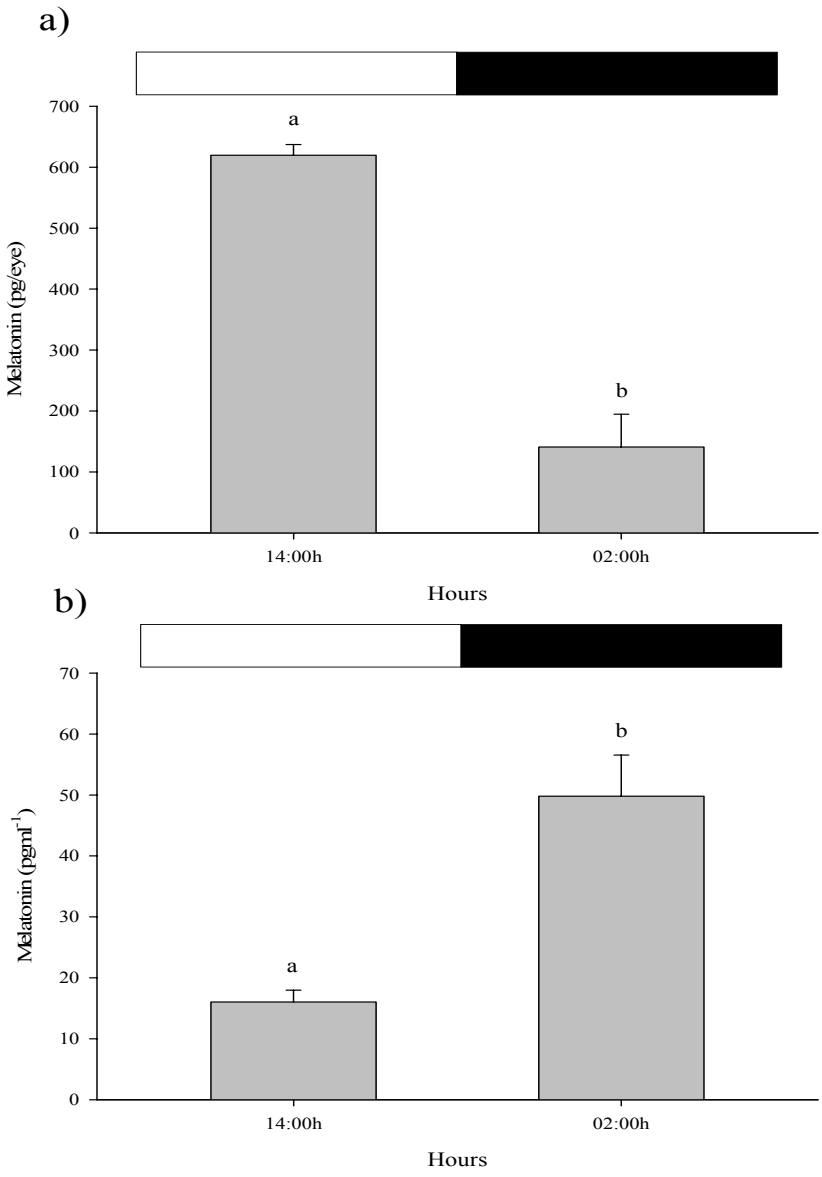
Figure 1. Mid-day and mid-night melatonin levels in a) eye cups and b) plasma of Nile tilapia (n=5). Data is presented as mean ± SE. Superscripts indicate significant (p<0.05) difference between sample points. Open and filled boxes indicate photophase and scotophase, respectively.

Figure 2. Mid-day and mid-night circulating plasma melatonin levels in fully enucleated (enucleated), partially enucleated (right/left) and non-enucleated (control) fish during 2 consecutive days, following surgical ophthalmectomy procedure. Data is presented as mean ± SE (n = 4). Superscripts indicate significant (p<0.05) difference between sample points and treatments. Open and filled boxes indicate photophase and scotophase, respectively.

Figure 3. Mean i*n vitro* melatonin levels produced by isolated Nile tilapia pineal glands maintained in a flow-through culture system during 24 h under LD. Each point represents mean ± SEM (n = 6). Superscripts (*) indicate levels below the sensitivity threshold of the assay. Open and filled boxes indicate photophase and scotophase, respectively.

Figure 4. Mid-day and mid-night circulating plasma melatonin levels in fully enucleated (enucleated) and intact (control) fish sampled during 36 hrs at night and day either following surgical ophthalmectomy or a week later. Data is presented as mean ± SE (n=4). Superscripts indicate significant (p<0.05) difference between sample points and treatments. Open and filled boxes indicate photophase and scotophase, respectively.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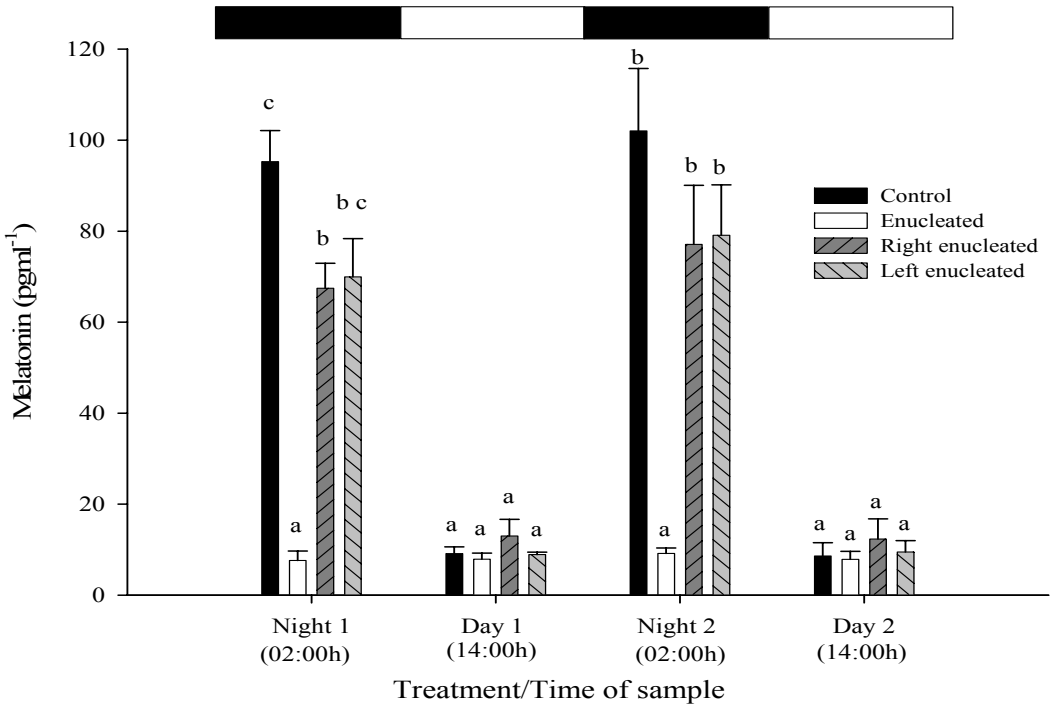
Hours

Figure 1.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Control
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Right enucleated
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Night 1
(02:00h)
Day 1
(14:00h)
Night 2
(02:00h)
Day 2
(14:00h)
Treatment/Time of sample

Figure 2.

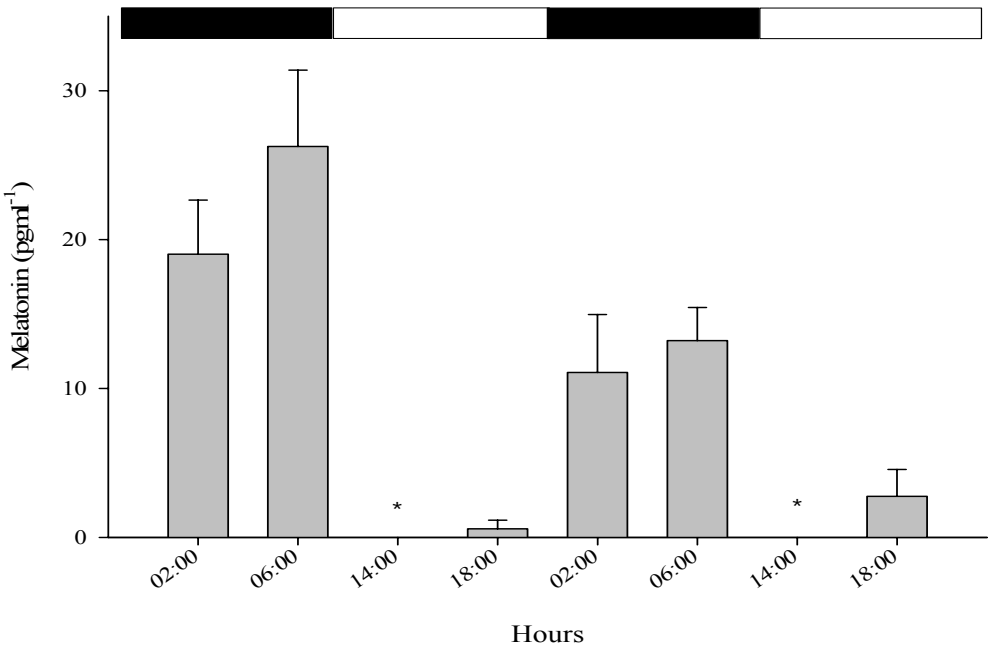
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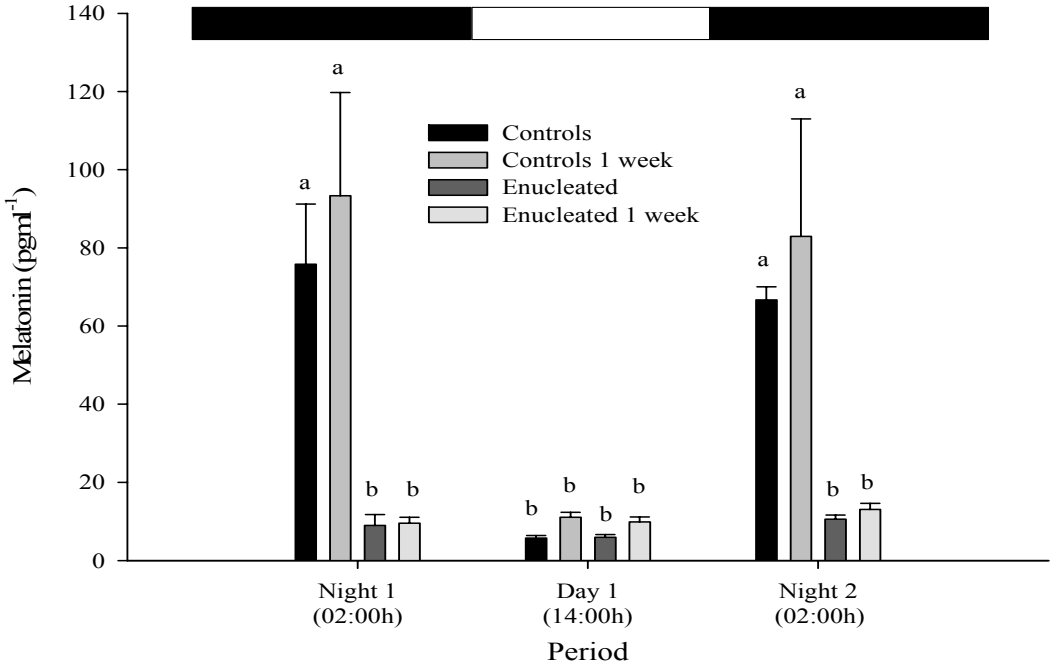
Figure 3.


140
120
100
80
60
40
20
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Melatonin (pgml-1)
Controls
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Enucleated
Enucleated 1 week
a
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b
b
b
a
a
b
b
Night 1
(02:00h)
Day 1
(14:00h)
Night 2
(02:00h)
Period

Figure 4.

References

Al-Khamees, S. (**2000**). Effects of handling and stocking density on indicators of stress in *Clarias gariepinus* and *Oreochromis niloticus*. MSc. thesis, Institute of Aquaculture, University of Stirling, Stirling, Scotland. pp.105.

Barcellos, L.J.G., Nicolaiewsky, S., de Souza, S.M.G. and Lulhier, F. (**1999**). The effects of stocking density and social interaction on acute stress response in Nile tilapia *Oreochromis niloticus* (L.) fingerlings. *Aquaculture Research* 30**,** 887-892.

Barrenetxe, J., Delagrange, P. and Martinez, J.A. (**2004**). Physiological and metabolic functions of melatonin. *Journal of Physiology and Biochemistry* 60**,** 61-72.

Barreto, R.E. and Volpato, G.L. (**2006**). Stress responses of the fish Nile tilapia subjected to electroshock and social stressors. *Brazilian Journal of Medical and Biological Research* 39**,** 1605-1612.

Barreto, R.E. and Volpato, G.L. (**2007**). Evaluating feeding as unconditioned stimulus for conditioning of an endocrine effect in Nile tilapia. *Physiology and Behavior* 92**,** 867-872.

Bayarri, M.J., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2002**). Influence of light intensity, spectrum and orientation on seabass plasma and ocular melatonin. *Journal of Pineal Research* 32**,** 34-40.

Bayarri, M.J., Rol de Lama, M.A., Madrid, J.A. and Sanchez-Vasquez, F.J. (**2003**). Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in seabass. *Brain Research* 969**,** 175-182.

Bayarri, M.J., Garcia-Allegue, R., Lopez-Olmeda, J.F., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2004**). Circadian melatonin release *in vitro* by European seabass pineal. *Fish Physiology and Biochemistry* 30**,** 87-89.

Begay, V., Falcon, J., Cahill, G.M., Klein, D.C. and Coon, S.L. (**1998**). Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebrafish, but not in trout. *Endocrinology* 139**,** 905-912.

Benyassi, A., Schwartz, C., Coon, S.L., Klein, D.C. and Falcon, J. (**2000**). Melatonin synthesis: Arylalkylamine N-Acetyltransferases in trout retina and pineal organ are different. *Neuroreport* 11**,** 255-258.

Besseau, L., Benyassi, A., Moller, M., Coon, S.L., Weller, J.L., Boeuf, G., Klein, D.C. and Falcon, J. (**2006**). Melatonin pathway: breaking the 'high-at-night' rule in trout retina. *Experimental Eye Research* 82**,** 620-627.

Beyer, C.E., Steketee, J.D. and Saphier, D. (**1998**). Antioxidant properties of melatonin-an emerging mystery. *Biochemical Pharmacology* 56**,** 1265-1272.

Bolliet, V., Ali, M.A., Lapointe, F.J. and Falcon, J. (**1996**). Rhythmic melatonin secretion in different teleost species: an *in vitro* study. *Journal of Comparative Physiology: Part B* 165**,** 677-683.

Bubenik, G.A. and Pang, S.F. (**1997**). Melatonin levels in the gastrointestinal tissues of fish, amphibians, and a reptile. *General and Comparative Endocrinology* 106**,** 415-419.

Cahill, G.M., Grace, M.S. and Besharse, J.C. (**1991**). Rhythmic regulation of retinal melatonin: metabolic pathways, neurochemical mechanisms and the ocular circadian clock. *Cellular and Molecular Neurobiology* 11**,** 529-558.

Cahill, G.M. (**1996**). Circadian regulation of melatonin production in cultured zebrafish pineal and retina. *Brain Research* 708**,** 177-181.

Campos-Mendoza, A., McAndrew, B.J., Coward, K. and Bromage, N. (**2004**). Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic

manipulation; effects on spawning periodicity, fecundity and egg size. *Aquaculture* 231**,** 299-314.

Coon, S.L., Begay, V., Deurloo, D., Falcon, J. and Klein, D.C. (**1999**). Two Arylalkylamine N-Acetyltransferase genes mediate melatonin synthesis in fish. *Journal of Biology and Chemistry* 274**,** 9076-9082.

Coon, S.L. and Klein, D.C. (**2006**). Evolution of Arylalkylamine N-Acetyltransferase: emergence and divergence. *Molecular and Cellular Endocrinology* 252**,** 2-10.

Drivenes, O., Soviknes, A.M., Ebbeson, L.O.E., Fjose, A., Seo, H. and Helvik, J.V. (**2003**). Isolation and charachterization of two teleost melanopsin genes and their differential expression within the inner retina and brain. *Journal of Comparative Neurology* 456**,** 84-93.

Ekstrom, P.M.H. (**2003**). Evolution of photosensory pineal organs in new light: the fate of neuroendocrine receptors. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 358**,** 1679-1700.

Ellis, T., James, J.D., Stewart, C. and Scott, A.P. (**2004**). A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. *Journal of Fish Biology* 65**,** 1233-1252.

Falcon, J., Marmillon, J.B., Claustrat, B. and Collin, J.P. (**1989**). Regulation of melatonin secretion in a photoreceptive pineal organ: an *in vitro* study in the pike. *Journal of Neuroscience* 9**,** 1943-1950.

Falcon, J., Gothilf, Y., Coon, S.L., Boeuf, G. and Klein, D.C. (**2003**). Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. *Journal of Neuroendocrinology* 15**,** 378-382.

Garcia-Allegue, R., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2001**). Melatonin rhythms in European seabass plasma and eye: influence of seasonal photoperiod and water temperature. *Journal of Pineal Research* 31**,** 68-75.

Gern, W.A. and Greenhouse, S.S. (**1988**). Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gardnieri*) pineal organs mantained under diel illumination or continuous darkness. *General and Comparative Endocrinology* 71**,** 163-174.

Gern, W.A., Owens, D.W. and Ralph, CH.L. (**1978**). The synthesis of melatonin by the trout retina. *Journal of Experimental Zoology* 206**,** 263-270.

Hardeland, R., Pandi-Perumal, S.R. and Cardinali, D.P. (**2006**). Melatonin. *The International Journal of Biochemistry and Cell Biology* 38**,** 313-316.

Herzog, E.D. and Tosini, G. (**2001**). The mammalian circadian clock shop. *Seminars in Cell and Developmental Biology* 12**,** 295-303.

Holmqvist, B., Ostholm, T. and Ekstrom, P. (**1992**). Retinohypothalamic projections and the suprachiasmatic nucleus of the teleost brain. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Press. Montreal, Quebec. pp.293-318.

Iigo, M., Kezuka, H., Aida, K. and Hanyu, I. (**1991**). Circadian rhythms of melatonin secretion from superfused goldfish (*Carassius auratus*) pineal glands *in vitro*. *General and Comparative Endocrinology* 83**,** 152-158.

Iigo, M., Furukawa, K.H.A., Ohtani-Kaneko, R., Hara, M., Suzuki, T., Tabata, M. and Aida, K. (**1997a**). Ocular melatonin rhythms in the goldfish, *Carassius auratus*. *Journal of Biological Rhythms* 12**,** 182-192.

Iigo, M., Sanchez-Vasquez, F.J., Madrid, J.A., Zamora, S. and Tabata, M. (**1997b**). Unusual responses to light and darkness of ocular melatonin in European seabass. *Neuroreport* 8**,** 1631-1635.

Iigo, M., Kitamura, S., Ikuta, K., Sanchez-Vazquez, F.J., Ohtani-Kaneko, R., Hara, M., Hirata, K., Tabata, M. and Aida, K. (**1998**). Regulation by light and darkness of melatonin secretion from the superfused masu salmon (*Oncorhynchus masou*) pineal organ. *Biological Rhythm Research* 29**,** 86-97.

Iigo, M., Mizusawa, K.Y.Ma., Hara, M., Ohtani-Kaneko, R., Tabata, M., Aida, K. and Hirata, K. (**2003**). *In vitro* photic entrainment of the circadian rhythm in melatonin release from the pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-through culture. *Brain Research* 982**,** 131-135.

Iigo, M., Fujimoto, Y., Gunji-Suzuki, M., Yokosuka, M., Hara, M., Ohtani-Kaneko, R., Tabata, M., Aida, K. and Hirata, K. (**2004**). Circadian rhythm of melatonin release from the photoreceptive pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-thorough culture. *Journal of Neuroendocrinology* 16**,** 45-51.

Iigo, M., Abe, T., Kambayashi, S., Oikawa, K., Masuda, T., Mizusawa, K., Kitamura, S., Azuma, T., Takagi, Y., Aida, K. and Yanagisawa, T. (**2007**). Lack of circadian regulation of *in vitro* melatonin release from the pineal organ of salmonid teleosts. *General and Comparative Endocrinology* 154**,** 91-97.

Iuvone, P.M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D.C. and Chaurasia, S.S. (**2005**). Circadian clocks, clock networks, Arylalkylamine N-Acetyltransferase, and melatonin in the retina. *Progress in Retinal and Eye Research* 24**,** 433-456.

Kezuka, H., Aida, K. and Hanyu, I. (**1989**). Melatonin secretion from golfish pineal gland in organ culture. *General and Comparative Endocrinology* 75**,** 217-221.

Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Begay, V., Falcon, J., Cahill, G.M., Cassone, V.M. and Baler, R. (**1997**). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-Acetyltransferase in the pineal gland. *Recent Program in Hormone Research* 52**,** 307-356.

Klein, D.C. (**2004**). The 2004 Aschoff/Pittendrigh Lecture: theory of the origin of the pineal gland-A tale of conflict and resolution. *Journal of Biological Rhythms* 19**,** 264-279.

Larson, E.T., Winberg, S., Mayer, I., Lepage, O., Summers, C.H. and Overli, O. (**2004**). Social stress affects circulating melatonin levels in rainbow trout. *General and Comparative Endocrinology* 136**,** 322-327.

Lee, H.S., Nelms, J.L., Nguyen, M., Silver, R. and Lehman, M.N. (**2003**). The eye is necessary for a circadian rhythm in the suprachiasmatic nucleus. *Nature Neuroscience* 6**,** 111-112.

Migaud, H., Taylor, J.F., Taranger, G.L., Davie, A., Cerda-Reverter, J.M., Carrillo, M., Hansen, T. and Bromage, N.R. (**2006**). A comparative *ex vivo* and *in vivo* study of day and night perception in teleosts species using the melatonin rhythm. *Journal of Pineal Research* 41**,** 42-52.

Migaud, H., Davie, A., Martinez Chavez, C.C. and Al-Khamees, S. (**2007**). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *Journal of Pineal Research* 43**,** 327-335.

Nicholson, C. (**1999**). Signals that go with the flow. *Trends in Neurosciences* 22**,** 143-145.

North, B.P., Turnbull, J.F., Ellis, T., Porter, M.J., Migaud, H., Bron, J. and Bromage, N.R. (**2006**). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 255**,** 466-479.

Okimoto, D.K. and Stetson, M.H. (**1999**). Presence of an intrapineal circadian oscillator in the teleostean family poeciliidae. *General and Comparative Endocrinology* 114**,** 304-312.

Overli, O., Sorensen, C., Pulman, K.G.T., Pottinger, T.G., Korzan, W., Summers, C.H. and Nilsson, G.E. (**2007**). Evolutionary background for stress-coping styles: relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. *Neuroscience and Biobehavioral Reviews* 31**,** 396-412.

Reiter, R.J., Richardson, B.A., Matthews, S.A., Lane, S.J. and Ferguson, B.N. (**1983**). Rhythms in immunoreactive melatonin in the retina and harderian gland of rats: persistence after pinealectomy. *Life Sciences* 32**,** 1229-1236.

Reiter, R.J. (**1993**). The melatonin rhythm: both a clock and a calendar. *Experientia* 49**,** 654-664.

Ron, B., Zohar, Y., Borski, R., Young, G. and Grau, E.G. (**1995**). Effects of dorsal aorta cannulation on cortisol and other stress parameters in the euryhaline tilapia, *Oreochromis mossambicus*. *Aquaculture* 135**,** 216-295.

Ron, B. (**2004**). *In vitro* melatonin rhythm reveals a clock controlled pineal in the European seabass, *Dicentrarchus Labrax*. *The Israeli Journal of Aquaculture-Bamidgeh* 56**,** 281-285.

Serino, I., D'Istria, M. and Monteleone, P. (**1993**). A comparative study of melatonin production in the retina, pineal gland and harderian gland of *Bufo viridis* and *Rana esculenta*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology* 106**,** 189-193.

Simonneaux, V. and Ribelayga, C. (**2003**). Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacology Reviews* 55**,** 325-395.

Siu, A.W., Maldonado, M., Sanchez-Hidalgo, M., Tan, D.X. and Reiter, R.J. (**2006**). Protective effects of melatonin in experimental free radical-related ocular diseases. *Journal of Pineal Research* 40**,** 101-109.

Skinner, D.C. and Malpaux, B. (**1999**). High melatonin concentrations in third ventricular cerebrospinal fluid are not due to galen vein blood recirculating through the choroid plexus. *Endocrinology* 140**,** 4399-4405.

Takemura, A., Ueda, S., Hiyakawa, N. and Nikaido, Y. (**2006**). A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish, *Siganus guttatus*. *Journal of Pineal Research* 40**,** 236-241.

Tamai, T.K., Vardhanabhuti, V., Arthur, S., Foulkes, N.S. and Whitmore, D. (**2003**). Flies and fish: birds of a feather. *Journal of Neuroendocrinology* 15**,** 344-349.

Tilgner, S., Lehmann, L. and Westphal, U.I. (**1990**). Retinohypothalamic connections in vertebrates. *Klin Mbl Augenheilkd* 197**,** 295-301.

Tosini, G. and Fukuhara, C. (**2003**). Photic and circadian regulation of retinal melatonin in mammals. *Journal of Neuroendocrinology* 15**,** 364-369.

Touitou, Y., Smolensky, M.H. and Portaluppi, F. (**2006**). Ethics, standards, and procedures of animal and human chronobiology research. *Chronobiology International* 23**,** 1083-1096.

Tricoire, H., Locatelli, A., Chemineau, P. and Malpaux, B. (**2002**). Melatonin enters the cerebrospinal fluid through the pineal recess. *Endocrinology* 143**,** 84-90.

Vijayan, M.M., Pereira, C., Grau, E.G. and Iwama, G.K. (**1997**). Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 116**,** 89-95.

Wullimann, M.F. and Meyer, D.L. (**1990**). Phylogeny of putative cholinergic visual pathways through the pretectum to the hypothalamus in teleost fish. *Brain Behaviour and Evolution* 36**,** 14-29.

Yu, H.S., Pang, S.F. and Tang, P.L. (**1981**). Increase in the level of retinal melatonin and persistence of its diurnal rhythm in rats after pinealectomy. *Journal of Endocrinology* 91**,** 477-481.

Zachmann, A., KniJff, S.C.M., Ali, M.A. and Anctil, M. (**1991**). Effects of photoperiod and different intensities of light exposure on melatonin levels in the blood, pineal organ, and retina of the brook trout (*Salvelinus fontinalis,* Mitchill). *Canadian Journal of Zoology* 70**,** 25-29.

Zachmann, A., Falcon, J., KniJff, S.C.M., Bolliet, V. and Ali, M.A. (**1992**). Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the white sucker (*Catostomus commersoni*) *in vitro*. *General and Comparative Endocrinology* 86**,** 26-33.

Zar J.H., (**1999**). Biostatistical analysis. 4th, New Jersey, USA. pp.929.

Zordan, M.A., Rosato, E., Piccin, A. and Foster, R. (**2001**). Photic entrainment of the circadian clock: from *Drosophila* to mammals. *Seminars in Cell and Developmental Biology* 12**,** 317-328.

5 Paper III: Clock-controlled endogenous melatonin rhythms in Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarias gariepinus*)

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

Carlos Christian Martinez-Chavez,[1] Sammi Al-Khamees,[1] Antonio Campos-Mendoza,[2] David James Penman,[1] and Herve Migaud[1]

[1]*Institute of Aquaculture, University of Stirling, Stirling, UK*
[2]*Facultad de Biología, Laboratorio de Acuicultura, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México*

The purpose of this work was to investigate the circadian melatonin system in two tropical teleost species characterized by different behavioral 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 acclimatized 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 photocycle. Additional research is clearly needed to further characterize the circadian axis in teleost species, identify and localize the circadian clocks, and better understand the environmental entrainment of fish physiology. (Author correspondence: hm7@stir.ac.uk)

Keywords Melatonin, Oscillator, Pineal, Tilapia, Catfish

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Carlos C. Martinez-Chavez and S. Al-Khamees contributed equally to this study.

Address correspondence to Dr. Herve Migaud, Institute of Aquaculture, University of Stirling, Stirling, UK. Tel.: +44 1786467878; Fax: +44 1786472133; E-mail: hm7@stir.ac.uk

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; Falcon et al., 2007; Fukada & Okano, 2002; Holzberg & Albrecht, 2003). As such, melatonin seems to play a major role in synchronizing many behavioral (e.g., locomotor, feeding, shoaling, and migration activities) and physiological (e.g., growth, reproduction, and 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 (Herzog & Tosini, 2001; Malpaux et al., 2001; Simonneaux & 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 rerio* (Cahill, 2002; Carr et al., 2006; Lopez-Olmeda et al., 2006; Vallone et al., 2005; Ziv & Gothilf, 2006). Furthermore, no clear pathway between the hormone melatonin and the seasonality of fish physiology has been demonstrated in fish (Falcon et al., 2007; Mayer et al., 1997) 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 that have been shown to affect melatonin production in fish (e.g., light, temperature, size, age, and previous photoperiod entrainment), highlighting the complexity of the melatonin system in fish and the diversity of experimental procedures used (e.g., melatonin administered by injection, feed or water, or pinealectomy; see Falcon et al., 2007; Mayer et al., 1997). 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 (Ekstrom & Miessl, 2003; Menaker et al., 1997). 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; Holzberg & Albrecht, 2003; Iuvone et al., 2005; Vígh et al., 2002). Importantly, in mammals, photo-entrainment is exclusively mediated by retinal photoreceptors, and as such, pineal photoreceptors have lost their direct light sensory abilities (Ekstrom & Miessl, 2003). In teleosts, research has predominantly focused on temperate, annual breeding

species such as salmonids, pike *Esox lucius* and sea bass *Dicentrarchus labrax* (Bayarri et al., 2002, 2003; Iigo et al., 1998). 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 eyes (retina), and may contain, depending on the species, an endogenous oscillator that can sustain in vitro melatonin rhythms (Cahill, 1996; Iigo et al., 2004; Okimoto & Stetson, 1999a; Zachmann et al., 1992a).

However, a recent study suggested a different type of circadian organization in Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*), characterized by a pineal gland that is not light sensitive, or far less sensitive than in previously studied teleost species, with no independent circadian pacemaker regulating melatonin production (Migaud et al., 2007). Studies of pineal glands in culture performed in both temperate and tropical teleosts have commonly demonstrated rhythmic melatonin production under light and dark (LD) periods (Gern & Greenhouse, 1988; Iigo et al., 1991; Kezuka et al., 1989; Migaud et al., 2006). However, some species such as Nile tilapia and African catfish seem to be exceptions to this generalized 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* (Iigo et al., 1991; Kezuka et al., 1989), white sucker, *Catostomus commersoni* (Zachmann et al., 1992b), zebrafish (Cahill, 1996), sailfin molly, *Poecilia velifera* (Okimoto & Stetson, 1999a, 1999b), golden rabbitfish, *Siganus guttatus* (Takemura et al., 2006), ayu, *Plecoglossus altivelis* (Iigo et al., 2004), and sea bass (Bayarri et al., 2004a; Bolliet et al., 1996; Migaud et al., 2006; Ron, 2004). However, no such endogenous circadian system has been shown to exist in salmonids (Gern & Greenhouse, 1988; Iigo et al., 2007; Migaud et al., 2006) and common dentex, *Dentex dentex* (Pavlidis et al., 1999).

Such endogenous rhythms are clearly entrained by circadian clocks that have not yet been fully characterized 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 entrained by light (mainly, BMAL, Clock, Per's, and Cry's) that maintain and synchronize self-sustained rhythms (Iuvone et al., 2005; Stehle et al., 2003; Zordan et al., 2001). 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 & 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 (Almazan-Rueda et al., 2004; Appelbaum & McGeer, 1998; Biswas et al., 2005; Campos-Mendoza et al., 2004; Rad et al., 2006; Ridha & Cruz, 2000), although the mechanisms by which photoperiod acts 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 confirm circadian melatonin rhythms, to examine endogenous melatonin rhythms under constant photic conditions (i.e., continuous light [LL] and darkness [DD]) and determine whether these rhythms are circadian in nature, and finally 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, 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 involving 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 by automatic feeders (Fish-mate F14 Pet-Mate, Surrey, UK). In all experiments, lights were switched on at 08:00 h and off at 20:00 h under a 12L:12D photoperiod. Similarly, lights for 6L:6D were switched on at 08:00 h and then switched off and on at 6 h intervals. For each species, experiments were done in closed water recirculation systems (27 $\pm$ 1°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, Ohio, 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 was sampled by venipuncture of the caudal vein using heparinized 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 included four and eight light-proof individual compartments with two tanks in each, respectively. Tank size was 40 × 120 × 40 cm (200 L) and 46 × 46 × 41 cm (86 L) for tilapia and catfish, respectively. Fish under a given experimental photoperiodic treatment could 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 the 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 2 h (tilapia) or every 3 h (catfish) during the scotophase. In this trial, Nile tilapia were held constant at 24 ± 1°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, first to confirm the inhibitory effect of LL on melatonin production and determine the profile of return to normal melatonin levels, and second 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 eight tanks (four fish/tank, isolated by Perspex sheets with flow through holes). Fish from each tank were blood sampled every 12 h (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 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 of blood sampling fish under anaesthesia from all tanks once (corresponding to eight sampling points, randomized design; fish were then allowed to recover in aerated water and returned to their tanks) and then sacrificing fish from each tank 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 (four 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 six tanks (30 fish/tank), corresponding to the three experimental photoperiodic regimes in duplicate (control, LL, and DD). Sampling consisted of sacrificing three fish per tank (six per treatment) every 12 h 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 withdrawal. 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 experiments 2 and 3 under DD (for tilapia and catfish, respectively) were 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 h 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, and 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 three consecutive subjective photocycles 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 × g for 15 min at 4°C (Jouan CT422, Buckinghamshire, UK), and plasma was stored at −70°C until analyzed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). Prior to the analyses, the kit was 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 were analyzed using MINITAB® Release 14.13 (Minitab Ltd., UK). When necessary, data were transformed using the natural logarithm to conform to normality and homogeneity of variance (Kolmogorov-Smirnov and Bartlett's tests). Melatonin levels were analyzed 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 were pooled, as no significant differences between duplicates were observed. Data are expressed as mean ± SEM values. Significant differences were determined at $p \leq 0.05$.

RESULTS

A clear diel rhythm was observed in Nile tilapia (see Figure 1a) and African catfish (see Figure 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 h 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

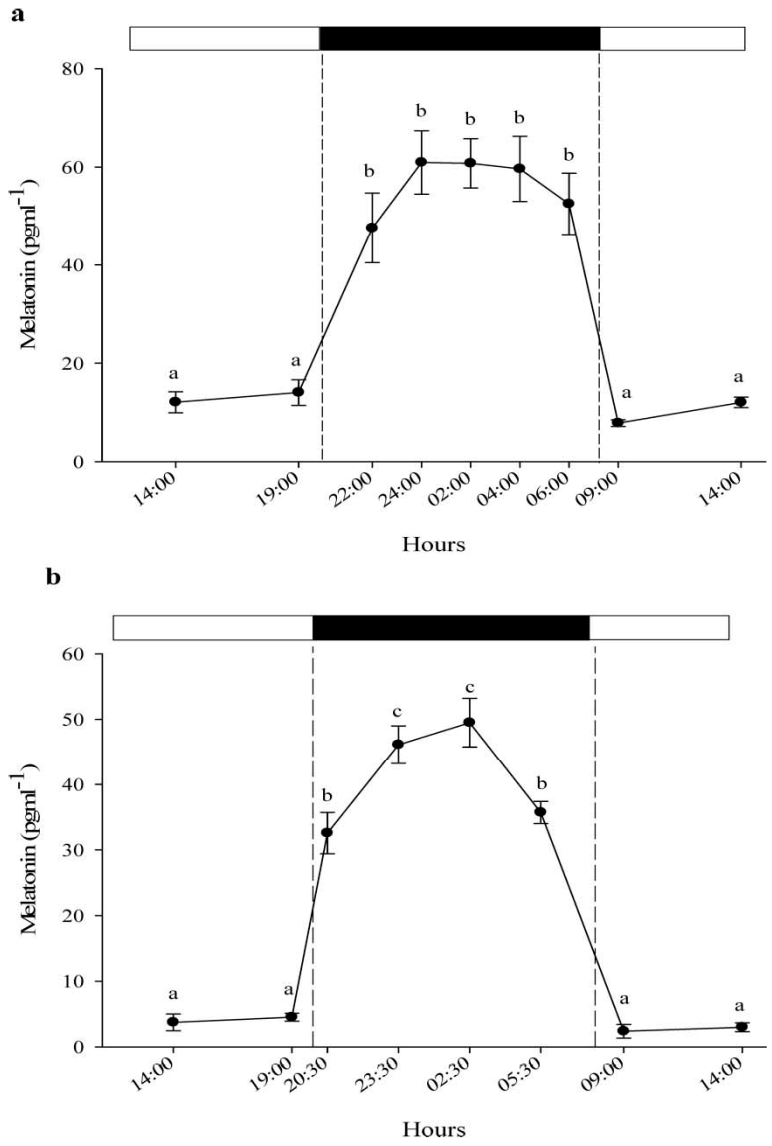

FIGURE 1 Circadian plasma melatonin profile in a) Nile tilapia (n = 10) and b) African catfish (n = 5). All values shown are mean ± SEM. Letters indicate significant ($p < 0.05$) difference between sample points. Open and filled boxes indicate photophase and scotophase, respectively.

levels in the following day sampling points (09:00 and 14:00 h), as depicted in Figure 1a. Similarly, melatonin levels in catfish remained basal during the photophase (14:00 and 19:00 h), then significantly increased 30 min after the start of the scotophase (20:30 h) to peak 3 h (23:30 h) later, and remain high at the following sampling point (02:30 h) (see Figure 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 to 07:45 h) in Nile tilapia were significantly reduced, although basal levels were only reached after the onset of photophase at 08:30 and 14:30 h (see Figure 2a). On the other hand, catfish melatonin levels were shown to decrease significantly during the scotophase 1 h 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 (see Figure 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

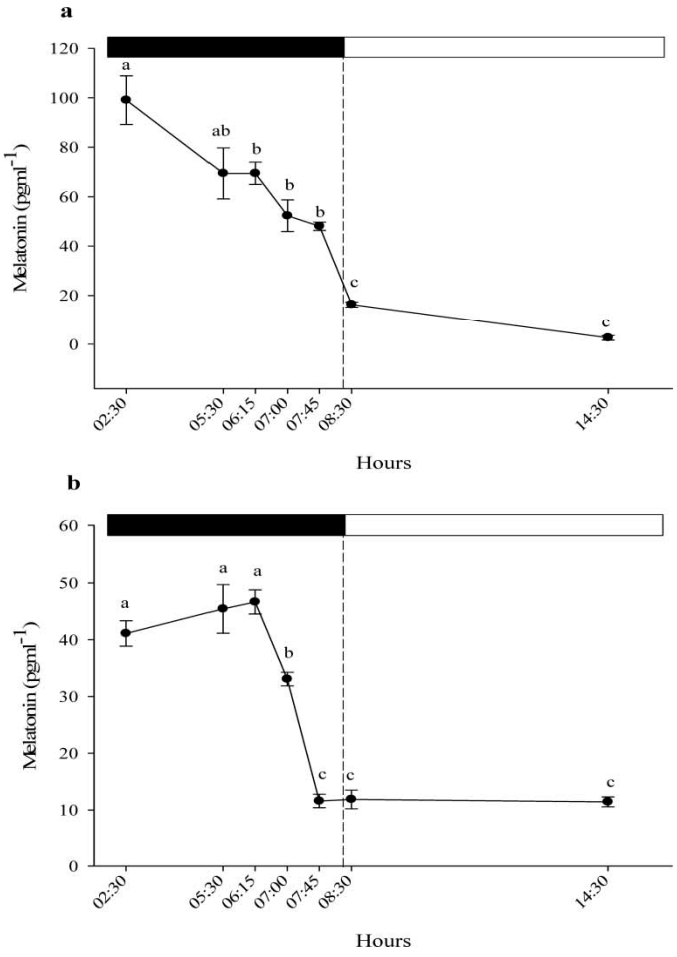

FIGURE 2 Anticipatory decrease of plasma melatonin levels prior to light onset in a) Nile tilapia (n = 5) and b) African catfish (n = 5). Values shown are mean ± SEM. Letters indicate significant ($p < 0.05$) difference between sampling points. Open and filled boxes indicate photophase and scotophase,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

the LL period (see Figure 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 (see Figure 3b). With the exception of photophase/scotophase levels on day 14, all other scotophase or subjective

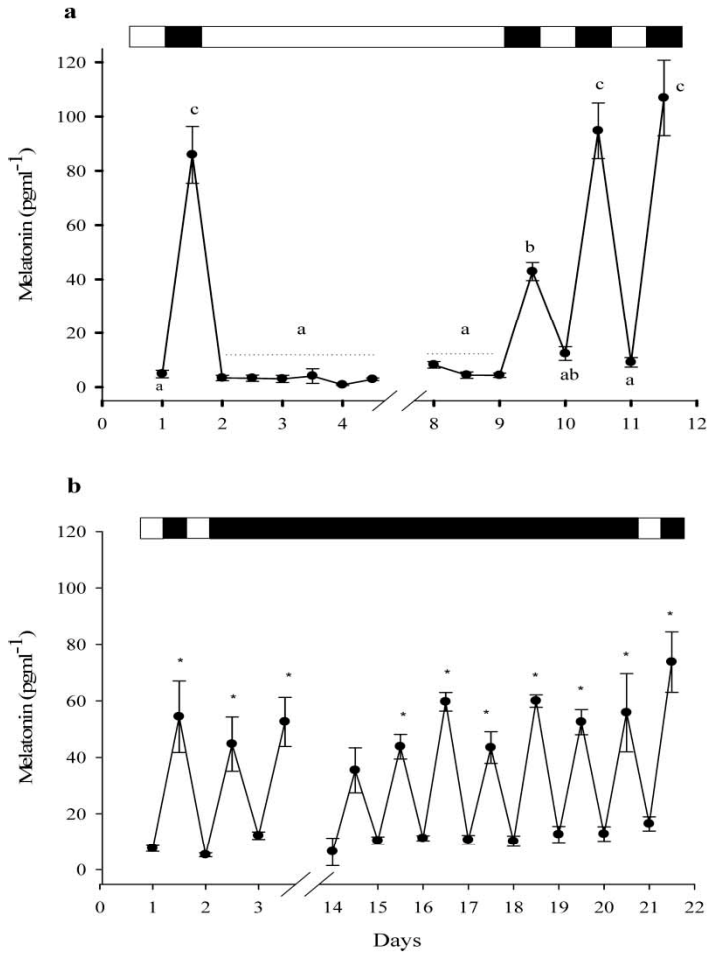

FIGURE 3 Plasma melatonin profile of Nile tilapia subjected to a) LL and b) DD regimes. Values shown are mean ± SEM (n = 4). Letters indicate significant ($p < 0.05$) difference between sampling points. Symbols (*) indicate significant differences with previous sampling point. Open and filled boxes indicate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

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 (see Figure 4a). When LL was applied, melatonin levels remained basal with no significant differences until a photo-cycle was restored, at which point melatonin significantly increased (see Figure 4b). However, when DD was applied, a

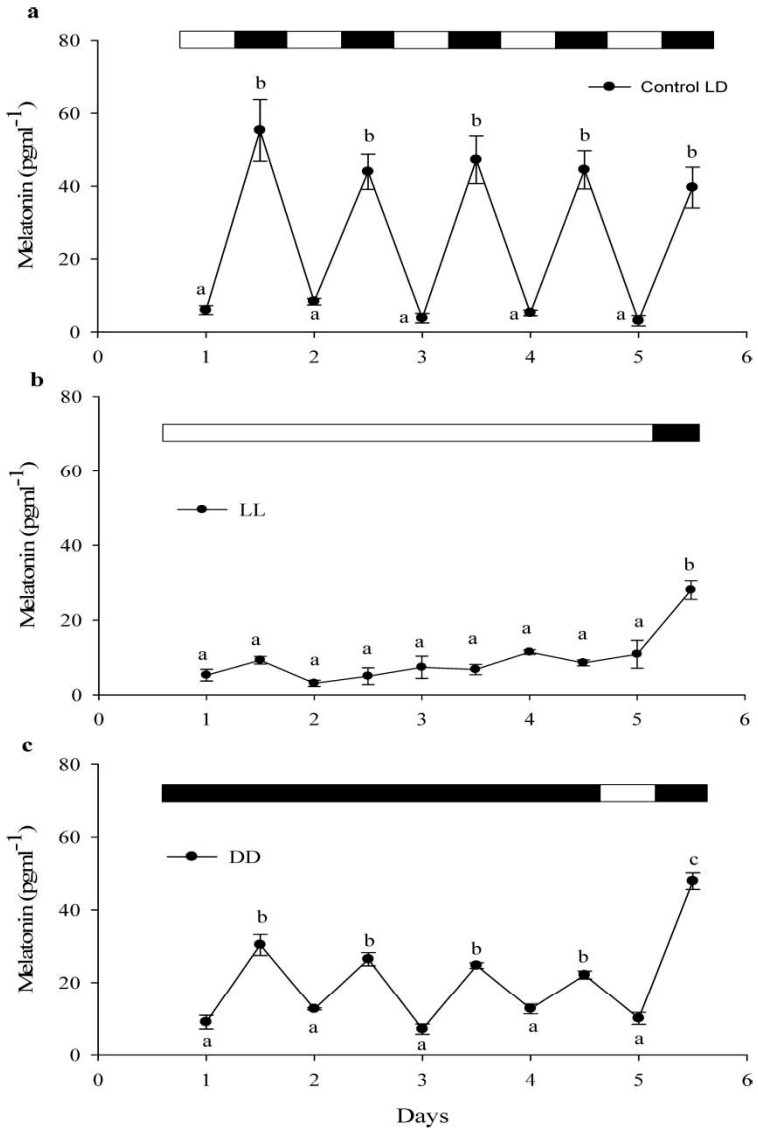

FIGURE 4 Plasma melatonin profile of African catfish subjected to a) 12L:12D, b) LL, and c) DD regimes. Values shown are mean ± SEM (n = 6). Letters indicate significant ($p < 0.05$) difference between sampling points. Open and filled boxes indicate photophase and scotophase, respectively.

robust daily rhythm of significantly lower amplitude (mean subjective scotophase levels of 26.0 ± 0.6 pg ml^{-1}) than that observed under a control 12L:12D regime (mean subjective scotophase levels of 48.8 ± 3.2 pg ml^{-1}) was maintained (see Figure 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 as 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, see Figure 5a), after which levels significantly increased during the subjective

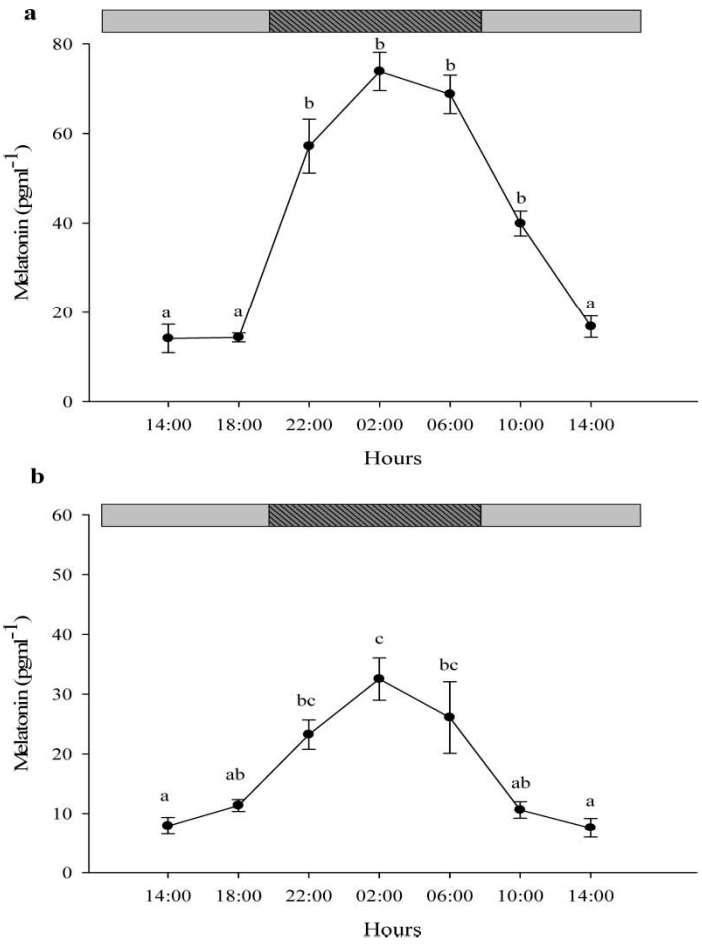

FIGURE 5 Circadian plasma melatonin profile in a) Nile tilapia and b) African catfish on the third day under DD. All values shown are mean ± SEM (n = 4). Letters indicate significant ($p < 0.05$) differences between sample points. Grey boxes indicate the subjective photophase periods and darker filled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

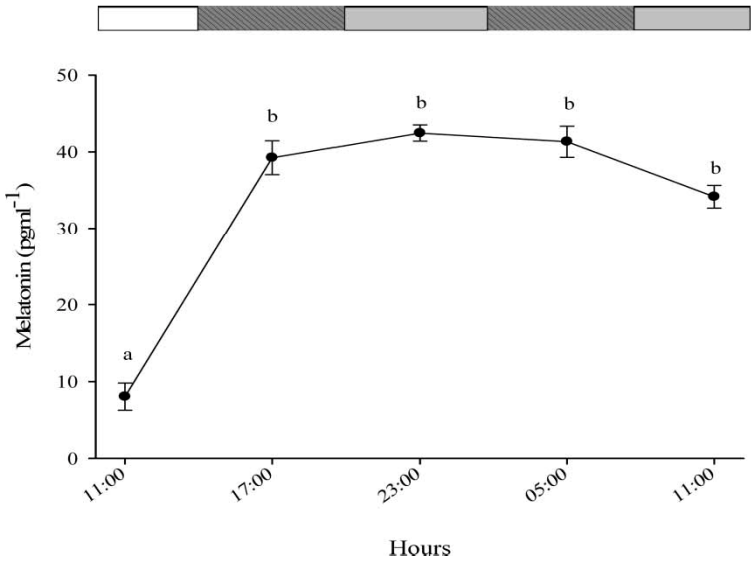

FIGURE 6 Melatonin levels of Nile tilapia acclimatized for two weeks to a 6L:6D photoperiod and sampled before and under DD for two subjective day-night cycles. Each point represents the mean ± SEM (n = 4). Letters indicate significant ($p < 0.05$) differences between sampling points. Open and filled boxes indicate photophase and scotophase, respectively.

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, Figure 5b), but they then showed an early tendency to increase toward 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 photocycle, 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 (see Figure 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 that 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 (Almazan-Rueda et al., 2004; Biswas et al., 2005; Campos-Mendoza et al., 2004; 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 showed a similar diel plasma melatonin profile in Nile tilapia and African catfish as that previously reported in most vertebrate species (Cassone, 1990; Hardeland et al., 2006; Mayer et al., 1997; Migaud et al., 2006; Pavlidis et al., 1999; Reiter, 1988). 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 that 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 that 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 (Appelbaum et al., 2005; Falcon et al., 2001; Klein et al., 1997). 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 (Appelbaum & Gothilf, 2006; Klein et al., 1997; Pando et al., 2001; Zordan et al., 2001).

In order to further characterize 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 (Falcon et al., 1989; Gern & Greenhouse, 1988; Kezuka et al., 1989; Migaud et al., 2006; Okimoto & Stetson, 1999a; 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 previously 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 desensitization 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 acclimatized to a 12L:12D photoperiod. A similar endogenous rhythm was also able to sustain itself for at least four days (duration of DD exposure) in African catfish. Importantly, results also demonstrated that the melatonin rhythm in both species exposed to DD for three days was circadian (i.e., cycling over approximately 24 h). However, from these trials, it is not possible to determine whether there was a phase-shift in the circadian rhythm later on, as only a single sampling point was performed in the middle of the subjective photophase/scotophase throughout the DD trials. Furthermore, although the full amplitude of melatonin oscillations was shown in tilapia under DD, melatonin levels during the 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 desensitized 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 of the present results, many in vitro studies suggest 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 (Bayarri et al., 2004b; Kazimi & Cahill 1999; Migaud et al., 2006; Oliveira et al., 2007; Pavlidis et al., 1999; Randall et al., 1995; Vera et al., 2007). A recent in vivo study performed on temperate fish species (sea bass, Atlantic salmon, and Atlantic cod) has shown that when acclimatized to a 12L:12D photoperiod at two different temperatures (10 and 18°C) and thereafter exposed to DD, no circadian endogenous melatonin rhythm was maintained, with levels remaining as high as during the 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°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 of 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 behavioral 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 were studied. This suggests that, irrespective of its location (pineal, retina, and/or brain), the endogenous melatonin oscillator was either unable to entrain to such short photo-cycles, or 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 & Kreitzman, 2005).

In the last decade or so, the 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 hypothesized 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 acclimatized 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 conditions (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, the results of these experiments 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 localization, 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 characterize the circadian axis in fish.

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REFERENCES

Almazan-Rueda P, Schrama JW, Verreth JAJ. (2004). Behavioural responses under different feeding methods and light regimes of the African catfish (*Clarias gariepinus*) juveniles. *Aquaculture* 231: 347–359.

Appelbaum L, Gothilf Y. (2006). Mechanism of pineal-specific gene expression: The role of E-box and photoreceptor conserved elements. *Mol. Cell. Endocrinol.* 252:27–33.

Appelbaum S, McGeer JC. (1998). Effect of diet and light regime growth and survival of African catfish (*Clarias gariepinus*) larvae and early juveniles. *Aquacult. Nutr.* 4:157–164.

Appelbaum L, Anzulovich A, Baler R, Gothilf Y. (2005). Homeobox-clock protein interaction in zebrafish: A shared mechanism for pineal-specific and circadian gene expression. *J. Biol. Chem.* 280: 11544–11551.

Arendt J. (1998). Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. *Rev. Reprod.* 3:13–22.

Bayarri MJ, Madrid JA, Sanchez-Vazquez FJ. (2002). Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. *J. Pineal Res.* 32:34–40.

Bayarri MJ, Rol de Lama MA, Madrid JA, Sanchez-Vasquez FJ. (2003). Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in sea bass. *Brain Res.* 969:175–182.

Bayarri MJ, Garcia-Allegue R, Lopez-Olmeda JF, Madrid JA, Sanchez-Vazquez FJ. (2004a). Circadian melatonin release *in vitro* by European sea bass pineal. *Fish Physiol. Biochem.* 30:87–89.

Bayarri MJ, Munoz-Cueto JA, Lopez-Olmeda JF, Vera LM, Rol de Lama MA, Madrid JA, Sanchez-Vazquez FJ. (2004b). Daily locomotor activity and melatonin rhythms in Senegal sole (*Solea senegalensis*). *Physiol. Behav.* 81:577–583.

Biswas AK, Morita T, Yoshizaki G, Maita M, Takeuchi T. (2005). Control of reproduction in Nile tilapia *Oreochromis niloticus* (L.) by photoperiod manipulation. *Aquaculture* 243:229–239.

Bolliet V, Ali MA, Lapointe FJ, Falcon J. (1996). Rhythmic melatonin secretion in different teleost species: An *in vitro* study. *J. Comp. Physiol. B* 165:677–683.

Bromage N, Roberts JR. (1995). *Broodstock Management and Egg and Larval Quality*. Edinburgh, UK: Blackwell Science..

Cahill GM. (1996). Circadian regulation of melatonin production in cultured zebrafish pineal and retina. *Brain Res.* 708:177–181.

Cahill G. (2002). Clock mechanisms in zebrafish. *Cell Tissue Res.* 309:27–34.

Campos-Mendoza A, McAndrew BJ, Coward K, Bromage N. (2004). Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation: Effects on spawning periodicity, fecundity and egg size. *Aquaculture* 231:299–314.

Carr AJ, Tamai TK, Young LC, Ferrer V, Dekens MP, Whitmore D. (2006). Light reaches the very heart of the zebrafish clock. *Chronobiol. Int.* 23:91–100.

Cassone VM. (1990). Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.* 13: 457–464.

Ekstrom P, Miessl H. (2003). Evolution of photosensory pineal organs in new light: The fate of neuroendocrine receptors. *Phil. Trans. R. Soc. Lond. B* 358:1679–1700.

Falcon J. (1999). Cellular circadian clocks in the pineal. *Prog. Neurobiol.* 58:121–162.

Falcon J, Marmillon JB, Claustrat B, Collin JP. (1989). Regulation of melatonin secretion in a photoreceptive pineal organ: An *in vitro* study in the pike. *J. Neurosci.* 9:1943–1950.

Falcon J, Galarneau KM, Weller JL, Ron B, Chen G, Coon SL, Klein DC. (2001). Regulation of arylalkylamine N-acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the fish pineal organ: Evidence for a role of proteasomal proteolysis. *Endocrinology* 142:1804–1813.

Falcon J, Besseau L, Sauzet S, Boeuf G. (2007). Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends Endocrinol. Metab.* 18:81–88.

Foster R, Kreitzman L. (2005). *Rhythms of Life*; London, UK: Profile Book.

Foulkes NS, Whitmore D, Sassone-Corsi P. (1997). Rhythmic transcription: The molecular basis of circadian melatonin synthesis. *Biol. Cell* 89:487–494.

Fukada Y, Okano T. (2002). Circadian clock system in the pineal gland. *Mol. Neurobiol.* 25:19–30.

Gern WA, Greenhouse SS. (1988). Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination or continuous darkness. *Gen. Comp. Endocrinol.* 71:163–174.

Hardeland R, Pandi-perumal SR, Cardinali DP. (2006). Melatonin. *Int. J. Biochem. Cell, Biol.* 38: 313–316.

Herzog ED, Tosini G. (2001). The mammalian circadian clock shop. *Sem. Cell Dev. Biol.* 12:295–303.

Holzberg D, Albrecht U. (2003). The circadian clock: A manager of biochemical processes within the organism. *J. Neuroendocrinol.* 15:339–343.

Iigo M, Kezuka H, Aida K, Hanyu I. (1991). Circadian rhythms of melatonin secretion from superfused goldfish (*Carassius auratus*) pineal glands *in vitro*. *Gen. Comp. Endocrinol.* 83:152–158.

Iigo M, Kitamura S, Ikuta K, Sanchez-Vazquez FJ, Ohtani-Kaneko R, Hara M, Hirata K, Tabata M, Aida K. (1998). Regulation by light and darkness of melatonin Secretion from the superfused masu salmon (*Oncorhynchus masou*) pineal organ. *Biol. Rhythm Res.* 29:86–97.

Iigo M, Fujimoto Y, Gunji-Suzuki M, Yokosuka M, Hara M, Ohtani-Kaneko R, Tabata M, Aida K, Hirata K. (2004). Circadian rhythm of melatonin release from the photoreceptive pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-thorough culture. *J. Neuroendocrinol.* 16:45–51.

Iigo M, Abe T, Kambayashi S, Oikawa K, Masuda T, Mizusawa K, Kitamura S, Azuma T, Takagi Y, Aida K, Yanagisawa T. (2007). Lack of circadian regulation of *in vitro* melatonin release from the pineal organ of salmonid teleosts. *Gen. Comp. Endocrinol.* 154:91–97.

Iuvone PM, Tosini G, Pozdeyev N, Haque R, Klein DC, Chaurasia SS. (2005). Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. *Prog. Ret. Eye Res.* 24: 433–456.

Kazimi N, Cahill GM. (1999). Development of a circadian melatonin rhythm in embryonic zebrafish. *Dev. Brain Res.* 117:47–52.

Kezuka H, Aida K, Hanyu I. (1989). Melatonin secretion from goldfish pineal gland in organ culture. *Gen. Comp. Endocrinol.* 75:217–221.

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R. (1997). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Rec. Prog. Horm. Res.* 52:307–356.

Lopez-Olmeda JF, Madrid JA, Sanchez-Vasquez FJ. (2006). Light and temperature cycles as zeitgebers of zebrafish (*Danio rerio*) circadian activity rhythms. *Chronobiol. Int.* 23:537–550.

Malpaux B, Migaud M, Tricoire H, Chemineau P. (2001). Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. *J. Biol. Rhythms* 16:336–347.

Mayer I, Bornestaf C, Borg B. (1997). Melatonin in non-mammalian vertebrates: Physiological role in reproduction? *Comp. Biochem. Physiol. A* 118:515–531.

Menaker M, Moreira LF, Tosini G. (1997). Evolution of circadian organization in vertebrates. *Braz. J. Med. Biol. Res.* 30:305–313.

Migaud H, Taylor JF, Taranger GL, Davie A, Cerda-Reverter JM, Carrillo M, Hansen T, Bromage N. (2006). A comparative *ex vivo* and *in vivo* study of day and night perception in teleosts species using the melatonin rhythm. *J. Pineal Res.* 41:42–52.

Migaud H, Davie A, Martinez-Chavez CC, Al-Khamees S. (2007). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *J. Pineal Res.* 43:327–335.

Okimoto DK, Stetson MH. (1999a). Presence of an intrapineal circadian oscillator in the teleostean family Poeciliidae. *Gen. Comp. Endocrinol.* 114:304–312.

Okimoto DK, Stetson MH. (1999b). Properties of the melatonin-generating system of the sailfin molly. *Poecilia velifera. Gen. Comp. Endocrinol.* 114:293–303.

Oliveira C, Ortega A, Lopez-Olmeda J, Vera LM, Sanchez-Vasquez FJ. (2007). Influence of constant light and darkness, light intensity, and light spectrum on plasma melatonin rhythms in Senegal sole. *Chronobiol. Int.* 24:615–627.

Pando MP, Pinchak AB, Cermakian N, Sassone-Corsi P. (2001). A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. *PNAS* 98:10178–10183.

Pavlidis M, Greenwood L, Paalavuo M, Molsa H, Laitinen JT. (1999). The effect of photoperiod on diel rhythms in serum melatonin, cortisol, glucose, and electrolytes in the common Dentex, *Dentex dentex*. *Gen. Comp. Endocrinol.* 113:240–250.

Pevet P. (2003). Melatonin: From seasonal to circadian signal. *J. Neuroendocrinol.* 15:422–426.

Rad F, Bozaoglu S, Ergene Gozukara S, Karahan A, Kurt G. (2006). Effects of different long-day photoperiods on somatic growth and gonadal development in Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture* 255:292–300.

Randall CF, Bromage N, Thorpe JE, Miles MS, Muir JS. (1995). Melatonin rhythms in Atlantic salmon (*Salmo salar*) maintained under natural and out-of-phase photoperiods. *Gen. Comp. Endocrinol.* 98: 73–86.

Reiter RJ. (1988). Comparative aspects of pineal melatonin rhythms in mammals. *Anim. Plant Sci.* 1: 111–116.

Ridha MT, Cruz EM. (2000). Effect of light intensity and photoperiod on Nile tilapia *Oreochromis niloticus* L. seed production. *Aquacult. Res.* 31:609–617.

Ron B. (2004). *In vitro* melatonin rhythm reveals a clock controlled pineal in the European sea bass, *Dicentrarchus labrax*. *Israeli J. Aquaculture* 56:281–285.

Simonneaux V, Ribelayga C. (2003). Generation of the melatonin endocrine message in mammals: A review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacol. Rev.* 55:325–395.

Stehle JH, von Gall C, Korf H-W. (2003). Melatonin: A clock-output, a clock-input. *J. Neuroendocrinol.* 15:383–389.

Takemura A, Ueda S, Hiyakawa N, Nikaido Y. (2006). A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish. *Siganus guttatus*. *J. Pineal Res.* 40:236–241.

Touitou Y, Smolensky MH, Portaluppi F. (2006). Ethics, standards and procedures in human and animal research in chronobiology. *Chronobiol. Int.* 23:1083–1096.

Vallone D, Lahiri K, Dickmeis T, Foulkes NS. (2005). Zebrafish cell clocks feel the heat and see the light!. *Zebrafish* 2:171–187.

Vera LM, De Oliveira C, Lopez-Olmeda JF, Ramos J, Mananos E, Madrid JA, Sanchez-Vazquez FJ. (2007). Seasonal and daily plasma melatonin rhythms and reproduction in Senegal sole kept under natural photoperiod and natural or controlled water temperature. *J. Pineal Res.* 43:50–55.

Vígh B, Manzano MJ, Zádori A, Frank CL, Lukáts A, Röhlich P, Szél A, Dávid C. (2002). Nonvisual photoreceptors of the deep brain, pineal organs and retina. *Histol. Histopathol.* 17:555–590.

Zachmann A, Ali MA, Falcon J. (1992a). Melatonin and its effects in fishes: An overview. In Ali MA (ed.) In *Rhythms in Fishes*. London: Plenum Publishing Corporation, pp. 149–165.

Zachmann A, Falcon J, Knijff SCM, Bolliet V, Ali MA. (1992b). Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the White Sucker (*Catostomus Commersoni*) *in vitro*. *Gen. Comp. Endocrinol.* 86:26–33.

Zar JH. (1999). *Biostatistical Analysis*. Upper Saddle River, NJ: Prentice Hall.

Ziv L, Gothilf Y. (2006). Period2 expression pattern and its role in the development of the pineal circadian clock in zebrafish. *Chronobiol. Int.* 23:101–112.

Zordan MA, Rosato E, Piccin A, Foster R. (2001). Photic entrainment of the circadian clock: From Drosophila to mammals. *Sem. Cell. Dev. Biol.* 12:317–328.

6 Paper IV: GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia

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GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia

Carlos C. Martinez-Chavez, Matteo Minghetti, Herve Migaud *

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

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Abstract

The Kiss1/GPR54 system has recently been shown to play a key role in the onset of puberty in mammals. Growing evidence suggests that this system is also conserved across vertebrates although very few studies so far have been performed in lower vertebrates. The aims of this study were firstly in the teleost Nile tilapia to screen tissues for GPR54 expression levels, secondly to measure the expression patterns of GPR54 and GnRH I receptor (rGnRH I) in whole brains during the onset of puberty and finally to determine the effects of continuous illumination (LL) on receptor expression levels. Results confirmed that GPR54 was predominantly expressed in the brain and pituitary of adult tilapia. Furthermore, a significant increase of GPR54 gene expression was found in tilapia brains at 11 weeks post hatch (wph) followed by rGnRH I at 13 wph just prior to the histological observation of vitellogenic oocytes and active spermatogenesis in ova and testes at 17 wph. These results suggest a correlation between the increase of GPR54 expression in the brain and the onset of puberty. Finally, a significant effect of LL was observed on GPR54 expression levels which were characterized by a delayed surge with significantly lower levels than those of control fish. The current study not only suggests a link between the Kiss1/GPR54 system and the onset of puberty in a tropical batch spawning teleost that would be a highly conserved feature across vertebrates but also that the transcriptional mechanisms regulating GPR54 expression could be directly or indirectly influenced by light.

Keywords: GPR54; GnRH I; Puberty; Light; Nile tilapia

1. Introduction

The mechanisms by which puberty is initiated in vertebrates have been a long sought question and the recent discovery of kisspeptin genes might help unravel this mystery. The brain–pituitary–gonadal (BPG) axis and the whole cascade of genes, receptors, enzymes and hormones involved in the control of puberty have been the object of many studies over the last ten years. Although such commitment clearly resulted in a better understanding of the GnRH and gonadotropin systems (for a detailed review, see Gore, 2002), the way by which the BPG axis is initiated remains unclear in mammals and unknown in teleosts.

To date, more than 20 forms of GnRH have been found in vertebrates, eight of which are present in teleosts (Lethimonier et al., 2004; Pawson and McNeilly, 2005). Of the three GnRH forms (I, II and III) found in brain of Nile tilapia and other perciforms, only GnRH I was found in the preoptic area of the hypothalamus and pituitary (hypophysiotropic) where it is also the most abundant (Parhar et al., 1996; Gothilf et al., 1996; Carolsfeld et al., 2000). GnRH I and its cognate receptor (rGnRH I) have thus been often used to study the mechanisms regulating gonadotropin release in many vertebrates.

The role played by environmental cues such as photoperiod are well reported across temperate vertebrate species and melatonin is known to be one of the key rhythmic signals entrained by light and used to synchronize physiological events such as reproduction in mammals (Malpaux et al., 2001; Simonneaux and Ribelayga, 2003; Pevet,

* Corresponding author. Fax: +44 1786472133.
E-mail address: hm7@stir.ac.uk (H. Migaud).

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2003). On the other hand, in teleosts, convincing evidence of the cellular mechanisms and pathways are yet to be found despite the fact that melatonin also seems to play a key role in mediating photoperiod effects (Mayer et al., 1997; Falcon et al., 2007). Recently, kisspeptin (also called metastin) a product of the gene Kiss1 and its cognate G protein-coupled receptor 54 (GPR54) have been suggested to play an important role in the initiation of puberty in higher vertebrates. Preliminary evidence of such a role was reported in 2003 when two independent research groups found that mutations of GPR54 caused hypogonadotropic hypogonadism in humans (de Roux et al., 2003) and mice (Funes et al., 2003). Since then, GPR54 has been cloned in other vertebrates including a few teleost species (Parhar et al., 2004; Nocillado et al., 2007; Mohamed et al., 2007) and have shown high homology of the amino acid sequence suggesting that the Kiss1/GPR54 system would be a conserved feature across vertebrates. Further studies have shown that kisspeptin could directly stimulate GnRH release via GPR54 in mouse (Messager et al., 2005). To date, this has not been shown in fish although GPR54 has been localized in GnRH neurons in tilapia (Parhar et al., 2004). In addition, kisspeptins also appear to coordinate the negative feedback loops of sex steroids in the hypothalamus of mammals (Rometo et al., 2007). Importantly, new evidence linking for the first time the Kiss1/GPR54 system and photoperiod has been reported in the hamster (Revel et al., 2006a). Many comprehensive reviews have recently been published (Colledge, 2004; Popa et al., 2005; Seminara, 2005; Aparicio, 2005; Murphy, 2005; Tena-Sempere, 2006; Kuohung and Kaiser, 2006; Smith et al., 2006a,b; Roa and Tena-Sempere, 2007; Roa et al., 2007). However, although the importance of this new system is without any doubt of prime importance, expression data in teleosts has been to date very scarce with only, to our knowledge, two published papers reporting studies performed in two seasonal marine species, the cobia (*Rachycentron canadum*) and grey mullet (*Mugil cephalus*). The latter have shown a positive correlation between the increase of GnRH expression and GPR54 during early development and puberty (Nocillado et al., 2007; Mohamed et al., 2007).

Further studies are clearly needed in a wider range of models to determine not only if the Kiss1/GPR54 system is highly conserved across vertebrates but also if such a system could be the missing link between perception of environmental cues and priming of the BPG axis. For this reason, teleosts could prove to be very good non-mammalian models due to the strong seasonality of their physiology, the range of reproductive strategies and the diversity of environmental niches they inhabit. Indeed, photoperiod has been shown to be the main signal used by most of the fish species to entrain and synchronize reproduction (review by Bromage et al., 2001). Although this has been mainly reported in temperate teleost species, recent reports have also shown significant effects of photoperiod on reproductive physiology in Nile tilapia (Campos-Mendoza et al., 2004; Biswas et al., 2005; Rad et al., 2006).

The objectives of this study were thus to (1) screen tissue expression levels of GPR54 in order to confirm potential action sites, (2) measure the developmental expression patterns of GPR54 and GnRH I receptor (rGnRH I) during the onset of puberty and (3) determine whether continuous illumination (LL) could have an effect on GPR54 and GnRH I receptor expression levels.

2. Materials and methods

2.1. Animals

Mixed sex red Nile tilapias (*O. niloticus niloticus*) were obtained from the tropical aquarium facilities at the Institute of Aquaculture (University of Stirling, Stirling, UK). All fish used in these experiments were from the same stock and were produced in the facilities. Experimental fish were reared in a light proof closed water recirculation system (27 ± 1 °C) as previously described in Campos-Mendoza et al. (2004). Light intensity was 0.75 W/m^2 at the water surface (measured by a single channel light sensor, Skye instruments, Powys, UK). Nitrate, nitrite, ammonia and pH were monitored throughout the experiments with aquarium water quality kits (C-Test kits, New Aquarium Systems, Mentor, USA) and levels remained within safe limits. Fry were hand fed to satiation three times a day at 9am, 1pm and 6pm with a crumb mix of two feeds (Nutra Trout Fry 02 and Standard Expander 40, Skretting, Cheshire, UK). All trials were carried out in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986, UK.

2.2. Experiments and sampling

In all experiments, fish were killed by a lethal dose of anesthetic (0.03 M benzocaine solution, SIGMA, Poole, UK), and then decapitated. Tissues were sampled swiftly to avoid RNA degradation following fish death, frozen in liquid nitrogen and stored at −70 °C until total RNA extraction.

2.2.1. Experiment 1: Tissue screening

To determine the potential action sites of GPR54, samples from seven different tissues were sampled at midday from two-year-old mature fish (three males and three females, mean weight of 135.8 ± 53.3 g): brain, pituitary, heart, kidney, liver, gonad (mostly ovarian membrane in females) and muscle. Fish were previously reared in normal high density stocking conditions under a 12L:12D photoperiod.

2.2.2. Experiment 2: Developmental expression of GPR54 and rGnRH I during onset of puberty

Nile tilapia fry/fingerlings reared under standard conditions in the facilities (27 ± 1 °C, 12L:12D photoperiod, fed three times a day *ad libitum*) were sampled from 3 wph (23 days post-fertilization) to 17 wph. Initially, swimming larvae were kept in 7L incubators within the recirculating light proof system. Half way through the experiment, fish were transferred into 40L aquaria within the same rearing system. Sampling consisted of sacrificing fish at each time point (every 2 weeks) precisely at the same time of the day (12pm). The number of fish sampled and pooled (from 5 pools of 15 larvae heads at 3 wph to 10 individual fish brains from 7 to 17 wph) varied depending on the size of the fish (see Table 1). Whole bodies (larval stage) and/or gonads of sampled fish were fixed in Bouin's fixative and processed for histological examination in order to determine sex and gonadal staging.

2.2.3. Experiment 3: Effects of constant illumination on GPR54 and rGnRH I gene expression during the onset of puberty

A different batch of fish, from the same origin, was reared following Experiment 2 under either 12L:12D photoperiod or constant illumination (LL) to test the effects of un-entrained photic conditions. LL regime was chosen as several authors reported the effects of such photoperiod or con-

Table 1
Experimental sampling structure of Nile tilapia

Stage (wph)	No. fish pooled	*n*	
		Female	Male
3	15 (heads)	5	
4	5 (heads)	5	
5	3 (heads)	5	
6	2 (heads)	5	
7	1 (brain)	5	5
9	1 (brain)	7	3
11	1 (brain)	7	3
13	1 (brain)	6	4
15	1 (brain)	5	5
17	1 (brain)	5	5

stant long-day regimes on growth and reproduction in Nile tilapia as well as other tropical and temperate species. Furthermore, Nile tilapia is a diurnal fish which feeds during the photophase. Sampling took place every 2 weeks starting at 7 wph until 13 wph (corresponding to the window where GPR54 and rGnRH I gene expression was shown to significantly increase in previous trial). Ten to twelve fish per treatment were randomly sampled at each time point in order to have a minimum of 5 males and 5 females however, in some cases only 4 individuals of one sex were sampled. Only eight individual fish brains (4 fish/sex) were analyzed for gene expression. All gonad samples were fixed as previously mentioned and analyzed by histology.

2.2.4. RNA extraction and cDNA synthesis

Frozen heads, brain samples and tissues were homogenized in 1 ml TRI reagent (Sigma Aldrich, St. Louis, MO, USA) solution per 100 mg of tissue. RNA pellets where reconstituted in 50 µl of MilliQ water, quality checks and measurements were performed with a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK) and running 1 µg of total RNA in a 1% agarose denaturating gel. cDNA was synthesized using 1 µg of total RNA, 1 µl of a blend (3:1) random hexamers (400 ng/µl) oligo dT (500 ng/µl) (ABgene®, Epsom, UK), respectively, 1 µl of 10 mM dNTPs, 1 µl (200 U/µl) of reverse transcriptase III (Invitrogen®, Paisley, UK) with provided buffers in a final volume of 20 µl. Following synthesis, 180 µl of MilliQ water was added to reach working dilution (1:10) and stored at −20 °C.

2.2.5. Primer design and quantitative RT-PCR (qPCR)

All primers were designed using using PrimerSelect Ver. 6.1 program (DNASTAR, www.dnastar.com) based on GenBank sequences of GPR54, rGnRH I (target genes) and β-actin, Accession Nos.: AB162143, AB111356 and EF206801, respectively. β-Actin has been selected as a reference gene following a preliminary gene expression study in the brain that showed remarkable stability under the experimental conditions tested. PCR products were then cloned into a pCR 2.1 vector (Invitrogen, Paisley, UK) and sequenced using a CEQ-8800 Beckman sequencer (Coulter Inc., Fullerton, USA). The identity of the cloned PCR products were then verified (100% overlapping) using BLAST (http://www.ncbi.nlm.nih.Gov/BLAST/). Primers used for qPCR were designed on these sequences and optimized (Table 2). In all cases, qPCR reactions containing 1 µl of each primer (7 pmol/µl), 5 µl cDNA (1:10 dilution), 10 µl Syber Green master mix (ABgene®, Epsom, UK) and 3 µl of MilliQ water in a final volume of 20 µl, were run in a thermocycler (Techne, Quantica, Cambridge, UK) using the following program: 95 °C for 15 min (Taq activation) followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s followed by a temperature ramp from 70 to 90 °C for melting curve analysis. Gene copy number in each reaction was calculated by comparison to a standard curve constructed from the results of a parallel set of reactions containing serial dilutions of linearized plasmids containing Nile tilapia GPR54, rGnRH I and β-actin cloned cDNA sequences. Concentrations of standards were determined spectrophotometrically and converted to copy number by consideration of plasmid size and DNA absorption coefficient. Samples were run in triplicate together with non-template controls, standards and internal controls to correct expression levels between plates. qPCR products were checked by sequencing confirming 100% overlap against GenBank sequences. Expression levels for tissue expression profile are shown as absolute copy numbers per µg of total RNA, due to the variability of β-actin expression in different tissues, whereas expression values for Experiments 2 and 3 were normalized against the reference gene (β-actin), multiplied by 100,000 (arbitrary number for graphs) and presented as relative expression.

2.2.6. Histological preparations and analysis

The fixed samples were trimmed and placed individually into cassettes and then dehydrated using methylated spirits, cleared in chloroform and impregnated with paraffin wax using an automated Tissue Processor (Thermo Fisher, Cheshire, UK). The samples were then embedded in molten paraffin wax using a histoembedder (Leica UK Ltd., Milton Keynes, UK). Once hardened the wax blocks were trimmed using a Rotary microtome (Leica UK Ltd., Milton Keynes, UK). When the samples consisted of whole fish it was necessary to surface decalcify the blocks for 1 h in Rapid Decalcifying solution (Cellpath, UK) before cutting. Using a Rotary Microtome, the blocks were sliced into 5 µm thick sections and transferred onto slides. These slides were stained according to a standard Haematoxylin and Eosin (H and E) protocol. The stage of oocyte development was visually determined and the leading oocytes were classified according to Coward and Bromage (1998) for oogenesis (chromatin nucleolar stage (1), early perinucleolar stage (2), late perinucleolar stage (3), cortical alveolar stage (4), vitellogenesis (5), maturation (6) and germinal vesicle migration (7)) and Babiker and Ibrahim (1978) for spermatogenesis (Immature (1), Maturing (2), Mature (3), Ripening (4), Ripe (5), Spawning (6) and Spent (7)), respectively.

2.3. Statistical analysis

Normality and homogeneity of variances were tested using Kolmogorov Smirnov (with Lilliefors' correction) and when appropriate, data was transformed using the natural logarithm. Data from the Experiment 1 (tissue GPR54 gene expression) and comparison of males *vs.* females for all trials were analyzed by *t*-tests. In addition, temporal gene expression data in Experiment 2 were analyzed by (a) a general lineal model (GLM) from 3 to 11 wph where no sex differences were observed (mean values) and by (b)

Table 2
Sequences of primers used for qPCR gene expression of developmental Nile tilapia

Primer name	Sequence	Product size (bp)	GenBank Accession No.
β-Actin F	5′-TCTCTTCCAGCCTTCCTTCC-3′	130	EF206801
β-Actin R	5′-GGTACCTCCAGACAGCACAGT-3′		
rGnRH I F	5′-GTGGCTTGCCGGAGACTTTG-3′	123	AB111356
rGnRH I R	5′-AGAGGGTTGAGGATGGCTGACT-3′		
GPR54 F	5′-ATGCCTGGCTGGTCCCTCTGTTCT-3′	136	AB162143
GPR54 R	5′-GGCGGCCAGGTTTGCTATGTA-3′		

a GLM per/sex from 7 to 17 wph. For purpose of clarity, temporal differences from 3 to 11 wph are not presented on Fig. 2. Data in Experiment 3 were also analyzed by a GLM including temporal and treatment factors (two way ANOVA) followed by multiple comparison test (Tukey). For all tests, significance was set at $p < 0.05$. All analyses were performed with Sigmastat (V. 3.11) and Minitab (V. 14.13). Values are presented as mean ± SEM.

3. Results

Raw gene expression data showed in all cases the same pattern of expression than the normalized results presented in this study. Nonetheless, normalization decreased sample variability showing that the reference gene used (β-actin) was appropriate in all cases.

In Experiment 1, GPR54 expression was found to be highest in brain of male and female fish with no significant differences between sexes (Fig. 1). Levels in the pituitary were shown to be lower (~14-fold) than in the brain and significantly higher in females than in males (at least 2-fold) although sampling size was small ($n = 3$). Expression levels were close to detection limits in the other tissue samples (heart, kidney, liver, gonad and muscle) ranging between 7 and 78 absolute copies/μg total RNA.

In Experiment 2, significant sex differences were found in GPR54 gene expression at 13 and 17 wph (Fig. 2a) with higher levels in females. Developmental GPR54 expression levels were generally low in the first 9 wph but displayed a significant increase between 3–4 and 6–7 wph which for purpose of clarity are not shown. Thereafter, GPR54 expression levels significantly increased in both sexes (~8.5-fold) from 9 to 11 wph and were maintained so (plateau) until the end of the study (17 wph) in females with males showing a significant reduction at 13 wph. GnRH I type receptor also produced a similar pattern of expression as GPR54 with small but significant increases between 3–4, 6–7 and 7–9 wph (Fig. 2b, not shown). Expression levels continued to increase significantly between 9–11 wph (~3.5-fold) and 13 wph (~2-fold) when peak expression levels were reached and maintained until 17 wph. Significant sex difference of rGnRH I expression levels were only found at 13 wph with higher levels in females. Fish had a final mean weight of 31.5 ± 4.4 ($n = 10$) at 17 wph. No significant correlations were found between GPR54/rGnRH I and growth (graphs not shown).

Gonadal histological observations showed that first sign of active oocyte development (cortical alveolar stage) appeared from 11 wph although all females sampled at 9 wph possessed oocytes at stage 2 (early perinucleolar) (Table 3). First vitellogenic oocytes were only observed at 17 wph. All males sampled up to 13 wph were at an immature stage with testes predominantly containing spermatogonia. First sign of active spermatogenesis was observed at 15 wph with testes possessing a few spermatids (stage 2). At 17 wph, 80% of the male fish sampled (4 fish) were at the ripening stage 4 characterized as active spermatogenesis and containing different stages with abundant spermatozoa.

In Experiment 3, GPR54 gene expression levels measured in the brain of fish exposed to 12L:12D (control) photoperiod and constant illumination (LL) were not significantly different at 7 wph (Fig. 3a). Thereafter, GPR54 expression levels increased significantly in fish from both treatments and reached significantly higher levels (2.2-fold) in control as compared to LL at 9 wph. Under the control photoperiod treatment, GPR54 expression levels remained elevated for the remainder of the study. However, under

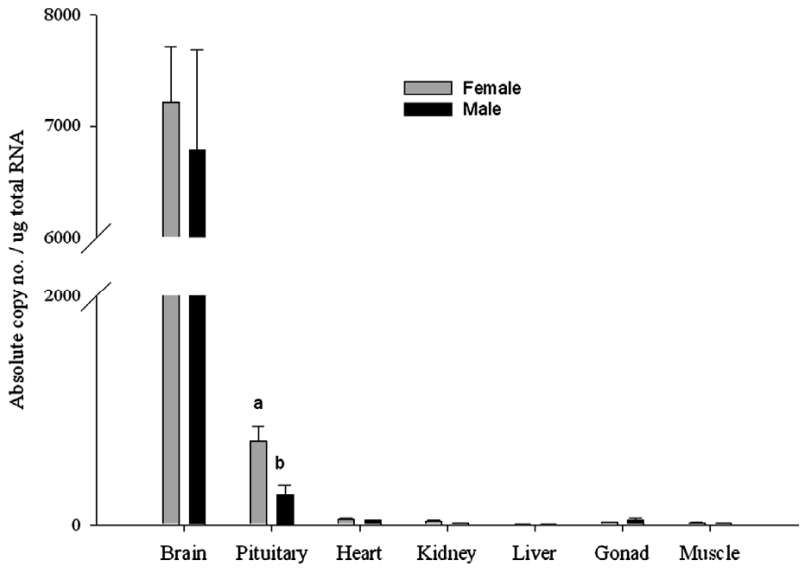

Fig. 1. Differential tissue expression of GPR54 (absolute copy numbers) in male and female Nile tilapia. Values expressed as mean ± SEM ($n = 3$). Superscripts denote significant difference between sexes in each tissue.

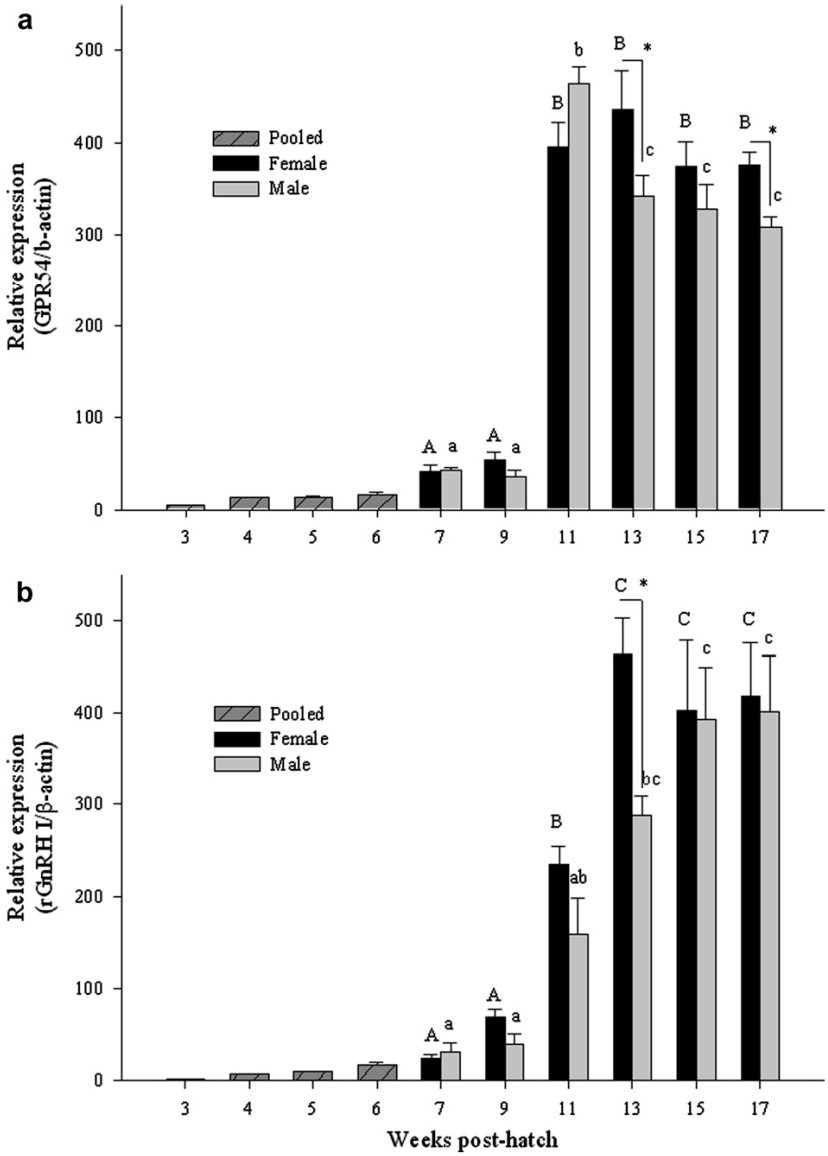

Fig. 2. Relative gene expression of GPR54 (a) and rGnRH I (b) during onset of puberty in Nile tilapia. Values expressed as mean ± SEM ($n = 3$ 7, see Table 1). Superscripts denote significant temporal differences for a given sex from 7 to 17 wph (capital and lower case letters for females and males, respectively) while asterisks (*) denote significant differences between sexes at given time points.

Table 3
Histological staging of male (M) and female (F) Nile tilapia sampled in Experiment 2 every two weeks from 7 to 17 wph

wph	7		9		11		13		15		17	
Stages	M	F	M	F	M	F	M	F	M	F	M	F
1	4	6	3		4		4					
2				7					4			
3						5		2	1	2	1	1
4						1		4		3	4	3
5												1
6												
Total	4	6	3	7	4	6	4	6	5	5	5	5

the LL treatment, GPR54 expression continued to increase between 9 and 11 wph, thereafter no differences were then observed. Expression levels between treatments at both 11 and 13 wph were not statistically different.

rGnRH I expression levels in the brain was similar in fish under both photoperiodic treatments throughout the study (from 7 to 13 wph) (Fig. 3b). Levels of both treatments significantly increased between 7 and 9 wph (2.5-fold) and then again between 9 and 11 wph (2.5-fold). Levels remained steady at 13 wph. No statistical differences were observed between treatments at both latter sampling points although mean rGnRH I expression lev-

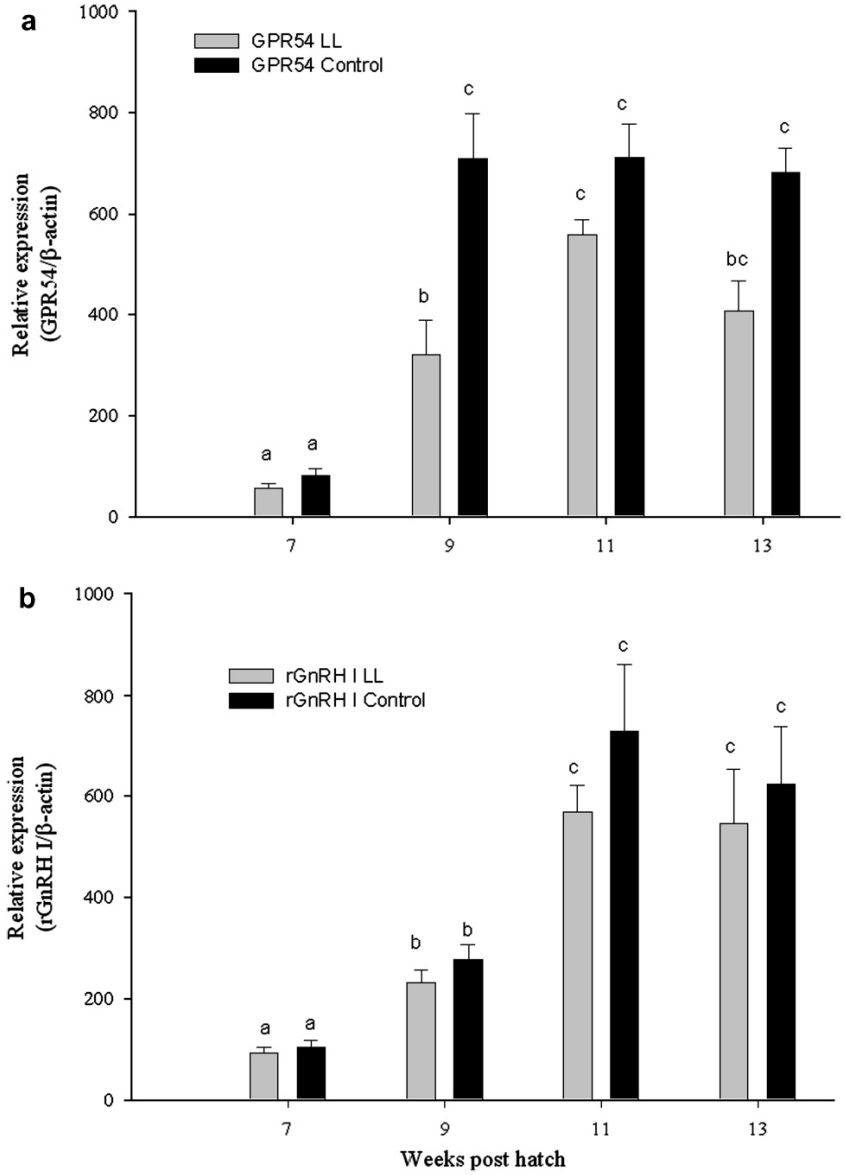

Fig. 3. Relative gene expression of GPR54 (a) and rGnRH I (b) during onset of puberty in Nile tilapia exposed to either 12L:12D (control) or constant illumination (LL) photoperiods from first feeding. Values expressed as mean ± SEM ($n = 8$). Superscripts denote significant differences between sampling points and treatments.

els appeared to be slightly higher in control fish. No significant sex differences were found in expression levels of both target genes throughout Experiment 3. No significant growth differences were observed throughout the duration of the experiment between photoperiodic treatments with mean end weight at 15 wph of 27.11 ± 1.33 g (control) and 28.97 ± 1.17 g (LL). Weight of the fish in both Experiments 2 and 3 were similar at 17 wph (31.6 ± 14.08 g, $n = 10$) and no significant correlations were found between GPR54/rGnRH I and growth (graphs not shown). Gonadal staging in this experiment showed no major differences between treatments although no statistical analyses could be performed due to the format of the data. Males in control group initiated spermatogenesis (stage 2) between 7 and 9 wph and reached advanced ripening stage in both treatments by 13 wph. Females started earlier at 7 wph reaching the early perinuclear stage (3) in control fish and the cortical alveolar stage (4) in the LL treatment. Females from the control group seemed to catch up with LL by 9 wph and the stages remained similar (late perinuclear to cortical alveolar) in both groups until 13 wph when the first females of both groups reached vitellogenesis (Table 4).

Table 4
Histological staging of male (M) and female (F) Nile tilapia exposed to either 12L:12D (control) or constant illumination (LL) photoperiods from first feeding (Experiment 3)

Treatments	Control								LL							
wph	7		9		11		13		7		9		11		13	
Stages	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	4	4	5		1				5		3					
2		3	2		5		2				2		8		1	
3				1	1	1	2			2		2		1	2	1
4				3		3	1	4		3		3		3	2	3
5								1								1
6																
Total	4	7	7	4	7	4	5	5	5	5	5	5	8	4	5	5

Sampling took place at 7, 9, 11 and 13 wph.

4. Discussion

The kisspeptin receptor GPR54 has been shown to stimulate GnRH secretion in mammals (Messager et al., 2005) and has been specifically localized in Nile tilapia GnRH neurons (Parhar et al., 2004) although to our knowledge, no studies have looked at its expression in tissues other than brain and gonads in fish. In the current study, the Nile tilapia kisspeptin receptor (GPR54) showed high tissue specificity (expressed in brain and pituitary) in accordance with its suggested role in the BPG axis (Seminara, 2005; Smith et al., 2006a). Although no significant differences between males and females were shown in brain tissues in Experiment 1, higher expression levels of GPR54 were observed in females in the pituitary. Such sexual dimorphism in expression levels have already been reported for rGnRH I in the same species (Levavi-Sivan et al., 2004). Interestingly, male brain expression levels showed more variation than females. This could be explained either by the small sample size ($n = 3$) or more likely by the social nature/behavior of this species (i.e. dominant males could potentially have higher GPR54 levels than their submissive counterparts). Such social interactions have been shown to exist in another cichlid (*Astatotilapia burtoni*) where territorial (dominant) males were shown to have significantly higher rGnRH I expression levels than non-territorial males (Au et al., 2006; Hofmann, 2006). Also, studies have reported that GnRH mRNA and its receptors are differentially regulated and sexually dimorphic during development (Gore, 2002; Levavi-Sivan et al., 2004). GPR54 levels in all other tissues were very low (especially taking into account the amount of cells present in ~100 mg tissue) and suggests that GPR54 might not play a functional role in these tissues, although it should be noted that in mammals, it has been reported that low occupancy (20%) of GnRH receptors (not shown for GPR54) is enough for a strong (80%) biological response (Naor et al., 1980). The low GPR54 transcripts found in the ovary in this study compared to those found in grey mullet (Nocillado et al., 2007) could be explained by the fact that in the present study, most of the oocytes were removed from the gonad to prevent the high fatty acid and glycoprotein content of tilapia eggs from potentially affecting RNA extraction, cDNA synthesis and qPCR sensitivity. Importantly when comparing expression levels between different studies, methodology aspects such as type (i.e. random hexamers, oligo dT), concentration of primers used during cDNA synthesis, final template dilution used and normalization strategy should be considered as they could all account for target copy variability (Ginzinger, 2002).

Furthermore, results in the current study clearly showed a GPR54/rGnRH I surge in whole brains of Nile tilapia, which correlated with the onset of puberty as shown through histological observations (Experiments 2 and 3). Indeed, in Experiment 2 male fish were at an immature stage (pre-gametogenesis) up to 13 wph while females appeared to initiate oogenesis soon after the first significant increases of GPR54/rGnRH I expression occurring between 7 and 9 wph. The highest surge of expression occurred by 11 wph and 13–15 wph for GPR54 and rGnRH I, respectively.

Although significant sex differences of GPR54 gene expression were observed at 13 and 17 wph with higher levels found in females, peak levels of expression were already reached for both sexes by 11 wph. If the role of GPR54 on the initiation of the BPG axis in tilapia is confirmed, these results could suggest that the timing of such stimulation would have occurred simultaneously in both sexes. Theses results contrast with those found by Mohamed et al. (2007) where male cobia displayed significantly higher GPR54 expression levels. Interestingly, rGnRH I expression levels significantly peaked at 13 wph for females and two weeks later in males (15 wph). Such sexual dimorphism, already reported in a tilapia hybrid, *O. niloticus* × *O. aureus* (Levavi-Sivan et al., 2004), correlate well with the later gonadal development observed in males as compared to females. However, we acknowledge that sample size might have been too low to depict further significant differences between sexes. Nonetheless, although females appeared to initiate gametogenesis earlier, both sexes only reached advanced stages of gonadal development (early to late vitellogenic stages in females and ripening testes in males)

by the end of the experiment (15–17 wph). This delay in male gonadal development most probably reflects the difference in time and energy required to accomplish full development in comparison to females.

Thus, in agreement with what has already been reported in other vertebrates, the onset of puberty in Nile tilapia would be correlated to increases in GPR54 and rGnRH I. Although these findings do not demonstrate a direct link between these two receptors and puberty, it suggests that such a connection could be at work as both receptors consecutively switch on prior to the onset of active gametogenesis. These results bring further evidences to recent data obtained in two marine teleost species (with longer life cycles and different reproductive strategies) in which a similar pattern of GPR54 expression at the respective gonadal stages has been shown (Mohamed et al., 2007; Nocillado et al., 2007). To date and to our knowledge, the present study is the first one to study the ontogeny of expression prior and throughout puberty in a batch spawner fish. In cobia, GPR54 expression was shown to peak when male fish were at an early stage of puberty followed by a rise in all three GnRH subtypes (Mohamed et al., 2007). Similarly, grey mullet showed higher GPR54 expression in brain during early gonadal development stages (Nocillado et al., 2007). In the present study (Experiment 2), the fact that oocytes in females were already at a late perinucleolar and cortical alveolar stages when GPR54 expression levels peaked suggests (a) that the intense surge in GPR54 and rGnRH I expression (observed at 11 and 13 wph, respectively, in the current work) might not be needed for the initial stages of oocyte development and/or (b) that kisspeptin, gonadotropin and sex steroid content previously released are enough to trigger onset of gametogenesis.

After the surge of gene expression in both GPR54 and rGnRH I, high levels were then maintained throughout the remaining of the experiments. This is in agreement with previous studies performed in higher vertebrates which have shown that puberty is associated with an increase in expression of GnRH mRNA and pulsatile releases of GnRH and LH which peak when reproductive function is attained and tends to remain steady (plateau) (Gore, 2002; Clarke and Pompolo, 2005). However, present results are in conflict with previous findings obtained in the grey mullet where expression levels were shown to decrease at intermediate and advanced gonadal stages (Nocillado et al., 2007). Differences in the profile of expression and sexual dimorphism could be due to the different reproductive strategies between species (continuous batch spawner in Nile tilapia vs. iteropare for grey mullet and cobia) and other factors such as the timing of sampling and interspecies differences.

Photoperiod is without any doubt one of the most powerful and noise free signal along with temperature that fish and other vertebrates can rely on to synchronize their reproductive physiology. However, there has not been any clear definition of the pathway through which photoperiod exerts its effects in fish physiology (Mayer et al., 1997; Falcon et al., 2007). The aim of Experiment 3 was firstly, to confirm findings of Experiment 2 and determine whether photoperiod could have an effect on the GPR54/rGnRH I expression patterns as well as gonadal development. Indeed, the pattern of GPR54 and rGnRH I expression observed in Experiment 3 reproduced very well what was shown in the Experiment 2 although there was a shift in the timing of the expression surge which occurred earlier (GPR54 peaking at 9 wph in control fish compared to 11 wph in Experiment 2) with a higher amplitude (almost 2-fold). Histology results in this experiment also confirmed those shown in the previous one with control males and females starting to develop after the initial surges in gene expression of both target genes (7–9 wph) and reaching more advanced stages by 11–13 wph. No evident differences could be observed in developmental stages between both control and LL treatments. However, males under the control photoperiodic regime matured earlier than males in Experiment 2 (stage 3 reached at 15 and 11 wph, respectively, for Experiments 2 and 3). Natural stock variability in addition to possible sex ratio interactions could explain these differences (Lorenzen, 2000).

Secondly, our results suggested an effect of photoperiod on GPR54 gene expression levels which were shown to be significantly reduced at 9 wph in fish exposed to LL compared to fish under 12L:12D control photoperiod. Although rGnRH I expression levels appeared to be lower in fish exposed to LL between 11 and 13 wph, no significant differences between treatments were observed. One possible explanation is that the GPR54 surge in the LL treatment would have been enough to trigger (gate) the GnRH cascade. These results would therefore suggest that the mechanism involved in the transcription of GPR54 and possibly its ligand Kiss1 (not tested in this study) could be affected by environmental cues as recently proposed in mammals (Revel et al., 2006b; Roa et al., 2007). These findings will obviously have to be confirmed. To our knowledge, data linking photoperiod effects in the Kiss/GPR54 system has only been recently reported in hamsters and sheep (Revel et al., 2006a; Greives et al., 2007; Wagner et al., 2007). Kiss1 mRNA (not GPR54) was shown to be expressed in higher quantities in long days (summer reproductive phase) rather than short days (winter inhibition) (Revel et al., 2006a). The reverse is true in sheep (Wagner et al., 2007). Most importantly, it was shown by Revel et al. (2006a) that pineal ablation did not reduce Kiss1 mRNA levels during short days as opposed to their control and sham counterparts. This suggests that melatonin would mediate the short day down regulation of Kiss1 expression in hamsters. Furthermore, photoperiod and the reproductive state of hamsters were shown to significantly affect the number and size of kisspeptin-immunoreactive neurons (Greives et al., 2007). Both these studies confirmed the importance of the Kiss1/GPR54 system in the interpretation of environmental stimuli and subsequent regulation of the reproductive axis in hamster. No such data is yet available in fish. The present study brings preliminary evidences suggesting for the first time in fish that light could act on the

Kiss1/GPR54 system. Although Nile tilapia is not a seasonal species as such, light has been shown to impact on its growth and reproductive physiology (Campos-Mendoza et al., 2004; Biswas et al., 2005; Rad et al., 2006) and mechanisms at work in higher vertebrates are likely to be conserved in Nile tilapia considered as one of the most evolved teleost species (perciforms). It could also be hypothesized that melatonin could have a direct or indirect role in regulating GPR54 expression levels as has been shown in higher mammals (Revel et al., 2006a; Greives et al., 2007; Wagner et al., 2007). As such, further co localization and regulatory studies in fish and vertebrates in general are clearly needed to help us better understand these regulatory mechanisms controlling puberty and reproduction in species with different reproductive strategies.

Overall, GPR54 and rGnRH I patterns of gene expression in Experiment 2 and control fish from Experiment 3 were comparable although in the latter, higher levels (circa 2-fold) were found throughout which could explain the more advanced stages of gametogenesis observed. The lack of strong correlation between expression levels and growth suggest that gene expression increased irrespectively of the size of the fish sampled and that growth (size) would not be a requirement for the onset of pubertal gene expression.

In conclusion, results showed a correlation between the GPR54, rGnRH I and the onset of puberty in a tropical batch spawning teleost. These findings are in accordance with results obtained in seasonal marine teleosts which further suggest a conserved role of this system across vertebrates. Moreover, the recent discovery of a functional Kiss1/GPR54 receptor in zebrafish (*Danio rerio*) provides a new tool to further study the Kiss system in teleosts (van Aerle et al., 2008). Furthermore, current results showed an effect of photoperiod on GPR54 expression with continuous illumination resulting in a reduced GPR54 expression compared to the control treatment. These preliminary findings suggest a potential link between environmental stimuli and the kisspeptin/GnRH systems. Further investigations are clearly required to test and confirm these results and demonstrate how such mechanisms would work.

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References

Aparicio, S.A.J.R., 2005. Kisspeptins and GPR54 The new biology of the mammalian GnRH axis. Cell Metab. 1, 293 296.

Au, T.M., Greenwood, A.K., Fernald, R.D., 2006. Differential social regulation of two pituitary gonadotropin-releasing hormone receptors. Behav. Brain Res. 170, 342 346.

Babiker, M.M., Ibrahim, H., 1978. Studies on the biology of reproduction in the cichlid *Tilapia nilotica* (L.): gonadal maturation and fecundity. J. Fish Biol. 14, 437 448.

Biswas, A.K., Morita, T., Yoshizaki, G., Maita, M., Takeuchi, T., 2005. Control of reproduction in Nile tilapia *Oreochromis niloticus* (L.) by photoperiod manipulation. Aquaculture 243, 229 239.

Bromage, N.R., Porter, M., Randall, C., 2001. The Environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63 98.

Campos-Mendoza, A., McAndrew, B.J., Coward, K., Bromage, N., 2004. Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effects on spawning periodicity, fecundity and egg size. Aquaculture 231, 299 314.

Carolsfeld, J., Powell, J.F.F., Park, M., Fischer, W.H., Craig, A.G., Chang, J.P., Rivier, J.E., Sherwood, N.M., 2000. Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology 141, 505 512.

Clarke, I.J., Pompolo, S., 2005. Synthesis and secretion of GnRH. Anim. Reprod. Sci. 88, 29 55.

Colledge, W.H., 2004. GPR54 and puberty. Trends Endocrinol. Metab. 15, 448 453.

Coward, K., Bromage, N., 1998. Histological classification of oocyte growth and dynamics of ovarian recrudescence in *Tilapia zillii*. J. Fish Biol. 53, 285 302.

De Roux, N., Genin, E., Carel, J.-C., Matsuda, F., Chaussain, J.-L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. PNAS 100, 10972 10976.

Falcon, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo pituitary axis in fish. Trends Endocrinol. Metab. 18, 81 88.

Funes, S., Hedrick, J.A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A., Yang, S., Monsma, F.J., Gustafson, E.L., 2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem. Biophys. Res. Commun. 312, 1357 1363.

Ginzinger, D.G., 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol. 30, 503 512.

Gore, A.C., 2002. GnRH: the master molecule of reproduction. Kluwer Academic, Dordrecht, The Netherlands.

Gothilf, Y., Munoz-Cueto, J.A., Sagrillo, C.A., Selmanoff, M., Chen, T.T., Kah, O., Elizur, A., Zohar, Y., 1996. Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. Biol. Reprod. 55, 636 645.

Greives, T.J., Mason, A.O., Scotti, M.A.L., Levine, J., Ketterson, E.D., Kriegsfeld, L.J., Demas, G.E., 2007. Environmental control of kisspeptin: implications for seasonal reproduction. Endocrinology 148, 1158 1166.

Hofmann, H.A., 2006. Gonadotropin-releasing hormone signaling in behavioral plasticity: signalling mechanisms. Curr. Opin. Neurobiol. 16, 343 350.

Kuohung, W., Kaiser, U.B., 2006. GPR54 and KISS-1: role in the regulation of puberty and reproduction. Rev. Endocr. Metab. Disord. 7, 257 263.

Lethimonier, C., Madigou, T., Munoz-Cueto, J.A., Lareyre, J.J., Kah, O., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen. Comp. Endocrinol. 135, 1 16.

Levavi-Sivan, B., Safarian, H., Rosenfeld, H., Elizur, A., Avitan, A., 2004. Regulation of gonadotropin-releasing hormone (GnRH)-receptor gene expression in tilapia: effect of GnRH and dopamine. Biol. Reprod. 70, 1545 1551.

Lorenzen, K., 2000. Population dynamics and management. In: Beveridge, M.C.M., McAndrew, B.J. (Eds.), Tilapias: Biology and Exploitation. Kluwer Academic, Dordrecht, The Netherlands, pp. 163 225.

Malpaux, B., Migaud, M., Tricoire, H., Chemineau, P., 2001. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J. Biol. Rhythms 16, 336 347.

Mayer, I., Bornestaf, C., Borg, B., 1997. Melatonin in non-mammalian vertebrates: physiological role in reproduction? Comp. Biochem. Physiol. 118, 515 531.

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R., Malinge, I., Lomet, D., Carlton, M.B.L., Colledge, W.H., Caraty, A., Aparicio, S.A.J.R., 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. PNAS 102, 1761 1766.

Mohamed, J.S., Benninghoff, A.D., Holt, G.J., Khan, I.A., 2007. Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. J. Mol. Endocrinol. 38, 235 244.

Murphy, K.G., 2005. Kisspeptins: regulators of metastasis and the hypothalamic pituitary gonadal axis. J. Neuroendocrinol. 17, 519 525.

Naor, Z., Clayton, R., Catt, K., 1980. Characterization of gonadotropin-releasing hormone receptors in cultured rat pituitary cells. Endocrinology 107, 1144 1152.

Nocillado, J.N., Levavi-Sivan, B., Carrick, F., Elizur, A., 2007. Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, *Mugil cephalus*. Gen. Comp. Endocrinol. 150, 278 287.

Parhar, I.S., Ogawa, S., Sakuma, Y., 2004. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. Endocrinology 145, 3613 3618.

Parhar, I.S., Pfaff, D.W., Schwanzel-Fukuda, M., 1996. Gonadotropin-releasing hormone gene expression in teleosts. Mol. Brain Res. 41, 216 227.

Pawson, A.J., McNeilly, A.S., 2005. The pituitary effects of GnRH. Anim. Reprod. Sci. 88, 75 94.

Pevet, P., 2003. Melatonin: from seasonal to circadian signal. J. Neuroendocrinol. 15, 422 426.

Popa, S.M., Clifton, D.K., Steiner, R.A., 2005. A KiSS to remember. Trends Endocrinol. Metab. 16, 249 250.

Rad, F., Bozaoglu, S., Ergene, G.S., Karahan, A., Kurt, G., 2006. Effects of different long-day photoperiods on somatic growth and gonadal development in Nile tilapia (*Oreochromis niloticus* L.). Aquaculture 255, 292 300.

Revel, F.G., Saboureau, M., Masson-Pevet, M., Pevet, P., Mikkelsen, J.D., Simonneaux, V., 2006a. Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Curr. Biol. 16, 1730 1735.

Revel, F.G., Saboureau, M., Masson-Pevet, M., Pevet, P., Mikkelsen, J.D., Simonneaux, V., 2006b. KISS-1: a likely candidate for the photoperiodic control of reproduction in seasonal breeders. Chronobiol. Int. 23, 277 287.

Roa, J., Tena-Sempere, M., 2007. KiSS-1 system and reproduction: comparative aspects and roles in the control of female gonadotropic axis in mammals. Gen. Comp. Endocrinol. 153, 132 140.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., 2007. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Front. Neuroendocrinol. 156, 48 69.

Rometo, A.M., Krajewski, S.J., Lou Voytko, M., Rance, N.E., 2007. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J. Clin. Endocrinol. Metab. 92, 2744 2750.

Seminara, S.B., 2005. Metastin and its G protein-coupled receptor, GPR54: critical pathway modulating GnRH secretion. Front. Neuroendocrinol. 26, 131 138.

Simonneaux, V., Ribelayga, C., 2003. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol. Rev. 55, 325 395.

Smith, J.T., Clifton, D.K., Steiner, R.A., 2006a. Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131, 623 630.

Smith, J.T., Popa, S.M., Clifton, D.K., Hoffman, G.E., Steiner, R.A., 2006b. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J. Neurosci. 26, 6687 6694.

Tena-Sempere, M., 2006. GPR54 and kisspeptin in reproduction. Hum. Reprod Update 12, 631 639.

van Aerle, R., Kille, P., Lange, A., Tyler, C.R., 2008. Evidence for the existence of a functional Kiss1/Kiss1 receptor pathway in fish. Peptides 29, 57 64.

Wagner, G.C., Johnston, J.D., Clarke, I.J., Lincoln, G.A., Hazlerigg, D.G., 2007. Redefining the limits of day length responsiveness in a seasonal mammal. Endocrinology. doi:10.1210/en.2007-0658.

7 Paper V: The effects of constant light on the growth performance and gonadal development of Nile tilapia (*Oreochromis niloticus niloticus*) juveniles

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The effects of constant light on the growth performance and gonadal development of Nile tilapia (*Oreochromis niloticus niloticus* L.) juveniles

Martinez-Chavez C. C., Parke D. V. and Migaud H.

Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA. UK.

ccm1@stir.ac.uk

Tel. +44 1786467878

Fax. +44 1786472133

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Abstract

A number of recent photoperiodic studies performed in tropical and subtropical marine and fresh water teleosts have shown that continuous lighting regimes can also be used to optimize growth and broodstock performance under culture. However, very few studies have been performed to test the effects of light intensity and spectral composition in teleosts. This work provides preliminary evidence of growth benefit of fish raised under constant illumination at different intensities (high, medium and low) compared to those under control regimes (12L:12D). Continuous illumination (LL) was applied in duplicate to 20 days post hatched (DPH) Nile tilapia larvae which were followed bi-weekly until 118 DPH. A control 12L:12D was used as a control. Mean length and weight of fish was significantly higher by the end of the experiment in all LL regimes irrespective of the intensities tested compared to the control groups with fish under the medium intensity treatment being the largest. SGR and FCR were very similar in all treatments although food consumed was higher in LL treatments suggesting that growth benefit might be due to increase in feed intake and/or energy conservation under LL. Furthermore, the effects of LL regimes on gonadal development appeared to be different between sexes with a slight delay observed in males whereas later stages of ovogenesis were advanced in females in comparison to controls. The results of this study thus suggest that LL regimes can influence both growth and gonadal development in Nile tilapia with no apparent differences between light intensities tested in this study. However, the mechanisms underlying such growth and gonadal development effects are still not understood and require further studies.

1 Introduction

Despite the significant improvement in growth performances observed in most commercially important aquaculture species over the last decades, there is still an increasing economic pressure to produce fish up to harvest in shorter periods. Growth improvement has been obtained through a series of species-specific management strategies such as selective breeding, suppression of maturation during ongrowing by photoperiodic manipulations, genetic manipulations (i.e. monosex and triploid culture) and largely improved diets and feeding regimes. These strategies are now routinely used within the industry for a range of commercially important species. Recent studies have also shown that photoperiod can significantly enhance growth performances independently of maturational control in temperate species. Indeed, in seasonal spawning species like rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), significant growth increase (up to 25 %) has been found when fish have been reared under constant long days (18L:6D) or continuous illumination (LL) as opposed to ambient conditions, with strong correlations between growth performance (growth rate, FCR), environment (temperature and photoperiod) and the hormones involved in the somatotropic axis (i.e. insulin-like growth factor-I (IGF-I), growth hormone (GH)) and reproduction (Bromage *et al*. 1994; Bromage *et al.*, 2001; Johnston *et al.*, 2003; Gabillard *et al.*, 2003a; Gabillard *et al.*, 2003b; Taylor *et al.*, 2005; Nordgarden *et al.*, 2005; Taylor *et al.*, 2006; Taylor *et al.*, 2008). To explain growth benefit effects in teleosts, two theories have been suggested; a shift in the endogenous growth rhythms (Duncan *et al.*, 1999; Oppedal *et al.*, 1999; Endal *et al.*, 2000) or a direct photostimulation of the growth axis through an increase of muscle fibre recruitment (Johnston *et al.*, 2003).

Similar growth enhancing and reproductive effects have been observed in tropical and subtropical seasonal breeding teleosts when exposed to constant long days or LL such as in snapper (*Pagrus auratus*), barramundi (*Lates calcarifer*), Mexican silverside *Menidia estor* (syn. *Chirostoma estor*) and red drum (*Sciaenops ocellatus*) (Barlow *et al.*, 1995; Leiner and MacKenzie, 2001; Tucker *et al.*, 2006; Martinez-Palacios *et al.*, 2007). However, very few studies investigating the photoperiodic effects on growth in non-seasonal (asynchronous; batch spawners) species have been performed. These include the Japanese medaka (*Oryzias latipes*) (Davis *et al.*, 2002) and the Nile tilapia (*Oreochromis niloticus niloticus*) where inconsistencies regarding LL growth effects in fry and fingerlings were reported (Biswas and Takeuchi, 2003; El-Sayed and Kawanna, 2004; Biswas *et al.*, 2004; Biswas *et al.*, 2005; Rad *et al.*, 2006). Importantly, despite the intensive use of photoperiod in most commercially important seasonal species by the industry and its clear effects, the mechanisms by which photoperiod acts is not yet fully understood (Boeuf and Le Bail, 1999; Boeuf and Falcon, 2001; Falcon *et al.*, 2007). However, recently, pineal (known to be the main site for non-visual light perception) light sensitivity has been shown to be different between Atlantic salmon, European sea bass and Atlantic cod suggesting that light should be applied in a species-specific manner (Migaud *et al.*, 2006; Migaud *et al.*, 2007). Importantly, for the lighting regimes to act on the fish physiology of a given species it must first be perceived at threshold levels and factors such as pineal window light penetration, pineal light sensitivity (thresholds) and spectral sensitivity could influence the degree of physiological effects observed. This might partly explain the inconsistencies observed in growth and reproduction following exposure to LL regimes (Boeuf and Le Bail, 1999; Migaud *et al.*, 2006; Migaud *et al.*, 2007). Thus it is clear that further studies on the effect of light on Nile tilapia growth and reproduction are clearly needed especially

with regards to light intensity prior to the potential implementation in tilapia aquaculture. The objective of this study was therefore to investigate the effects of LL regimes with different light intensities on growth and gonadal maturation as compared to a 12L:12D control photoperiod.

2 Materials and Methods

2.1 Animals and husbandry

A single batch of fertilized eggs of mixed sex red Nile tilapia (*O. niloticus niloticus*) was obtained from the tropical aquarium facilities at the Institute of Aquaculture (University of Stirling, Scotland). Experiments were perfromed in closed water recirculation systems at a constant temperature (27 ± 1 ^{o}C) as previously described (Campos-Mendoza *et al.*, 2004). Flow rate in each tank was controlled using valves in each tank and was set at approximately 60 litres h^{-1} $tank^{-1}$ (7 L round tanks), increasing to 100 litres h^{-1} $tank^{-1}$ at 62 days post hatch (DPH) when fry were released into the larger tanks (200 L aquariums filled to a 40 L volume) within the same compartments for the remainder of the experiment. Water temperature was monitored twice a day. 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. 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). All fish were hand fed to satiation three times a day at 09:00 hrs, 13:00 hrs and 18:00 hrs. Larvae were weaned on a 1:1 crumb mix (52%, Nutra Trout Fry 02 and 40%, Standard Expander 40, Skretting UK) until 72 DPH after which it was replaced by normal Nutra Trout Fry 02. Light intensity was measured at the beginning, middle and

end of the trial, at both the bottom and water surface using a Watt and Lux meter sensors (Skye Instruments, Ltd, Powys, UK).

2.2 Experimental design

A total of 150 fry / treatment (75 / replicate) of a same batch raised under 12L:12D (up to 20 DPH) were used. Weight and length of larvae at the beginning of the trial (20 DPH) was 0.06 g and 1.30 ± 0.04 cm respectively. Fish were exposed to either a 24 hour continuous light (LL) at three different light intensities (LL-High 100 %, LL-Medium 15-17 % and LL-Low 1-1.3 %) or a control 12L:12D treatment in duplicate (total of eight tanks). The trial ended at 118 (DPH). The control (12L:12D) tank had the same light intensity as the medium treatment. To achieve the different intensities required, number of bulbs and differing wattage were used in each compartment. For the LL-High (LL-H) treatment, four 100 watt light units (ScrewFix Direct, Yeovil, UK) were fitted into the compartment used. The LL-Medium (LL-M) light intensity treatment and the 12L:12D treatment had a single 60 watt light unit / compartment whereas the LL-Low (LL-L) intensity treatment had a 10 watt bulb with additional filtering to achieve the intensity levels shown in Table 1. The 12L:12D control photoperiod was controlled using a digital timer (Smiths Industries, London, UK).

Sampling took place every two weeks over a period of four months, at 20, 34, 48, 62, 76, 90, 104 and 118 DPH. At the first two sample points (20 and 34 DPH) only terminal sampling (n=10 / replicate / treatment) took place as the fish were too small to be anesthetised safely. Thereafter (48 – 118 DPH) both weight-length monitoring (all remaining fish at each time point) and terminal sampling (n=5-6 / replicate/ treatment) were carried out at each sample point. Sex ration (male/female) of remaining fish at 118 DPH was slightly variable between treatments (0.5, 0.9, 1.3 and 0.6 for LL-H, LL-M,

LL-L and 12L:12D respectively) whereas sex ratio estimated from all fish remaining and sampled throughout the experiment was similar in all treatments (1.1, 1.2, 1.4 and 1.1 for LL-H, LL-M, LL-L and 12L:12D respectively) .

2.3 Analysis

Histological samples were collected and prepared as previously described in Martinez-Chavez *et al.* (2008a). Histological slides of gonads from each fish sampled were used to identify the sex and maturational stage of development using a bifocal microscope (Olympus Optical Co., London, UK.). The stage of oocyte development was visually determined and the leading oocytes were classified according to a classification adapted from Coward and Bromage (1998) for oogenesis and Babiker and Ibrahim (1978) for spermatogenesis (Table 2a and 2b).

Image analysing software (ImageProPlus, Media Cybernetics Inc., MD, USA) was used to measure the long and short axis of 30 oocytes / replicate / treatment / sample point (mean values are presented). Specific growth rate was calculated by the formula SGR= $(e^g - 1) \times 100$, where $g = [\ln(W_2) - \ln(W_1)] \times (t_2 - t_1)^{-1}$, where W_2 and W_1 are the weight a time t_2 and t_1, respectively. The equation of Fulton's condition factor (K) (Ricker, 1975) was used using standard length (SL) as opposed to total length through the formula $K = 100 \times (W \times SL^{-3})$, where W is whole body weight and SL is standard length. The feed conversion ratio was calculated by the formula FCR = Kg food delivered x Kg weight gain^{-1}. Gonadosomatic index measurements were carried out for all fish at the end of the trial by the formula GSI = (wet gonad weight (gr) x wet body weight^{-1} (gr)) x 100. Survival was also estimated.

2.4 Statistical analysis

Due to the fact that equal variance was not met even after transformation attempts, data were analysed by non parametric statistical test (Kruskal-Wallis ANOVA on Ranks) before a Dunn's pair wise comparison at each sample point using SigmaStat Version 3. All statistical data was expressed as mean ± SE. All graphs were created using Sigma Plot Version 10.0 (Systat Software Inc, London, UK). For all tests, significance was set at $p<0.05$. As no significant differences were observed between replicates, data were pooled.

3 Results

3.1 Growth and feeding

No significant differences up to 118 DPH were found with regards to weight at which point fish in the LL-M intensity treatment were significantly heavier (50.8 ± 2.3 g) than 12L:12D fish (40.7 ± 2.2 g) with no other significant differences between LL-M and LL-H / LL-L (Table 3, Fig. 1a). Length measurements showed only significant differences between the LL-H (10.5 ± 0.2 cm) and the 12L:12D (9.9 ± 0.2 cm) groups also at 118 DPH (Table 2, Fig. 1b). Condition factor was significantly different between treatments at 48, 62, 90 and 104 DPH, with the LL-H treatment significantly different from all other treatments at 48 and 90 DPH (Fig. 1c). Total food consumed was very similar between all three light intensity treatments (LL-H, 3539.1 g, LL-M, 3533.7 g, LL-L, 3468.8 g) but 13-14 % lower in the 12L:12D group (3050.5 g). Overall (20-118 DPH) SGR (7.54 to 7.64 % day^{-1}) and FCR (0.71 to 0.76) were very similar between treatments (Table 3). Final biomass was found to be slightly higher in the LL-M treatment compared to the LL-H and LL-L treatments while the 12L:12D group was the lowest (see Table 3).

Little mortality was recorded over the period of the trial in LL-M, LL-L intensity treatments (2.0 ± 0.7 % and 1.0 ± 0.7 %, respectively) and control 12L12D group (5.3 ± 1.3 %). However, a higher mortality rate was observed in LL-H treatment (12.0 ± 2.7 %) although this was traced to a single event between 90 DPH and 104 DPH (Table 3).

The degree and timing of pigmentation of fish was also observed to be different, with LL-H fish acquiring pigmentation up to three weeks before any of the other treatments with red blotches appearing earlier than black ones (data not presented). The intensity of the blotches also appeared to be less vivid in the 12L:12D group compared to LL treatments. Unfortunately, no quantitative data is available.

3.2 Gonadal development

Gonadal staging for both sexes was assessed from 62-118 DPH. Females of all treatments were at pre-vitellogenic (S1) and early vitellogenic (S2) stages between 62 and 76 DPH. Late vitellogenic oocytes appeared first at 90 DPH in one female exposed to LL-H and 104 DPH in 12L:12D (only one female) and 118 DPH for both remaining treatments (LL-M, LL-L). At 118 DPH, 33 to 50 % of females sacrificed were at a mature stage (S4) in all LL treatments but none had reached this stage of gonadal development under 12L:12D in which 66 % were at late vitellogenic stage (S3).

Male gonads at 62 DPH were at an immature stage (S1) with only few individuals in LL-H, LL-M and 12L:12D at a maturating stage (S2). First signs of ripening (S4) was observed in the control group at 76 DPH. At 90 DPH, all treatments show 40-60% of sacrificed individuals in stage 4 with no apparent differences between treatments. However, by 104 DPH while most fish sampled in the 12L:12D treatment were spermiating (S5), only 10-33 % of the fish under LL treatments were spermiating with the remaining fish still at a ripening stage (S4). At 118 DPH, the percentage of

spermiating fish increased slightly in the LL treated groups (33-50 %) and importantly fish at stage 3 (all LL treatments) remained mature as opposed to the 12L:12D where fish were found only at S4-S5 stages (Fig. 4).

No significant GSI differences between treatments were observed from either sex. However, GSI of female fish exposed to LL-M and LL-L appeared higher while in males GSI of fish exposed to LL-M and LL-L appeared higher.

4 Discussion

In the present study, clear growth and gonadal development effects were observed between fish exposed to continuous illumination (LL-H, LL-H, LL-L) compared to a 12L:12D photoperiod. Fish under LL-H and LL-M treatments were significantly heavier (~20 %) than the fish under 12L:12D photoperiod by the end of the trial (118 DPH). Previous work done in the same species is limited to a couple of reports which appear inconsistent with the findings of the current study. In the first case, authors (Rad *et al.*, 2006) have reported a significantly higher (59 %) growth increase between Nile tilapia reared under LL (800 lux) compared to 12L:12D for a period of 168 days. Importantly, fish in the current study achieved double the weight of fish in Rad *et al.* (2006) approximately 50 days earlier. It is interesting to mention that despite the fact that fish in this previous study did not perform as well as the fish in the current study, a greater enhancement of growth was observed in fish exposed to LL. The difference observed in the percentage of growth improvement between both studies could relate to the overall performances of the fish populations. Indeed, in the study performed by Rad *et al.* (2006), fish appeared to not grow as well as in the present study however LL growth enhancement was much larger. This could suggest, as previously stated (Migaud, pers. com.), that LL effects on growth would depend on the growth rate at the time of

exposure with small enhancing effects when fish grow at their maximum capacities and larger effects when their growth rate is lower. Further studies are required to confirm such a hypothesis. Poor growth performances can be due to a variety of factors such as temperature during winter in temperate species, poor water quality or welfare among many others. In another study also performed in the Nile tilapia (El-Sayed and Kawanna, 2004), a similar percentage of increase (23 %) to that in the current study was found when fish were reared from 0.02 g for 60 days under LL (2500 lux). However, as in the first case, fish in the current study appeared 60-70 % heavier when similar stages (60 days) were compared. However when fish were reared under the same conditions from 2.4 g for 90 days a lower percentage of growth benefit was observed (6 %) (El-Sayed and Kawanna, 2004) suggesting that only fish exposed at earlier stages are affected by LL. Importantly, the degree of growth enhancement will vary depending on the stage of development at which LL is tested and the light intensity the fish are exposed to (Taylor *et al.*, 2006) however, in the present study no correlation between light intensities and growth performances have been observed. On the other hand, results in the current study are in agreement with those found in seasonal species like the rainbow trout were fish under long photoperiods (18L:6D) were significantly heavier (17.5 %) than fish reared under simulated natural photoperiods (Taylor *et al.*, 2005).

It is thus clear when comparing the current results to those in previous Nile tilapia studies (El-Sayed and Kawanna, 2004; Rad *et al.*, 2006) that fish in both cases were clearly not performing as well as in the current study and thus not reaching their full potential growth during the length of the trials. The reasons for most of these differences can be attributed to differences in the methods used such as feeding regime (i.e. feeding time and frequency), feeding type (i.e. % biomass vs. satiation), nutritional

composition of feed, stocking density and light intensities used, but also to more inconspicuous differences such as the genetic background and strain of fish. This illustrates the difficulties of comparing dataset from different trials even in a single species.

Importantly, in Atlantic salmon, growth patterns show a tendency towards greater skeletal growth during the winter months, providing the frame for muscle gain in spring. When fish are exposed to LL during winter, a shift towards greater muscle fibre recruitment (during the first 40 days of exposure) is observed subsequently followed by muscle hypertrophy (Bjornsson *et al.*, 2000; Johnston *et al.*, 2003). Unfortunately however, in this study data on muscle fibre recruitment is unavailable and future studies should determine its possible contribution to weight gain in Nile tilapia as seems to be the case of other species

Condition factor data in this study showed similar patterns of growth for all except the LL-H treatment which displayed higher amplitude of changes although the nature of the data (unequal variances) didn't allow us to determine if these changes were significant. However significant differences were seen at specific time points. Also, feed consumed by fish under LL treatments was found to be higher in all cases, while FCR and SGR were found to be similar between all treatments suggesting that continuous lighting would not enhance any of these parameters but rather act by stimulating the appetite of fish. Interestingly, this evidence contradicts what has been found in temperate seasonal fish such as rainbow trout where an increase in FCR and SGR was observed in fish exposed to LL (Taylor *et al.*, 2006). This would suggest that LL might not affect inbuilt rhythms and patterns of growth in Nile tilapia, if such patterns exist in a tropical species, but more likely directly stimulate the growth axis through an increased appetite. Further studies are required at both endocrine and molecular levels, to confirm such a

theory especially focusing on the somatotropic axis which controls feed intake and somatic growth.

Importantly, in this study no significant effects on growth were observed when exposing fish to LL with increasing light intensities. This again would seem in contradiction with rainbow trout findings previously reported where fish performed significantly better under higher light intensities (Taylor *et al.*, 2006). However comparisons between both studies are not straightforward as fish were reared in completely different environments and rearing systems (outdoor cages with day/night light intensity rhythms due to the superimposition of artificial lighting system on to the ambient illumination *vs* indoor recirculation tanks with constant light intensity throughout the day) (Taylor *et al.*, 2006).

The current results thus demonstrate that light intensity seems to not be playing an important role in the growth enhancement effect observed in fish exposed to LL. This has implications for the use of artificial lighting in tilapia culture as low to medium intensity LL regimes confer a cost-effective solution to higher intensity regimes at this stage. However long term studies are needed as it is possible that higher light intensities could have a greater effect in latter growth stages as shown in salmonids where in some cases growth effects where only shown 126 days after onset of LL (Johnston *et al.*, 2003).

Not only growth was influenced by the LL regimes tested in the present study but also gonadal development. Importantly, an important question must be addressed as to whether growth effects were linked to differences in gonadal development or the latter was due to growth effects. Male and female gonads reached the final stages of maturation at 104 and 118 DPH respectively, correlating well with growth improvement observed in fish under LL also by 118 DPH. In agreement with previous studies in Nile

tilapia (Rad *et al.*, 2006) and seasonal species such as Senegalese sole (*Solea senegalensis*) and European seabass (*Dicentrarchus labrax*) (Garcia-Lopez *et al.*, 2006), male gonads in this study seemed delayed under LL regimes compared to 12L:12D. In contrast, females in this study appeared to be at more advanced gonadal stages under all LL treatments compared to the 12L:12D photoperiod, although we acknowledge that in some cases, sample size might have been too low to make any firm conclusions. However, a reversed effect has also been observed in the Japanese medaka (*Oryzias latipes*) where 89.3 % of females in a long photoperiod (16L:8D) were significantly heavier and sexually mature by 19 weeks of age compared to 7.4 % of females in a shorter photoperiod (8L:16D) (Davis *et al.*, 2002). In both cases irrespective of the gonadal effects observed, weight increase is probably caused by an appetite stimulation of fish under LL and through energy conservation (Biswas *et al.*, 2002; Biswas and Takeuchi, 2002). It is also important to acknowledge that sexual growth dimorphism exists in Nile tilapia with male fish growing quicker than female fish. However this could not be monitored for several reasons. Firstly fish were too small to externally identify sexes and secondly fish numbers were limited and sampling was performed randomly. However it was possible to suggest that growth effects observed were not due to unbalanced sex ratios as they were similar in all treatments at the end of the trial.

The fact that in this species, females under LL treatments seem to reach advanced stages of maturation earlier is quite interesting as most of the previous studies have been performed in seasonal spawners that have clear windows of oocyte recruitment and maturational commitment that can be suppressed or delayed with the use of long days and/or LL depending on the species (Bromage *et al.*, 2001; Hansen *et al.*, 2001; Davie *et al.*, 2007). Thus, the mechanisms (i.e. developmental clock, size, age, nutritional status) controlling the initiation of puberty and maturation in Nile tilapia as in other

batch spawner species could be less sensitive to light than seasonal spawning species simply because of the different environments inhabited with less pronounced seasonal photoperiodic changes in the tropics as compared to temperate latitudes and differences in the reproductive strategies and/or social interactions (Coward and Bromage, 2000; Au *et al.*, 2006; Hofmann, 2006). Recently however, other important factors which might play a role in the differential effect of light in growth and reproduction have been suggested. Recent evidence has suggested significant differences in the basic light perception and entrainment pathways among teleosts including Nile tilapia. This evidence would suggest that factors such as light sensitivity (intensity and wavelength), percentage of light skull penetration and circadian organization might affect how light is perceived in teleost species (Migaud *et al.*, 2006; Migaud *et al.*, 2007; Ziv *et al.*, 2007; Martinez-Chavez *et al.*, 2008b). Moreover, it is also important to mention the lack of information on the specific molecular and cellular mechanisms (i.e. light thresholds) that ultimately control growth and reproduction in fish which could also vary significantly between species. In this respect, a recent molecular study performed in Nile tilapia (Martinez-Chavez *et al.*, 2008a) showed that despite an observed delay in gene expression of GPR54 (recently discovered to be a gater of reproduction) by LL, the expression of the type I GnRH receptor was unaltered suggesting that the cascade of hormones leading to gonadal maturation was in motion in this species and thus not affected by light. Further molecular and proteomic approaches on fish exposed to extreme photoperiods such as LL and 6L:6D which have shown to influence reproduction (Campos-Mendoza *et al.*, 2004; Biswas *et al.*, 2005) will be invaluable in elucidating the different mechanisms at work which could explain some of the seemingly contradictory findings across teleosts.

In brief, this study suggests that LL regimes of a low-medium intensity are ideal to attain a significant growth benefit in Nile tilapia and that no significant growth benefit is observed if higher intensities are applied at this stage (118 WPH). Also, this work provided preliminary evidence of differential effects of photoperiod on gonadal development in this species. However, the mechanisms underlying growth benefit and gonadal development are not known and further studies are required. Further studies are also required at a commercial scale to assess the potential economic benefits and viability of using artificial lighting regimes.

Acknowledgements

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Figure 1. a) Weight, b) length and c) condition factor over time in Nile tilapia raised up to 118 days post hatch under different light intensities (High LL, Medium LL, Low LL and Control 12L:12D). Values are expressed as mean ± SE (n = 33-75 / replicate). Superscripts indicate significant differences between treatments at a given time point.

Figure 2. Mean food consumed (gr/fish/week) under different light intensities (High LL, Medium LL, Low LL and Control 12L:12D). Values are expressed as mean (n = 33-75 / replicate).

Figure 3. Relative proportion of each stage of oogenesis in Nile tilapia fry reared under different light intensities (High LL, Medium LL, Low LL and Control 12L:12D) from 62 DPH to 118 DPH. The number of individuals is indicated on the graph in the bars.

Figure 4. Relative proportion of each stage of spermatogenesis in Nile tilapia fry reared under different light intensities (High LL, Medium LL, Low LL and Control 12L:12D) from 62 DPH to 118 DPH. The number of individuals is indicated on the graph in the bars.

Table 1. Light intensities in Watts m^{-2} and lux (mean ± SE) measured at the bottom and surface of the tanks for each experimental treatment during day time.

Treatment	Watts m^{-2} (Bottom/Surface)	Lux (Bottom/Surface)
LL High	3.0 ± 0.2 / 4.6 ± 0.6	684.0 ± 32.0 / 1031.0 ± 104.0
LL Med	0.5 ± 0.1 / 0.7 ± 0.1	141.5 ± 17.5 / 172.5 ± 10.5
LL Low	0.04 ± 0.0 / 0.0 ± 0.0	4.5 ± 0.5 / 8.0 ± 1.0
Control	0.7 ± 0.1 / 0.9 ± 0.2	172.5 ± 22.5 / 190.5 ± 30.5

Table 2a. Classification scheme used to identify the stages of oogenesis showing stages S1, S2, S3 and S4. Adapted from Coward and Bromage (1998).

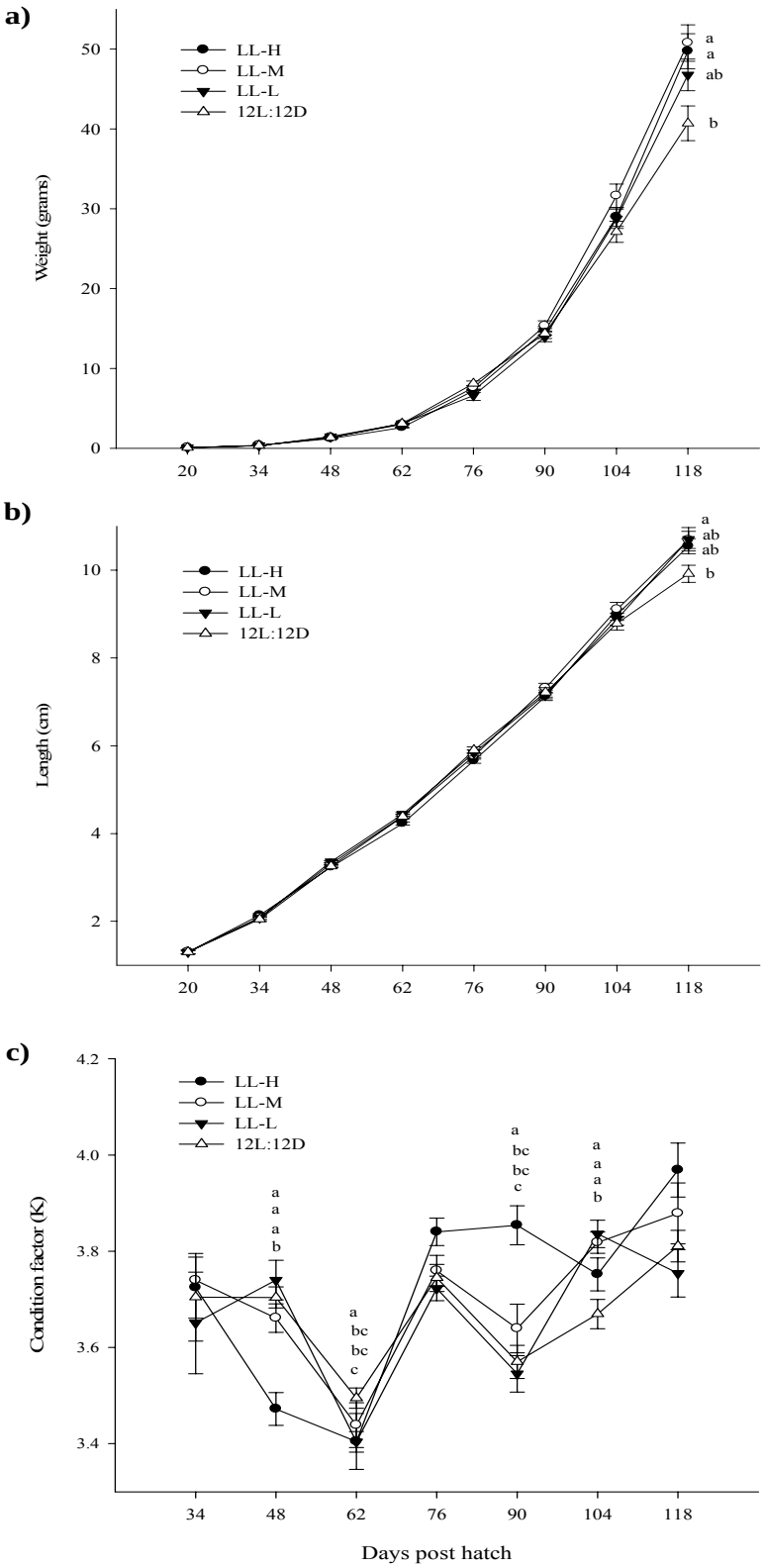
Stage	Definition	Size Range (μm)	Appearance
S1	Pre-vitellogenic	7-345	Nucleus containing chromatin strands. Developing follicular layer. Vesicle at near edge of oocyte. Stains dark pink reducing to pale pink as less basophilic.
S2	Early stages of vitellogenesis	224-658	Small yolk granules starting at periphery. Vesicles seen throughout oocyte. Follicular layer can be seen to be more developed.
S3	Late stages of vitellogenesis	428-1416	Yolk granules become larger yolk globules and empty vacuoles throughout oocyte. Very developed follicular wall. Nucleus central.
S4	Mature	428-1416	Same as S3 but vesicle migration can be seen.

Table 2b. Classification scheme used to identify stages of spermatogenesis 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 final (118 DPH) weights, lengths, sex ratio, GSI and overall (20-118 DPH) SGR, FCR, food consumed and mortality. Values are expressed as mean ± SE where appropriate. Superscripts indicate significant differences where appropriate. * Mortality traced back to a single unknown event.

	Treatments			
	High	**Medium**	**Low**	**Control**
Weight[F]	49.7 ± 2.2[a]	50.8 ± 2.3[a]	46.8 ± 2.0[ab]	40.7 ± 2.2[b]
Length[F]	10.5 ± 0.2[a]	10.7 ± 0.2[ab]	10.7 ± 0.3[ab]	9.9 ± 0.2[b]
Total food consumed (g)	3539	3533	3468	3050
SGR	7.6	7.6	7.5	7.4
FCR	0.7	0.8	0.7	0.7
GSI (F/M)	0.9 / 0.4	1.4 / 0.6	2.0 / 0.5	1.1 / 0.4
% Mortality	12.0 ± 2.7[*]	2.0 ± 0.7	1.0 ± 0.7	5.3 ± 1.3
Sex ratio (M/F) at 118 DPH	0.5	0.9	1.3	0.6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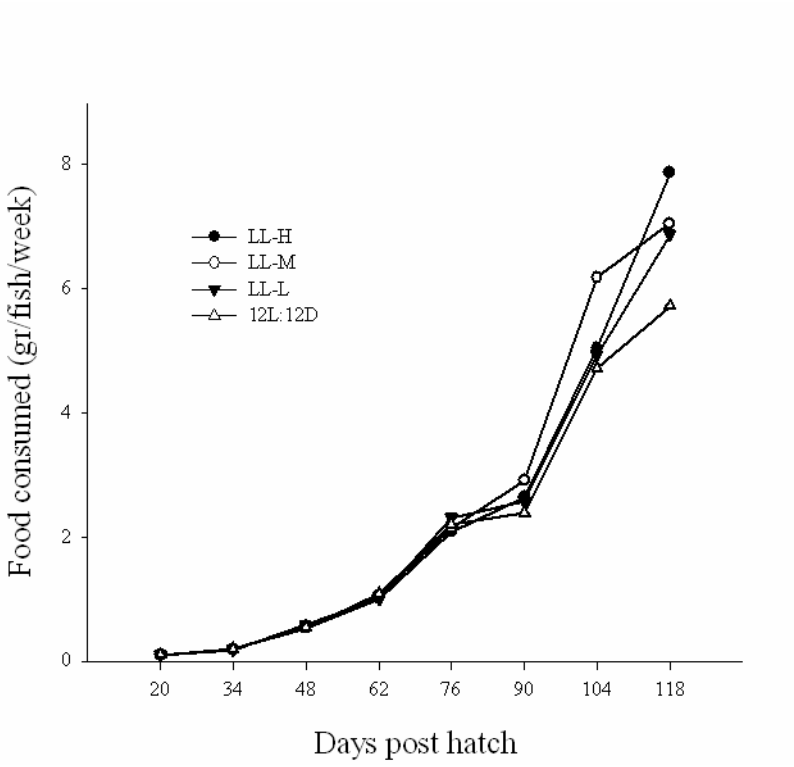
Days post hatch

Figure 1.


Food consumed (gr/fish/week)
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118
Days post hatch

Figure 2.

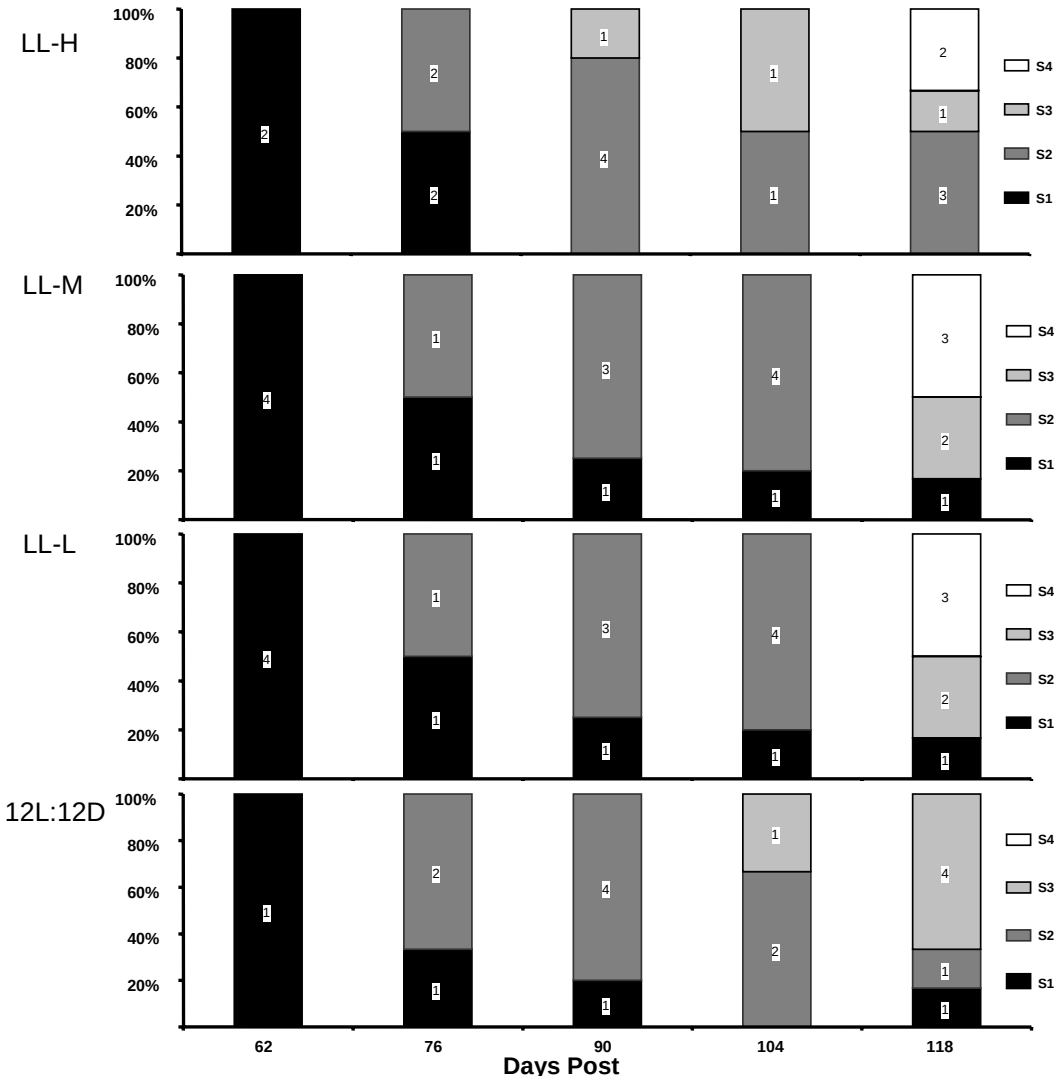
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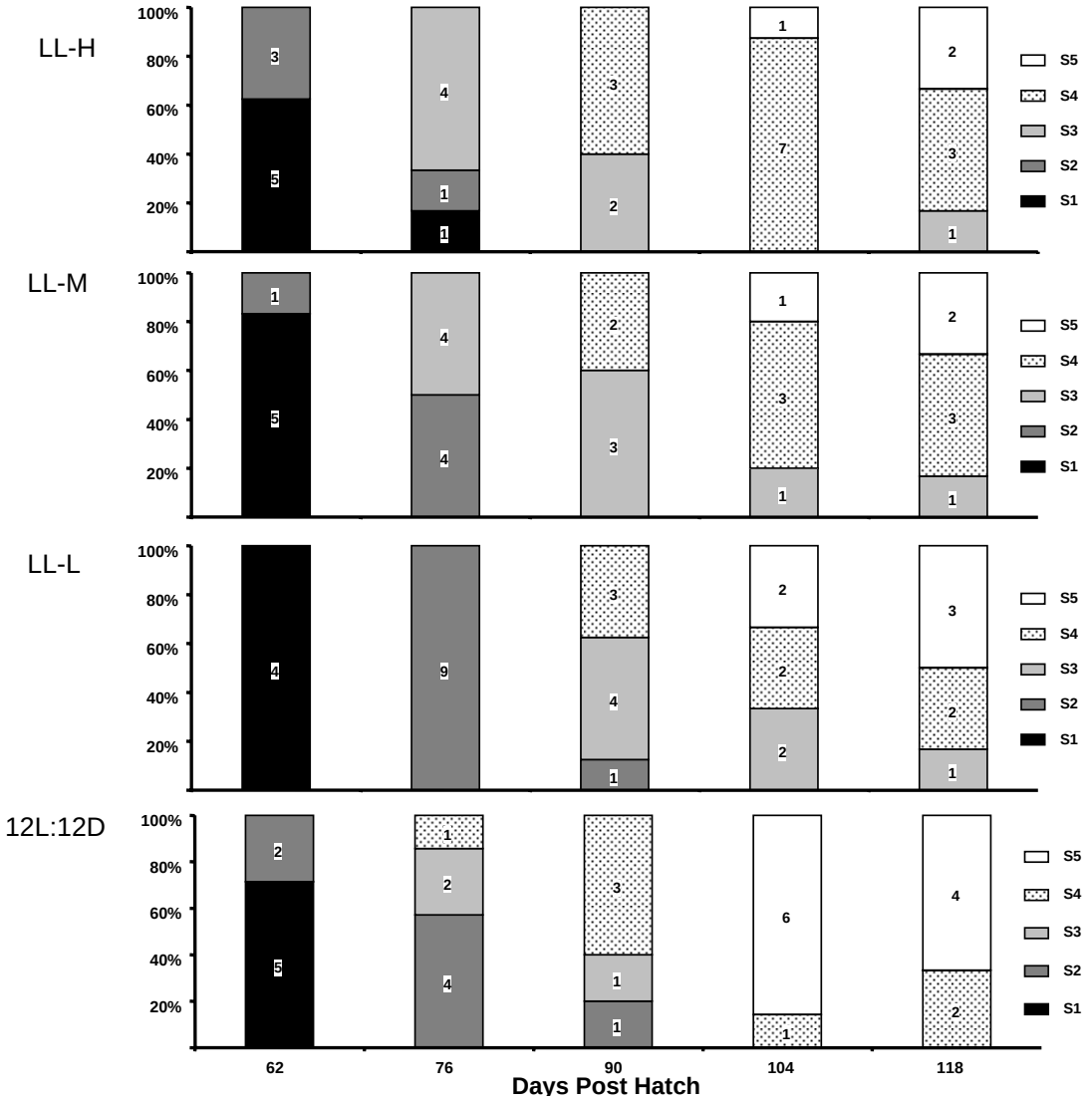
Figure 3.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Days Post Hatch

Figure 4.

References

Au, T.M., Greenwood, A.K. and Fernald, R.D. (**2006**). Differential social regulation of two pituitary gonadotropin-releasing hormone receptors. *Behavioural Brain Research* 170**,** 342-346.

Babiker, M.M. and Ibrahim, H. (**1978**). Studies on the biology of reproduction in the cichlid *Tilapia nilotica* (L.): gonadal maturation and fecundity. *Journal of Fish Biology* 14**,** 437-448.

Barlow, C.G., Pearce, M.G., Rodgers, L.J. and Clayton, P. (**1995**). Effects of photoperiod on growth, survival and feeding periodicity of larval and juvenile barramundi *Lates calcarifer* (Bloch). *Aquaculture* 138**,** 159-168.

Biswas, A.K. and Takeuchi, T. (**2002**). Effect of different photoperiod cycles on metabolic rate and energy loss of fed and unfed adult tilapia *Oreochromis niloticus*: Part II. *Fisheries Science* 68**,** 543-553.

Biswas, A.K., Endo, M. and Takeuchi, T. (**2002**). Effect of different photoperiod cycles on metabolic rate and energy loss of both fed and unfed young tilapia *Oreochromis niloticus*: Part I. *Fisheries Science* 68**,** 465-477.

Biswas, A.K. and Takeuchi, T. (**2003**). Effects of photoperiod and feeding interval on food intake and growth rate of Nile tilapia *Oreochromis niloticus* L. *Fisheries Science* 69**,** 1010-1016.

Biswas, A.K., Maita, M., Yoshizaki, G. and Takeuchi, T. (**2004**). Physiological responses in Nile tilapia exposed to different photoperiod regimes. *Journal of Fish Biology* 65**,** 811-821.

Biswas, A.K., Morita, T., Yoshizaki, G., Maita, M. and Takeuchi, T. (**2005**). Control of reproduction in Nile tilapia *Oreochromis niloticus* (L.) by photoperiod manipulation. *Aquaculture* 243**,** 229-239.

Bjornsson, B.T., Hemre, G.I., Bjornevik, M. and Hansen, T. (**2000**). Photoperiod regulation of plasma growth hormone levels during induced smoltification of underyearling Atlantic Salmon. *General and Comparative Endocrinology* 119**,** 17-25.

Boeuf, G. and Falcon, J. (**2001**). Photoperiod and growth in fish. *Vie Et Milieu-Life and Environment* 51**,** 247-266.

Boeuf, G. and Le Bail, P.Y. (**1999**). Does light have an influence on fish growth? *Aquaculture* 177**,** 129-152.

Bromage, N.R., Randall, C.F., Duston, J., Thrush, M. and Jones, J. (**1994**). Environmental control of reproduction in salmonids. In: *Recent advances in Aquaculture IV*, Eds. Muir, J.F. and Roberts, R.J., Blackwell Scientific Publications. London. pp.55-65.

Bromage, N.R., Porter, M. and Randall, C. (**2001**). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* 197**,** 63-98.

Campos-Mendoza, A., McAndrew, B.J., Coward, K. and Bromage, N. (**2004**). Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effects on spawning periodicity, fecundity and egg size. *Aquaculture* 231**,** 299-314.

Coward, K. and Bromage, N. (**1998**). Histological classification of oocyte growth and dynamics of ovarian recundescence in *Tilapia zillii*. *Journal of Fish Biology* 53**,** 285-302.

Coward, K. and Bromage, N.R. (**2000**). Reproductive physiology of female tilapia broodstock. *Reviews in Fish Biology and Fisheries* 10**,** 1-25.

Davie, A., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2007**). The role of seasonally altering photoperiod in regulating physiology in Atlantic cod (*Gadus morhua*). Part I. Sexual maturation. *Canadian Journal of Fisheries and Aquatic Sciences* 64**,** 84-97.

Davis, C.R., Okihiro, M.S. and Hinton, D.E. (**2002**). Effects of husbandry practices, gender, and normal physiological variation on growth and reproduction of Japanese medaka, *Oryzias latipes*. *Aquatic Toxicology* 60**,** 185-201.

Duncan, N., Mitchell, D. and Bromage, N. (**1999**). Post-smolt growth and maturation of out-of-season 0+ Atlantic salmon (*Salmo salar*) reared under different photoperiods. *Aquaculture* 177**,** 61-71.

El-Sayed, A.M. and Kawanna, M. (**2004**). Effects of photoperiod on the performance of farmed Nile tilapia *Oreochromis niloticus*: I. Growth, feed utilization efficiency and survival of fry and fingerlings. *Aquaculture* 231**,** 393-402.

Endal, H.P., Taranger, G.L., Stefansson, S.O. and Hansen, T. (**2000**). Effects of continuous additional light on growth and sexual maturity in Atlantic salmon, *Salmo salar*, reared in sea cages. *Aquaculture* 191**,** 337-349.

Falcon, J., Besseau, L., Sauzet, Sa. and Boeuf, G. (**2007**). Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends in Endocrinology and Metabolism* 18**,** 81-88.

Gabillard, J.C., Weil, C., Rescan, P.Y., Navarro, I., Gutierrez, J. and Le Bail, P.Y. (**2003a**). Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* 133**,** 233-242.

Gabillard, J.C., Weil, C., Rescan, P.Y., Navarro, I., Gutierrez, J. and Le Bail, P.Y. (**2003b**). Environmental temperature increases plasma GH levels independently of nutritional status in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* 133**,** 17-26.

Garcia-Lopez, A., Pascual, E., Sarasquete, C. and Martinez-Rodriguez, G. (**2006**). Disruption of gonadal maturation in cultured Senegalese sole *Solea senegalensis* (Kaup) by continuous light and/or constant temperature regimes. *Aquaculture* 261**,** 789-798.

Hansen, T., Karlsen, O., Taranger, G.L., Hemre, G.I., Holm, J.C. and Kjesbu, O.S. (**2001**). Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. *Aquaculture* 203**,** 51-67.

Hofmann, H.A. (**2006**). Gonadotropin-releasing hormone signaling in behavioral plasticity: signalling mechanisms. *Current Opinion in Neurobiology* 16**,** 343-350.

Johnston, I.A., Manthri, S., Smart, A., Campbell, P., Nickell, D. and Alderson, R. (**2003**). Plasticity of muscle fibre number in seawater stages of Atlantic salmon in response to photoperiod manipulation. *Journal of Experimental Biology* 206**,** 3425-3435.

Leiner, K.A. and MacKenzie, D.S. (**2001**). The effects of photoperiod on growth rate and circulating thyroid hormone levels in the red drum, *Sciaenops ocellatus*: evidence for a free-running circadian rhythm of T4 secretion. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology* 130**,** 141-149.

Martinez-Chavez, C.C., Minghetti, M. and Migaud, H. (**2008a**). GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. *General and Comparative Endocrinology* 156**,** 224-233.

Martinez-Chavez, C.C., Al-Khamees, S., Campos-Mendoza, A., Penman, D.J. and Migaud, H. (**2008b**). Clock-controlled endogenous melatonin rhythms in Nile

tilapia (*Oreochromis niloticus niloticus*) and African Catfish (*Clarias gariepinus*). *Chronobiology International* 25**,** 31-49.

Martinez-Palacios, C.A., Chavez-Sosa, J.C., Santoyo-Guzman, V.O., Campos-Mendoza, A., Martinez-Chavez, C.C. and Ross, L.G. (**2007**). The effect of photoperiod on the reproduction of *Chirostoma estor estor* Jordan 1879 from Lago de Patzcuaro, Mexico. *Journal of Applied Ichthyology* 23**,** 621-623.

Migaud, H., Taylor, J.F., Taranger, G.L., Davie, A., Cerda-Reverter, J.M., Carrillo, M., Hansen, T. and Bromage, N.R. (**2006**). A comparative *ex vivo* and *in vivo* study of day and night perception in teleosts species using the melatonin rhythm. *Journal of Pineal Research* 41**,** 42-52.

Migaud, H., Davie, A., Martinez Chavez, C.C. and Al-Khamees, S. (**2007**). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *Journal of Pineal Research* 43**,** 327-335.

Nordgarden, U., Hansen, T., Hemre, G.I., Sundby, A. and Bjornsson, B.T. (**2005**). Endocrine growth regulation of adult Atlantic salmon in seawater: the effects of light regime on plasma growth hormone, insulin-like growth factor-I, and insulin levels. *Aquaculture* 250**,** 862-871.

Oppedal, F., Taranger, G.L., Juell, J.E. and Hansen, T. (**1999**). Growth, osmoregulation and sexual maturation of underyearling Atlantic salmon smolt S*almo salar* L. exposed to different intensities of continuous light in sea cages. *Aquaculture Research* 30**,** 491-499.

Rad, F., Bozaoglu, S., Ergene Gozukara, S., Karahan, A. and Kurt, G. (**2006**). Effects of different long-day photoperiods on somatic growth and gonadal development in Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture* 255**,** 292-300.

Ricker, W.E. (**1975**). Computation and interpretation of biological statistics of fish populations. *Bulletin of the Fisheries Research Board of Canada* 191**,** 1-382.

Taylor, J.F., Migaud, H., Porter, M.J.R. and Bromage, N.R. (**2005**). Photoperiod influences growth rate and plasma insulin-like growth factor-I levels in juvenile rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* 142**,** 169-185.

Taylor, J.F., North, B.P., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2006**). Photoperiod can be used to enhance growth and improve feeding efficiency in farmed rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 256**,** 216-234.

Taylor, J.F., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2008**). Relationships between environmental changes, maturity, growth rate and plasma insulin-like growth factor-I (IGF-I) in female rainbow trout. *General and Comparative Endocrinology* 155**,** 257-270.

Tucker, B.J., Booth, M.A., Allan, G.L., Booth, D. and Fielder, D.S. (**2006**). Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper *Pagrus auratus*. *Aquaculture* 258**,** 514-520.

Ziv, L., Tovin, A., Strasser, D. and Gothilf, Y. (**2007**). Spectral sensitivity of melatonin suppression in the zebrafish pineal gland. *Experimental Eye Research* 84**,** 92-99.

8 Summary of findings

In this section, the main findings of each paper are summarized and shown at which physiological level each study was done (Figure 6).

Paper I

Evidence for differential photic regulation of pineal melatonin synthesis in teleosts.

- Preliminary evidence of night time melatonin suppression in ophthalmectomized Nile tilapia suggesting the existence of a third type (eye dependant) of light circadian organization in teleosts.

- Preliminary pineal *in vitro* melatonin levels in Nile tilapia suggesting a pineal less light sensitive compared to previously studied teleost species.

- Evidence of a high light skull penetration in Nile tilapia compared to other species tested especially towards near-infrared wavelengths.

Paper II

Confirmation that Nile tilapia eyes are required for night time plasma melatonin

- Phase shifted melatonin synthesis in the Nile tilapia retina.

- Confirmation that total but not partial ophthalmectomy suppresses night-time melatonin production in Nile tilapia.

- Preliminary evidence that the absence of night-time melatonin production would not be caused by post surgical stress through behavioural observations and cortisol measurements.

- Confirmation of unexpected low melatonin levels produced at night *in vitro*.

Paper III

Clock-controlled endogenous melatonin rhythms in Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*)

- Confirmation of a diel melatonin rhythm in tilapia as seen in other teleosts.
- Demonstration of robust circadian endogenous melatonin rhythms under darkness in tilapia acclimated to 12L:12D for at least 3 weeks.
- Evidence of a clock controlled diel melatonin profile with anticipation of the photophase.
- Preliminary evidence that the endogenous melatonin oscillator is not capable of entraining to a 6L:6D photoperiod.

Paper IV

GPR54 and rGnRH I gene expression during onset of puberty in Nile tilapia

- Correlation of Kisspeptin receptor (GPR54) and GnRH I receptor during the onset of puberty in Nile tilapia suggesting a similar role of the Kiss 1/GPR54 system in tilapia as recently found in mammals.
- First evidence to date in fish (2nd in vertebrates) of photoperiodic (LL) effects on the Kiss/GPR54 signalling mechanisms.

Paper V

The effects of constant light and light intensities on the growth performance and gonadal development of Nile tilapia (*Oreochromis niloticus niloticus* L.) juveniles

- Demonstration of a growth enhancement in Nile tilapia exposed to LL regimes with no significant effects of light intensities.

- Suggested sex dimorphisms in the timing to first maturation between males (delay) and females (advancement) of Nile tilapia reared under LL.

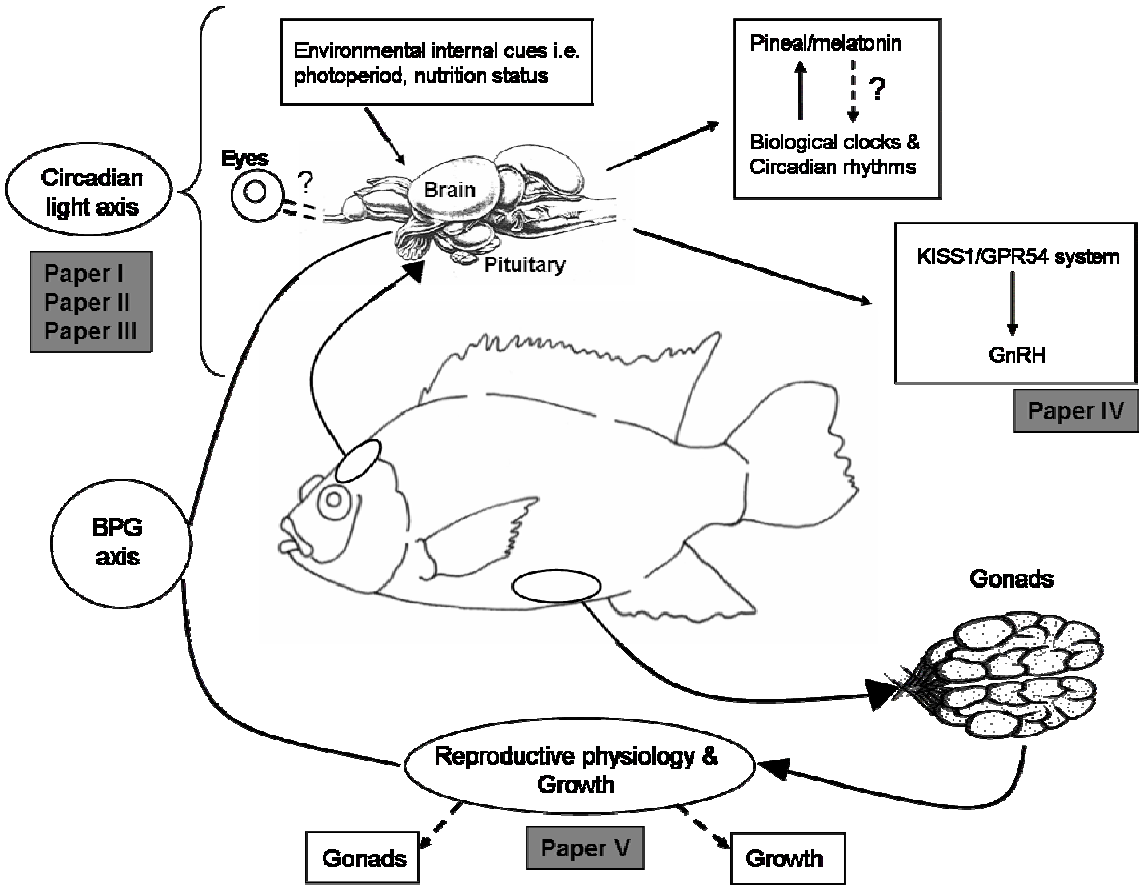


Figure 6.- Schematic presentation of the studies performed along the circadian and BPG axes in Nile tilapia.

9 General Discussion

Most of the physiological, circadian and reproductive studies involving environmental cues such as photoperiod have been performed in temperate seasonal teleost species. This is due to a high level of research investment due to their large scale commercial importance. However, although there is a natural tendency to generalise results that one finds in a given fish species to the whole teleost phylogeny. 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 (iteropare *vs.* continuous spawner), it is unlikely that one unique circadian organization (retina-pineal gland network) exist in fish. Thus, the overall aim of this work was to expand our knowledge on circadian biology and environmental physiological effects in tropical species. The selection of Nile tilapia as a model was thus very relevant for this study due to the divergent nature of the species in terms of its reproductive strategy, social habits, environmental niche (i.e. subtropical, freshwater) and phylogenetic position being considered as one of the most "evolved" teleosts. A number of studies were therefore performed in Nile tilapia during this PhD project which resulted in five manuscripts (*published* or *to be submitted*) addressing the circadian light axis (manuscripts I-III), the control of the BPG axis (manuscript IV) and finally, the reproductive and growth effects of photoperiod (manuscript V). The aims of this final section is to review the main conclusions arising from these studies, integrate them in a more general context and highlight the need for future work in these fields.

Circadian light axis

Despite being one of the most important species for the worldwide aquaculture (over 2.5 million tones in 2005; Josupeit, 2007) and an important teleost model for many research areas (i.e. nutrition, genetics), to our knowledge, Nile tilapia has not been used in any kind of chronobiological studies. The first focus of this PhD project was thus to define the circadian light axis in Nile tilapia in comparison to other teleosts species (**Paper I).** Preliminary evidence of a different circadian light axis organization in Nile tilapia, characterised by a complete dependence of the pineal gland and more specifically melatonin production on the eyes, was shown. This came initially as a surprise as it was the first time this was observed in any teleost species which were considered to have a decentralized organization with no or little dependence of the melatonin synthesis by the pineal gland on the eyes. This type of organisation has only been previously seen in higher vertebrates, such as mammals, with light perception taking place solely in the eyes with regards to the control of pineal melatonin production. This could partially explain why fish were not often considered to a greater extent as models for clinical/chronobiological studies. These preliminary findings were undoubtedly very interesting but required further confirmation which was given by following studies **(Paper II)** in which total and partial ophthalmectomy was performed and the potential effect of post-surgery stress tested. Totally eye enucleated fish were again unable to synthesize and release melatonin into the blood circulation during the dark phase even after a week post-surgery. Interestingly, fish with only one eye remaining were able to produce normal melatonin levels at night although with slightly lower levels suggesting that basal levels observed in totally enucleated fish were not due to post-surgery stress and that one eye might even be enough for normal light entrainment. This claim was indirectly supported by behavioural observations showing

that fish returned to a "normal" behaviour (i.e. feeding behaviour and dominance display as observed prior to surgery) within 24 to 48hrs following ophthalmectomy. Although stress levels, through the measurement of cortisol were monitored, it clearly appears that the sampling procedure was not appropriate as high levels were recorded in ophthalmectomized fish as well as intact control fish. Indeed, when analysing the data, a clear effect of sampling time between the four individual fish sampled at each time point was observed with low levels (up to 50 $ngml^{-1}$) in the first fish sampled irrespective of the photoperiod and increasing levels in the following fish (see Appendix 2). According to previous studies performed in the same species, such levels, although higher than in other species (e.g. salmonids, $<5ngml^{-1}$, North *et al.*, 2006) are observed in non stressed fish (Vijayan *et al.*, 1997; Barreto and Volpato, 2006). Furthermore, no difference was observed between controls and ophthalmectomized first sampled fish. Further studies are needed to better assess the potential effects of stress on melatonin production.

Additional evidence of this new circadian organization in fish was also given by *in vitro* culture of Nile tilapia pineal glands (**Paper I and Paper II**). Results showed that pineal glands in isolation were not able to normally produce melatonin at night as seen in other species studied so far, with lower amplitude levels. This indirectly confirms that pineal glands photo-sensitivity might be different than in other teleosts. However, we acknowledge that further optimization of the technique for this specific species might still be required despite the coherence of these findings with those observed *in vivo*.

Nile tilapia showed further discrepancies when cranial (pineal window) light transmission was compared between species. In this respect, Nile tilapia continued to surprise, as it had by far the highest percentage of light penetration of all species

analysed with higher light transmission for longer wavelengths as observed in other species (600-700 nm) Prior to performing this comparative study, we originally hypothesised that light penetration through the skull of Nile tilapia would be lower than in the other species tested due to a) the thickness of the cranial bones and the small size of the pineal window and b) the exposure to stronger light intensities in their natural environment compared to other species. It is difficult to explain at this stage why the pineal gland in this species would be less sensitive than in other studied species although being exposed to more light. The localisation and definition of deep brain photoreceptors might help understand the latter as they might play an important role in the light entrainment pathway of this species. More questions arise regarding the importance of this network of photoreceptive sites (retinal, pineal and deep brain) for which not much is known yet in fish. However in birds different circadian organizations have been suggested across species and removal or ablation of the different photoreceptive components have been shown to affect different species to a higher or lesser degree. It is then just logical to suggest that the largest group of vertebrates (teleosts represent 46% of vertebrates in comparison to 10% for mammals, (Kardong, 2002), would also have developed more than one single circadian organization type in response to the range of environment inhabited and 500 millions years of evolution. .

In **Paper II**, we have also described the phase shifted melatonin production in the retina with higher levels being produced during the day than at night. Although this has been observed in other teleost species, (European seabass, Iigo *et al.*, 1997; Garcia-Allegue *et al.*, 2001 and trout, Gern *et al.*, 1978; Zachmann *et al.*, 1992; Besseau *et al.*, 2006), the regulatory mechanisms are still unclear. To date, it is believed that high levels of AANAT and melatonin could act as detoxifying agents in the retina protecting it from harmful free radicals caused by day light in the eye together with other known

roles as photoreceptor adaptation to darkness and retinal circadian physiology. AANAT is essential in the success of ancestral life forms and so important that has lingered throughout the course of evolution of life on earth (Ganguly *et al.*, 2001; Klein, 2006). The basis of this is supported by the potent antioxidant properties of AANAT and evidence in non-vertebrates that melatonin synthesis was not its original function (Coon and Klein, 2006; Klein, 2006). Such an important role of AANAT as a free radical protector and key component of melatonin synthesis in the eyes of vertebrates would explain the out of phase pattern of AANAT and melatonin found in Nile tilapia and other species of fish. However further studies on the divergent evolution of AANAT expression patterns are needed in order to explain the specific roles and advantages of phase shifted melatonin patterns in the eyes of some vertebrates. The recent discovery of several AANAT subtypes should help to further elucidate these divergences (Besseau *et al.*, 2006; Coon and Klein, 2006; Zilberman-Peled *et al.*, 2006). The current hypothesis would thus suggest, that in fish, genome duplication allowed one enzyme type (retinal AANAT1) to keep a broad ancestral activity (involving acetylation of a wide range of arylalkylamine substrates important in amine detoxification) and the other enzyme (pineal AANAT2), to become highly specialized in serotonin acetylation and melatonin production (Zilberman-Peled *et al.*, 2004). Due to the relevance of AANAT in this study, it was also our intention in this study to describe AANAT activity in parallel to that of melatonin synthesis. To do so we initiated an investigation to test whether we could quantify AANAT protein using rat antibodies know previously to work for salmonids. We also intended to clone and sequence AANAT in Nile tilapia to do gene expression and localization studies. However, due to technical and time constraints, we could not pursue this any further.

Irrespective of the light entrainment pathway (i.e. eyes and/or pineal) in this novel circadian organization, we set out to describe the circadian melatonin rhythm system of Nile tilapia in terms of circulating melatonin release. In **Paper III**, we reported on the low at day and high at night melatonin levels found in Nile tilapia which is also found in all teleosts and vertebrates studied to date. 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. Moreover, we also showed evidence of how this light/dark melatonin rhythm is constantly entrained by light. This occurs by the suppression of circulating melatonin levels during the day and will not resume until darkness appears as confirmed by exposing fish to continuous illumination. This mechanism is thought to be controlled in other vertebrates directly by light at the transcriptional level through specialized light regulated promoter regions (e.g. E-box) in the AANAT gene. More importantly, we also showed the presence of a strong endogenous melatonin rhythm that could drive melatonin production in constant darkness. This endogenous melatonin rhythm found in Nile tilapia was also confirmed to be circadian in nature. Endogenous melatonin rhythms like this have been found in every other teleost studied except salmonids (Iigo *et al.*, 2007). Of more relevance however, is the fact that to our knowledge, this is the longest running *in vivo* melatonin rhythm shown to date in any species. It is important to mention that these recent observations together with other research group findings would suggest that fish are much more sensitive to light/dark changes than previously thought, thus presenting an even more particular challenge when performing constant darkness studies. To this respect we cannot stress how important is to confirm lightproofness of any system used in such studies as we have done in this work. In this work our DD studies were carried out in completely light controlled rooms, where if needed, background aquarium lights

were left on 24 h with the exception of the short sampling periods where they were turned off to get access to the fish. This allowed us to confirm that in fact our system was completely lightproof as otherwise melatonin synthesis in fish would be suppressed by outside (background) lighting. It is important to mention the constant use of an infrared camera system to monitor fish exposed to DD. Interestingly, a recent paper by Matsumoto and Kawamura (2005) has provided evidence that the eyes, but not the pineal, of Nile tilapia are sensitive to near infrared (865 nm) wavelength when tested at high intensities (50.6 μmol,$^{-2}$s^{-1}). In their study, cardiac conditioning experiments in which pineal window covered fish exposed to 865 nm wavelengths showed cardiac conditioned response which were confirmed by histological retina slides where incomplete darkness adaptation was found. In our study however, despite the fact that the LED's of the infrared cameras used gave a weak red glow (below sensitivity of our light scanning equipment), fish behaviour suggested they did not perceive it even when they were turned on and off during DD. Moreover, despite being on constantly (24h), fish were capable of producing circadian endogenous melatonin rhythms, suggesting that infrared light even if perceived by the eyes and or pineal gland would not be perceived by the light entrainment pathway which would ultimately affect melatonin synthesis (Hanifin *et al.*, 2006). Importantly, isolation of any other type of environmental input (i.e. light, temperature and feeding) other than the one studied is a key step in the design and outcome of LL/DD experiments. This can prove to be particularly challenging as specialized holding systems (i.e. light–temperature-feed controlled) are needed which limit the size of the experiment. In our respective experiments, as briefly mentioned earlier, lightproofness of the system was confirmed by performing DD experiments in completely light and temperature controlled rooms. Additionally, the photoperiodic system was also temperature controlled (27^0C ± 1).

Feeding on the other hand has proven to synchronize patterns of behaviour (i.e. locomotor) and physiology in different fish species including other perciforms (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 known to be independent (Meijer and Rietveld, 1989). To our knowledge, in fish, no evidence of feeding entrainment affecting the melatonin synthesis rhythm system has been found, in fact, melatonin levels are thought to be among the least sensitive circadian rhythms to the entraining effects of feeding (Holloway *et al.*, 1979; Inouye, 1982; Spieler, 1992). However this possibility could not be overlooked and thus, round the clock automatic feeders were used in all experiments testing the presence/absence of endogenous melatonin rhythms in this species. The idea (as we could not stop feeding the fish), was to avoid as much as possible any potential feed input to entrain circadian fish rhythms.

Circadian endogenous melatonin rhythms in Nile tilapia reared under simulated natural photoperiods (i.e. 12L:12D) seemed to be very strong and robust (**Paper III**). However when fish were acclimatized in to a 6L:6D photoperiod, despite being able to produce diel melatonin rhythms under these short LD periods, when exposed to DD, the endogenous melatonin rhythm system previously found to mimic the photoperiod (12L:12D) under which fish were acclimatised under DD has lost this endogenous rhythmicity. This finding would suggest this photocycle (6L:6D) to be too short for the endogenous clock to entrain irrespective of its location which exposes some of the mechanisms abilities and limitations to cope with extreme (unnatural) photoperiods known to affect other vertebrates (Foster and Kreitzman, 2005). Such short photo-cycle could prove useful in further dissecting components of the biological clocks especially if they are confirmed to cause endocrine disruption as suggested by a previous studies in

Nile tilapia. In this later study, female broodstock exposed to 6L:6D photoperiods were shown to prevent spawning and had significantly lower E_2 (estradiol) levels than their counterparts exposed to 12L:12D and 14L:10D (Biswas *et al.*, 2005a). This together with the findings of the present work would suggest that fish may be unable to entrain to such short photoperiods. This however is only a hypothesis which requires further investigation.

In this work we have described the light circadian axis and endogenous melatonin rhythms of Nile tilapia and provided evidence of a new form of circadian organization in teleosts which has recently also been confirmed in African catfish (*C. gariepinus*) by another PhD project performed in parallel. However further studies are clearly needed to confirm the mechanisms, advantages and similarities of this circadian organization compared to the mammalian one, which would prove very interesting in the chronobiology field. Irrespective of the phylogenetic comparisons between model species and their divergence, the information gathered in this work might help to better understand the mechanisms involved in light entrainment and reproductive physiology of Nile tilapia and other teleosts.

The control of the BPG axis

As briefly mentioned earlier, not much is known about the specific pathways and mechanisms that link the perception of environment signals (i.e. photoperiod, temperature) and the entrainment of physiology of teleosts especially with regards to reproduction. In fact until recently, the onset of the GnRH cascade through 'spontaneous' stimulation of GnRH neurons during puberty was regarded as the uppermost level of the BPG axis in vertebrates including fish. It was not until the beginning of this decade that the Kiss1/GPR54 system has been described in mammals

as a gater of reproductive events sitting at the top of the BPG axis. What we now know from mammals including humans is that kisspeptins (product of the Kiss 1 gene) stimulate GnRH neurons during puberty through the kisspeptin transmembrane receptor (GPR54) found in GnRH secreting cells. As such, the Kiss1/GPR54 system appears to be an essential endocrine component at the top of the BPG axis which must be activated to stimulate GnRH signalling, sexual development and successive reproductive events in mammals. This was demonstrated by the fact that loss of Kiss1 or GPR54 signalling cannot be overcome by compensatory mechanisms (d'Anglemont de Tassigny *et al.*, 2007; Roa *et al.*, 2008). During the progress of this PhD project, the first cloning of a GPR54 sequence in a teleost fish (Nile tilapia) occurred. This encouraged us and gave us the initial tool to develop a qPCR protocol for gene expression which would enable us firstly to correlate its activity with its novel known function as a gater of reproduction in mammals and support the idea that this system is conserved in vertebrates. However, we were also interested in finding a link between the environmental light stimuli and the reproductive physiology of teleosts. Initially, we intended to do this through melatonin receptor expression for which some primers were available but we preferred instead to focus on the Kiss1/GPR54 system due to its novelty, availability of the GPR54 sequence for tilapia and 'upward' approach compared to melatonin where so many efforts have been previously unsuccessful.

Because at the time the only information available on the Kiss1/GPR54 system in teleosts was the actual sequence and colocalization of GPR54 in Nile tilapia GnRH neurons (Parhar *et al.*, 2004), the first logical step was thus, to screen its expression across several adult Nile tilapia tissues in order to determine further potential active sites **(Paper IV)**. The fact that relevant expression is only observed in brain and pituitary tissues is in agreement with its potential conserved role in fish as a gater of

reproduction at the top of the BPG axis. In this paper, we also demonstrated how a parallel surge of GPR54 and rGnRH I gene expression occurs in juvenile Nile tilapia brains after 7 weeks post hatch which, importantly, is closely correlated with gonadal development. This would indirectly suggest a connection between reproductive signalling activity in the brain and the onset of puberty and first maturation although at this stage our data are only correlative. Thus, this work suggests for the first time a similar role of the Kiss1/GPR54 system in a tropical batch spawner teleost. Interestingly, more evidence in teleosts has recently been published during my PhD project by other research groups in four different species (grey mullet, cobia, fathead minnow and zebrafish) with similar results (Nocillado *et al.*, 2007; Mohamed *et al.*, 2007; van Aerle *et al.*, 2008; Filby *et al.*, 2008).

The work carried out in this PhD project also provided, for the first time in teleosts, very interesting preliminary evidence of photoperiodic effects on the GPR54 expression and brain-pituitary-gonadal (BPG) axis **(Paper IV)**. Indeed fish exposed to continuous illumination showed a delayed surge in GPR54 expression compared to fish exposed a 12L:12D photoperiod. This suggests for the first time a potential effect of light in a specific reproductive signalling pathway which is in agreement with the observed effects of delay or inhibition of maturation by long or continuous illumination in a number of seasonal fish species (Amano *et al.*, 2000; Bromage *et al.*, 2001; Berrill *et al.*, 2003). However this effect has not yet been confirmed in Nile tilapia and different physiological mechanisms might be at work due to the divergent nature of the reproductive strategy compared to seasonal species in which light may not have such marked effects. Thus in order to confirm the direct/indirect effect of light at the Kiss1/GPR54 transcriptional level, it would be interesting to see if a similar pattern of delayed expression or suppression of GPR54 and GnRH activity is also observed in

seasonal species in which LL is known to delay reproduction. More evidence could be gathered by determining if the Kiss1/GPR54 genes have promoter regions likely to be influenced by light as happens with other genes like AANAT. Further work is also needed to confirm that effectively a surge of kisspeptin and GPR54 activity are actively stimulating GnRH neurons at the brain and pituitary levels and initiate the GnRH cascade. It is important to determine, if the Kiss1/GPR54 activation is needed at each reproductive cycle or if it is solely influencing the timing of puberty and first maturation. For this, further molecular and proteomic studies are needed in order to first, clone and sequence the Kiss1 gene in these species and secondly to determine to a greater extent the patterns and mechanisms of gene activation through qPCR, *in situ* hybridization and gene silencing/knockout studies *in vivo* and *in vitro*. The recent evidence of the existence and characterization of the Kiss1 gene in zebrafish is a step forward in this direction (van Aerle *et al.*, 2008).

Although the finding of the Kiss1/GPR54 system will undoubtedly unravel many questions regarding the stimulatory mechanisms leading to the onset of the GnRH cascade in vertebrates, some of the initial questions remain as to how this system is triggered and controlled. Also, of further interest would be to compare in more detail potential signalling differences between species of different reproductive strategies and life cycles (seasonal vs. continuous spawners, protandrous vs. dioeic etc) which could give us an insight into conserved roles and mechanisms within teleost evolution.

Reproductive and growth effects of photoperiod

As said previously, the specific mechanisms involved in the regulation of environmental signals and teleost reproductive physiology are still unclear. What is clear however is that photoperiod is known to affect growth and reproduction in all fish

species studied to date. Interestingly, the selection of fish species used for this purpose has been driven mainly by their commercial importance and to a lesser extent by the diversity of habitats inhabited and life strategies. As a result, there are many reports on the effects of photoperiod on the growth and sexual maturation of salmonids in general and other temperate seasonal species. It is important to mention that even between closely related species (i.e. salmon vs. trout, cod vs. haddock), significant differences in photoperiodic effects have been observed. It is thus of particular interest to compare the effects of very divergent species regarding life cycles, environment and phylogeny. Until very recently Nile tilapia had been overlooked both as a model in this particular area of research and also by its commercial importance being currently the most productive aquaculture species worldwide. Recent efforts however have focused on determining potential reproductive and growth effects by photoperiod manipulation in Nile tilapia and other tropical and subtropical species (Barlow *et al.*, 1995; Davis *et al.*, 2002; El-Sayed and Kawanna, 2004; Rad *et al.*, 2006; Tucker *et al.*, 2006). The objectives of this last section of the work were to first confirm initial discrepancies about the effects of constant illumination on the growth and sexual development of Nile tilapia. Additionally, we tested if different light intensities of LL had an effect on these important aspects as pineal light sensitivity has been shown to be different between species including Nile tilapia (Migaud *et al.*, 2006; Ziv *et al.*, 2007; Migaud *et al.*, 2007a). Results of this work presented and discussed in **Paper V** are in agreement with previous growth studies regarding an enhanced effect of growth in this species (El-Sayed and Kawanna, 2004; Rad *et al.*, 2006). However the percentage of growth enhancement of light treated fish (LL) and the final weight of the fish between this and previous studies made apparent the importance of experimental design and the particular complications (fish performance, sexual growth dimorphisms and sample

limitations) of light growth studies in this particular species. By the end of the trial (118 DPH) a significant improvement in growth was apparent in all fish exposed to LL irrespective of the intensities used. This confirmed that photoperiod in this species can enhance growth although the mechanisms of action (whether through direct stimulation of the growth axis, muscle recruitment or feeding stimulus) remain unclear. However, preliminary evidence provided by this study suggested that feed intake was stimulated in fish exposed to LL with feed conversion efficiency (measured through FCR) remaining similar between treatments. Similar findings have also been observed in two other perciforms such as red sea bream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*) (Kohbara *et al.*, 2003; Biswas *et al.*, 2005b). Moreover sexual growth dimorphisms (males>females) observed in this study and others could be due in part, to different stress-related coping mechanisms such as compensatory growth which occurs in this species (Jauncey, 1998; Barreto *et al.*, 2003). In fact, a greater compensatory growth mechanism has been observed in male tilapia (Barreto *et al.*, 2003). In such study, although both sexes observed similar feed conversion efficiency rates after being food deprived for eleven days it was the males who attained a higher growth at the end of the re-feeding, period possibly due to greater hyperphagia (overeating) than females (Jauncey, 1998; Barreto *et al.*, 2003). However, in no circumstances firm conclusions can be made based on the present data as feed intake has not been directly monitored and fish were only fed to satiation and feed delivered recorded with no collection of waste.

Regarding sexual development, LL appeared to have effects on the timing of reproduction with a delay observed in males whereas later stages of the reproductive cycle appeared advanced in females which has also been observed in Japanese medaka, *O. latipes* (Davis *et al.*, 2002). Together, these results (precocity of males and delay of

females) are in accordance with the sexual growth dimorphisms observed in this species where males reach maturation size quicker than females. This however is only a hypothesis which will need to be confirmed as no strong evidence of different sex related growth/reproduction mechanisms are known yet.

It is clear that in order to increase our knowledge on the photoperiodic effects on growth and reproduction in teleosts, it is vital that we broaden the range of fish models used. This will provide important information on the diversity of systems at work and their adaptations to a multitude of environments.

Potential applications for the Industry and conclusions

With respect to Nile tilapia, this work supports the idea that photoperiod might be beneficial to the culture of this species, especially in intensive culture, however further studies are needed to determine the net cost benefits at a farmed scale. Also, short photoperiods have potential in Nile tilapia culture if further studies confirm reproductive/growth advantages. Importantly however, it would seem that in order to achieve maximum growth performance in this species, the combination of several husbandry tools might produce better results than photoperiod manipulation alone. So for example the combination of monosex culture (either by genetic manipulations or hormonal sex reversal), good husbandry practices (good water quality, feed and feeding regimes, stocking densities) and photoperiod manipulations (i.e. LL or 6L:6D) during the on growing phase could have the potential of considerably shortening the time required for the fish to reach market size. Interestingly, another aspect which has the potential to further improve farming performance of this species would be to investigate the potential of species-specific feeding strategies which could enhance the feed efficiency (Azzaydi *et al.*, 2007). Ultimately the greater knowledge of the basic

mechanisms leading to growth and reproductive physiology will lead to the development of stock management tools which could further increase the performance of farmed aquaculture species.

10 References

Albrecht, U. (**2002**). Functional genomics of sleep and circadian rhythm: Invited review: Regulation of mammalian circadian clock genes. *Journal of Applied Physiology* 92**,** 1348-1355.

Amano, M., Iigo, M., Ikuta, K., Kitamura, S., Yamada, H. and Yamamori, K. (**2000**). Roles of melatonin in gonadal maturation of underyearling precocious male masu salmon. *General and Comparative Endocrinology* 120**,** 190-197.

Amano, M., Iigo, M., Ikuta, Ka., Kitamura, S. and Yamamori, K. (**2003a**). Daily variations in melatonin binding sites in the masu salmon brain. *Neuroscience Letters* 350**,** 9-12.

Amano, M., Iigo, M., Ikuta, M., Kitamura, S. and Yamamori, K. (**2003b**). Characterization and maturational differences of melatonin binding sites in the masu salmon brain. *General and Comparative Endocrinology* 131**,** 338-344.

Appelbaum, L. and Gothilf, Y. (**2006**). Mechanism of pineal-specific gene expression: The role of E-box and photoreceptor conserved elements. *Molecular and Cellular Endocrinology* 252**,** 27-33.

Aranda, A., Madrid, J.A., Zamora, A., Sanchez-Vasquez, S. and Sanchez-Vasquez, F.J. (**1999a**). Synchronizing effect of photoperiod on the dual phasing of demand-feeding rhythms in seabass. *Biological Rhythm Research* 30**,** 392-406.

Aranda, A., Sanchez-Vazquez, F.J. and Madrid, J.A. (**1999b**). Influence of water temperature on demand-feeding rhythms in seabass. *Journal of Fish Biology* 55**,** 1029-1039.

Aranda, A., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2001**). Influence of light on feeding anticipatory activity in goldfish. *Journal of Biological Rhythms* 16**,** 50-57.

Aronson, B.D., Bell-Pedersen, D., Block, G.D., Bos, N.P.A., Dunlap, J.C., Eskin, A., Garceau, N.Y., Geusz, M.l.E., Johnson, K.A. and Khalsa, S.B.S. (**1993**). Circadian rhythms. *Brain Research Reviews* 18**,** 315-333.

Au, T.M., Greenwood, A.K. and Fernald, R.D. (**2006**). Differential social regulation of two pituitary gonadotropin-releasing hormone receptors. *Behavioural Brain Research* 170**,** 342-346.

Azzaydi, M., Martinez, F.J., Zamora, S., Sanchez-Vazquez, F.J. and Madrid, J.A. (**2000**). The influence of nocturnal vs. diurnal feeding under winter conditions on growth and feed conversion of European seabass (*Dicentrarchus labrax,* L.). *Aquaculture* 182**,** 329-338.

Azzaydi, M., Rubio, V.C., Martinez Lopez, F.J., Sanchez-Vasquez, F.J., Zamora, S. and Madrid, J.A. (**2007**). Effect of restricted feeding schedule on seasonal shifting of daily demand-feeding pattern and food anticipatory activity in European seabass (*Dicentrarchus labrax* L.). *Chronobiology International* 24**,** 859-874.

Babiker, M.M. and Ibrahim, H. (**1978**). Studies on the biology of reproduction in the cichlid *Tilapia nilotica* (L.): gonadal maturation and fecundity. *Journal of Fish Biology* 14**,** 437-448.

Barlow, C.G., Pearce, M.G., Rodgers, L.J. and Clayton, P. (**1995**). Effects of photoperiod on growth, survival and feeding periodicity of larval and juvenile barramundi *Lates calcarifer* (Bloch). *Aquaculture* 138**,** 159-168.

Barrenetxe, J., Delagrange, P. and Martinez, J.A. (**2004**). Physiological and metabolic functions of melatonin. *Journal of Physiology and Biochemistry* 60**,** 61-72.

Barreto, R.E., Moreira, P.S.A. and Carvalho, R.F. (**2003**). Sex specific compensatory growth in food deprived Nile tilapia. *Brazilian Journal of Medical and Biological Research* 36**,** 477-483.

Barreto, R.E. and Volpato, G.L. (**2006**). Stress responses of the fish Nile tilapia subjected to electroshock and social stressors. *Brazilian Journal of Medical and Biological Research* 39**,** 1605-1612.

Barrett, P., Conway, S. and Morgan, P.J. (**2003**). Digging deep structure function relationships in the melatonin receptor family. *Journal of Pineal Research* 35**,** 221.

Bayarri, M.J., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2002**). Influence of light intensity, spectrum and orientation on seabass plasma and ocular melatonin. *Journal of Pineal Research* 32**,** 34-40.

Bayarri, M.J., Garcia-Allegue, R., Lopez-Olmeda, J.F., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2004a**). Circadian melatonin release *in vitro* by European seabass pineal. *Fish Physiology and Biochemistry* 30**,** 87-89.

Bayarri, M.J., Munoz-Cueto, J.A., Lopez-Olmeda, J.F., Vera, L.M., Rol de Lama, M.A., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2004b**). Daily locomotor activity and melatonin rhythms in Senegal sole (*Solea senegalensis*). *Physiology and Behavior* 81**,** 577-583.

Benyassi, A., Schwartz, C., Coon, S.L., Klein, D.C. and Falcon, J. (**2000**). Melatonin synthesis: Arylalkylamine N-Acetyltransferases in trout retina and pineal organ are different. *Neuroreport* 11**,** 255-258.

Berrill, I.K., Porter, M.J.R., Smart, A., Mitchell, D. and Bromage, N.R. (**2003**). Photoperiodic effects on precocious maturation, growth and smoltification in Atlantic salmon, *Salmo salar*. *Aquaculture* 222**,** 239-252.

Besseau, L., Benyassi, A., Moller, M., Coon, S.L., Weller, J.L., Boeuf, G., Klein, D.C. and Falcon, J. (**2006**). Melatonin pathway: breaking the 'high-at-night' rule in trout retina. *Experimental Eye Research* 82**,** 620-627.

Beyer, C.E., Steketee, J.D. and Saphier, D. (**1998**). Antioxidant properties of melatonin-an emerging mystery. *Biochemical Pharmacology* 56**,** 1265-1272.

Biswas, A.K. and Takeuchi, T. (**2003**). Effects of photoperiod and feeding interval on food intake and growth rate of Nile tilapia *Oreochromis niloticus* L. *Fisheries Science* 69**,** 1010-1016.

Biswas, A.K., Maita, M., Yoshizaki, G. and Takeuchi, T. (**2004**). Physiological responses in Nile tilapia exposed to different photoperiod regimes. *Journal of Fish Biology* 65**,** 811-821.

Biswas, A.K., Morita, T., Yoshizaki, G., Maita, M. and Takeuchi, T. (**2005a**). Control of reproduction in Nile tilapia *Oreochromis niloticus* (L.) by photoperiod manipulation. *Aquaculture* 243**,** 229-239.

Biswas, A.K., Seoka, M., Inoue, Y., Takii, K. and Kumai, H. (**2005b**). Photoperiod influences the growth, food intake, feed efficiency and digestibility of red sea bream (*Pagrus major*). *Aquaculture* 250**,** 666-673.

Boeuf, G. and Le Bail, P.Y. (**1999**). Does light have an influence on fish growth? *Aquaculture* 177**,** 129-152.

Boeuf, G. and Falcon, J. (**2001**). Photoperiod and growth in fish. *Vie Et Milieu-Life and Environment* 51**,** 247-266.

Bolliet, V., Ali, M.A., Lapointe, F.J. and Falcon, J. (**1996**). Rhythmic melatonin secretion in different teleost species: an *in vitro* study. *Journal of Comparative Physiology: Part B* 165**,** 677-683.

Borjigin, J., Sun, X. and Wang, M.M. (**2007**). Is NAT the rate-limiting enzyme in melatonin synthesis? In: *Treatise on pineal gland and melatonin*, Eds. Haldar, Ch., Singaravel, M. and Kumar M, S., Science Publishers. Plymouth, UK. pp.423-460.

Boujard, T. and Leatherland, J.F. (**1992**). Circadian rhythms and feeding time in fishes. *Enviromental Biology of Fishes* 35**,** 109-131.

Bromage, N.R., Elliott, J.A.K., Springate, J.R.C. and Whitehead, C. (**1984**). The effects of constant photoperiods on the timing of spawning in the rainbow trout. *Aquaculture* 43**,** 213-223.

Bromage, N. (**1995**). Broodstock managements and seed quality-general considerations. In: *Broodstock Management and Egg and Larval Quality*, Eds. Bromage, N. and Roberts, J.R., Blackwell Science. Edinburgh, UK. pp.1-24.

Bromage, N.R., Porter, M. and Randall, C. (**2001**). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* 197**,** 63-98.

Bubenik, G.A. and Pang, S.F. (**1997**). Melatonin levels in the gastrointestinal tissues of fish, amphibians, and a reptile. *General and Comparative Endocrinology* 106**,** 415-419.

Cahill, G.M., Grace, M.S. and Besharse, J.C. (**1991**). Rhythmic regulation of retinal melatonin: metabolic pathways, neurochemical mechanisms and the ocular circadian clock. *Cellular and Molecular Neurobiology* 11**,** 529-558.

Cahill, G.M. and Besharse, J.C. (**1995**). Circadian rhythmicity in vertebrate retinas: Regulation by a photoreceptor oscillator. *Progress in Retinal and Eye Research* 14**,** 267-291.

Cahill, G.M. (**1996**). Circadian regulation of melatonin production in cultured zebrafish pineal and retina. *Brain Research* 708**,** 177-181.

Cahill, G.M. (**1997**). Circadian melatonin rhythms in cultured zebrafish pineals are not affected by catecholamine receptor agonists. *General and Comparative Endocrinology* 105**,** 270-275.

Cahill, G. (**2002**). Clock mechanisms in zebrafish. *Cell Tissue Research* 309**,** 27-34.

Campos-Mendoza, A., McAndrew, B.J., Coward, K. and Bromage, N. (**2004**). Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effects on spawning periodicity, fecundity and egg size. *Aquaculture* 231**,** 299-314.

Cariboni, A., Maggi, R. and Parnavelas, J.G. (**2007**). From nose to fertility: the long migratory journey of gonadotropin-releasing hormone neurons. *Trends in Neurosciences* 30**,** 638-644.

Carolsfeld, J., Powell, J.F.F., Park, M., Fischer, W.H., Craig, A.G., Chang, J.P., Rivier, J.E. and Sherwood, N.M. (**2000**). Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. *Endocrinology* 141**,** 505-512.

Carr, A.J., Tamai, T.K., Young, L.C., Ferrer, V., Dekens, M.P. and Whitmore, D. (**2006**). Light reaches the very heart of the zebrafish clock. *Chronobiology International* 23**,** 91-100.

Chen, W. and Baler, R. (**2000**). The rat Arylalkylamine N-Acetyltransferase E-box: differential use in a master vs. a slave oscillator. *Molecular Brain Research* 81**,** 43-50.

Chong, K.L., Koh, M. and Melamed, P. (**2005**). Molecular regulation of gonadotropin gene expression in teleosts. In: *Hormones and their receptors in fish reproduction*, Eds. Melamed, P. and Sherwood, N.M., World Scientific Publishing. London. pp.76-104.

Conti, A., Tettamanti, Ch., Singaravel, M., Haldar, Ch., Pandi-Perumal, R.S. and Maestroni, G.J.M. (**2002**). Melatonin: an ubiquitous and evolutionary hormone. In: *Treatise on the Pineal Gland and Melatonin*, Eds. Haldar, Ch., Singaravel, M. and Kumar, M.S., Science Publisher. USA.

Coon, S.L., Begay, V., Deurloo, D., Falcon, J. and Klein, D.C. (**1999**). Two Arylalkylamine N-Acetyltransferase genes mediate melatonin synthesis in fish. *Journal of Biology and Chemistry* 274**,** 9076-9082.

Coon, S.L. and Klein, D.C. (**2006**). Evolution of Arylalkylamine N-Acetyltransferase: emergence and divergence. *Molecular and Cellular Endocrinology* 252**,** 2-10.

Coward, K. and Bromage, N. (**1998**). Histological classification of oocyte growth and dynamics of ovarian recundescence in *Tilapia zillii*. *Journal of Fish Biology* 53**,** 285-302.

Coward, K. and Bromage, N.R. (**2000**). Reproductive physiology of female tilapia broodstock. *Reviews in Fish Biology and Fisheries* 10**,** 1-25.

d'Anglemont de Tassigny, X., Fagg, L.A., Dixon, J.P.C., Day, K., Leitch, H.G., Hendrick, A.G., Zahn, D., Franceschini, I., Caraty, A., Carlton, M.B.L., Aparicio, S.A.J.R. and Colledge, W.H. (**2007**). Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. *PNAS* 104**,** 10714-10719.

Dardente, H., Klosen, P., Pevet, P. and Masson-Pevet, M. (**2003**). MT1 melatonin receptor mRNA expressing cells in the pars tubelaris of the European hamster: effect of photoperiod. *Journal of Neuroendocrinology* 15**,** 778-786.

Davie, A., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2007a**). The role of seasonally altering photoperiod in regulating physiology in Atlantic cod (*Gadus morhua*). Part I. Sexual maturation. *Canadian Journal of Fisheries and Aquatic Sciences* 64**,** 84-97.

Davie, A., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2007b**). The role of seasonally altering photoperiod in regulating physiology in Atlantic cod (*Gadus morhua*). Part II. Somatic growth. *Canadian Journal of Fisheries and Aquatic Sciences* 64**,** 98-112.

Davies, B., Bromage, N. and Swanson, P. (**1999**). The brain-pituitary-gonadal axis of female rainbow trout *Oncorhynchus mykiss*: effects of photoperiod manipulation. *General and Comparative Endocrinology* 115**,** 155-166.

Davies, B., Hannah, L.T., Randall, C.F., Bromage, N. and Williams, L.M. (**1994**). Central melatonin binding sites in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* 96**,** 19-26.

Davies, B., Randall, C.F. and Bromage, N.R. (**1992**). Absolute daylength and the entrainment of an endogenous clock controlling reproduction in the female rainbow trout. *Aquaculture* 100**,** 321-322.

Davis, C.R., Okihiro, M.S. and Hinton, D.E. (**2002**). Effects of husbandry practices, gender, and normal physiological variation on growth and reproduction of Japanese medaka, *Oryzias latipes*. *Aquatic Toxicology* 60**,** 185-201.

Davis, K.B., Goudie, C.A., Simco, B.A., MacGregor, R. and Parker, N.C. (**1986**). Environmental regulation and influence of the eyes and the pineal gland on the gonadal cycle and spawning in channel catfish (*Ictalurus punctatus*). *Physiological Zoology* 59**,** 717-724.

Day, J.R. and Taylor, M.H. (**2005**). Environmental control of the annual cycle of *Fundulus heteroclitus* L.: the pineal organ and eyes. *Journal of Experimental Zoology* 227**,** 453-458.

de Roux, N., Genin, E., Carel, J.-C., Matsuda, F., Chaussain, J.-L. and Milgrom, E. (**2003**). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *PNAS* 100**,** 10972-10976.

Decressac, S., Grechez-Cassiau, A., Lenfant, J., Falcon, J. and Bois, P. (**2002**). Cloning, localization and functional properties of a cGMP-gated channel in photoreceptor cells from fish pineal gland. *Journal of Pineal Research* 33**,** 225-233.

Dekens, M.P.S., Santoriello, C., Vallone, D., Grassi, G., Whitmore, D. and Foulkes, N.S. (**2003**). Light regulates the cell cycle in zebrafish. *Current Biology* 13**,** 2051-2057.

Devlin, R.H. and Nagahama, Y. (**2002**). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208**,** 191-364.

Dubocovich, M.L., Cardinali, D.P., Guardiola-Lemaitre, B., Hagan, R.M., Krause, D.N., Sugden, D., Yocca, F.D. and Vanhoutte, P.M. (**1998**). Melatonin receptors. In: *The IUPHAR Compendium o f Receptor Characterization and Classification*,IUPHAR Media. London, UK. pp.87-193.

Dufour, S., Lopez, E., Le Menn, F., Le Belle, N., Baloche, S. and Fontaine, Y.A. (**1988**). Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. *General and Comparative Endocrinology* 70**,** 20-30.

Dufour, S., Weltzien, F.A., Sebert, M.E., Le Belle, N., Vidal, B., Vernier, P. and Pasqualini, C. (**2005**). Dopaminergic inhibition of reproduction in teleost fishes. *Annals of the New York Academy of Sciences* 1040**,** 9-22.

Dunlap, J.C. (**1999**). Molecular bases for circadian clocks. *Cell* 96**,** 271-290.

Duston, J. and Bromage, N.R. (**1987**). Constant photoperiod regimes and the entrainment of the annual cycle of reproduction in the female rainbow trout (*Salmo gardnieri*). *General and Comparative Endocrinology* 65**,** 373-384.

Ekstrom, P.M.H. (**1997**). The pineal organ of teleost fishes. *Reviews in Fish Biology and Fisheries* 7**,** 199-284.

Ekstrom, P.M.H. (**2003**). Evolution of photosensory pineal organs in new light: the fate of neuroendocrine receptors. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 358**,** 1679-1700.

El-Sayed, A.M. and Kawanna, M. (**2004**). Effects of photoperiod on the performance of farmed Nile tilapia *Oreochromis niloticus*: I. Growth, feed utilization efficiency and survival of fry and fingerlings. *Aquaculture* 231**,** 393-402.

El-Sayed A.M., (**2006**). Tilapia culture. Wallingford, UK. pp.277.

Endal, H.P., Taranger, G.L., Stefansson, S.O. and Hansen, T. (**2000**). Effects of continuous additional light on growth and sexual maturity in Atlantic salmon, *Salmo salar*, reared in sea cages. *Aquaculture* 191**,** 337-349.

Falcon, J., Marmillon, J.B., Claustrat, B. and Collin, J.P. (**1989**). Regulation of melatonin secretion in a photoreceptive pineal organ: an *in vitro* study in the pike. *Journal of Neuroscience* 9**,** 1943-1950.

Falcon, J., Begay, V., Besse, C. and Ravault, J.P.C.J.P. (**1992**). Pineal photoreceptor cells in culture: fine structure, and light/ dark control of cyclic nucleotide levels and melatonin secretion. *Journal of Neuroendocrinology* 4**,** 641-651.

Falcon, J., Barraud, S., Thibault, C. and Begay, V. (**1998**). Inhibitors of messenger RNA and protein synthesis affect differently serotonin Arylalkylamine N-Acetyltransferase activity in clock-controlled and non clock-controlled fish pineal. *Brain Research* 797**,** 109-117.

Falcon, J. (**1999**). Cellular circadian clocks in the pineal. *Progress in Neurobiology* 58**,** 121-162.

Falcon, J., Galarneau, K.M., Weller, J.L., Ron, B., Chen, G., Coon, S.L. and Klein, D.C. (**2001**). Regulation of Arylalkylamine N-Acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the fish pineal organ: evidence for a role of proteasomal proteolysis. *Endocrinology* 142**,** 1804-1813.

Falcon, J., Gothilf, Y., Coon, S.L., Boeuf, G. and Klein, D.C. (**2003**). Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. *Journal of Neuroendocrinology* 15**,** 378-382.

Falcon, J., Besseau, L. and Boeuf, G. (**2006**). Molecular and cellular regulation of pineal organ responses. In: *Fish Physiology Sensory Systems Neuroscience*, Ed. Toshiaki, J.H.a.B., Academic Press. pp.243-306.

Falcon, J., Besseau, L., Sauzet, Sa. and Boeuf, G. (**2007**). Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends in Endocrinology and Metabolism* 18**,** 81-88.

Filby, A.L., van Aerle, R., Duitman, J. and Tyler, C.R. (**2008**). The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. *Biology of Reproduction* 78**,** 278-289.

Foster, R.G., Grace, M.S., Provencio, I., Degrip, W.J. and Garcia-Fernandez, J.M. (**1994**). Identification of vertebrate deep brain photoreceptors. *Neuroscience and Behavioural Reviews* 18**,** 541-546.

Foster, R.G. and Hankins, M.W. (**2002**). Non-rod, non-cone photoreception in the vertebrates. *Progress in Retinal and Eye Research* 21**,** 507-527.

Foster, R.G. and Bellingham, J. (**2004**). Inner retinal photoreceptors (IRPs) in mammals and teleost fish. *Photochemical and Photobiological Sciences* 3**,** 617-627.

Foster R. and Kreitzman L., (**2005**). Rhythms of Life. London, UK. pp.320.

Foulkes, N.S., Whitmore, D. and Sassone-Corsi, P. (**1997**). Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Biology of the Cell* 89**,** 487-494.

Fu, Y., Liao, H.W., Do, M.T.H. and Yau, K.W. (**2005**). Non-image-forming ocular photoreception in vertebrates. *Current Opinion in Neurobiology* 15**,** 415-422.

Fukada, Y. and Okano, T. (**2002**). Circadian clock system in the pineal gland. *Molecular Neurobiology* 25**,** 19-30.

Futter, C.W. (**2002**). Perception of light by rainbow trout (*Oncorhynchus mykiss* W.) and its role in development and maturation. PhD thesis, Institute of Aquaculture, University of Stirling, Stirling, Scotland, UK.

Ganguly, S., Mummaneni, P., Steinbach, P.J., Klein, D.C. and Coon, S.L. (**2001**). Characterization of the *Sacharomyces cerevisiae* homolog of the melatonin rhythm enzyme Arylalkylamine N-Acetyltransferase (EC 2.3.1.87). *Journal of Biological Chemistry* 276**,** 47239-47247.

Garg, S.K. (**1989**). Effect of pinealectomy, eye enucleation, and melatonin treatment on ovarian activity and vitellogenin levels in the catfish exposed to short photoperiod or long photoperiod. *Journal of Pineal Research* 7**,** 91-104.

Gerkema, M.P. (**1992**). Biological rhythms: mechanisms and adaptative values. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Publishing Corporation. New York, USA. pp.27-61.

Gern, W.A., Owens, D.W. and Ralph, CH.L. (**1978**). The synthesis of melatonin by the trout retina. *Journal of Experimental Zoology* 206**,** 263-270.

Gern, W.A. and Greenhouse, S.S. (**1988**). Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gardnieri*) pineal organs mantained under diel illumination or continuous darkness. *General and Comparative Endocrinology* 71**,** 163-174.

Gore A.C., (**2002**). GnRH: the master molecule of reproduction. New York, USA. pp.324.

Gothilf, Y., Munoz-Cueto, J.A., Sagrillo, C.A., Selmanoff, M., Chen, T.T., Kah, O., Elizur, A. and Zohar, Y. (**1996**). Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. *Biology of Reproduction* 55**,** 636-645.

Grace, M.S., Cahill, G.M. and Besharse, J.C. (**1991**). Melatonin deacetylation: retinal vertebrate class distribution and *Xenopus laevis* tissue distribution. *Brain Research* 559**,** 56-63.

Gubitz, A.K. and Reppert, S.M. (**1999**). Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X (Gpr50). *Genomics* 55**,** 248-251.

Gupta, B.B.P. and Premabati, Y. (**2002**). Fish pineal: structure, function and regulation. In: *Treatise on Pineal Gland and Melatonin*, Eds. Haldar, C., Singaravel, M. and Kumar M, S., Science Publishers. Plymouth, UK. pp.77-102.

Gur, G., Bonfil, D., Safarian, H., Naor, Z. and Yaron, Z. (**2001**). GnRH receptor signaling in tilapia pituitary cells: role of mitogen-activated protein kinase (MAPK). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 129**,** 517-524.

Gur, G., Bonfil, D., Safarian, H., Naor, Z. and Yaron, Z. (**2002**). GnRH signaling pathways regulate differentially the tilapia gonadotropin subunit genes. *Molecular and Cellular Endocrinology* 189**,** 125-134.

Haldar C., Singaravel M. and Maitra K.S., (**2002**). Treatise on pineal gland and melatonin. USA. pp.580.

Hanifin, J.P., Stewart, K.T., Smith, P., Tanner, R., Rollag, M. and Brainard, G.C. (**2006**). High-intensity red light supresses melatonin. *Chronobiology International* 23**,** 251-268.

Hastings, M.H., Duffield, G.E., Ebling, F.J., Kidd, A., Maywood, E.S. and Schurov, I. (**1997**). Non-photic signalling in the suprachiasmatic nucleus. *Biology of the Cell* 89**,** 495-503.

Hellqvist, A., Bornestaf, C., Borg, B. and Schmitz, M. (**2004**). Cloning and sequencing of the FSH-β and LH-β-subunit in the three-spined stickleback, *Gasterosteus aculeatus*, and effects of photoperiod and temperature on LH-β and FSH-β mRNA expression. *General and Comparative Endocrinology* 135**,** 167-174.

Herzog, E.D. and Tosini, G. (**2001**). The mammalian circadian clock shop. *Seminars in Cell and Developmental Biology* 12**,** 295-303.

Hilsdorf, A.W.S., Penman, D.J., Cunha Farias, E. and McAndrew, B. (**2002**). Melanophore appearance in wild and red tilapia embryos. *Pigment Cell Research* 15**,** 57-61.

Hofmann, H.A. (**2006**). Gonadotropin-releasing hormone signaling in behavioral plasticity: signalling mechanisms. *Current Opinion in Neurobiology* 16**,** 343-350.

Holloway, W.R., Tsui, H.W., Grota, L.J. and Brown, G.M. (**1979**). Melatonin and corticosterone regulation: feeding time or the light:dark cycle? *Life Sciences* 25**,** 1837-1842.

Holmqvist, B., Ostholm, T. and Ekstrom, P. (**1992**). Retinohypothalamic projections and the suprachiasmatic nucleus of the teleost brain. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Press. Montreal, Quebec. pp.293-318.

Holzberg, D. and Albrecht, U. (**2003**). The circadian clock: a manager of biochemical processes within the organism. *Journal of Neuroendocrinology* 15**,** 339-343.

Humphries, A., Wells, T., Baler, R., Klein, D.C. and Carter, D.A. (**2007**). Rodent aanat: intronic E-box sequences control tissue specificity but not rhythmic expression in the pineal gland. *Molecular and Cellular Endocrinology* 270**,** 43-49.

Hurd, M.W., Debruyne, J., Straume, M. and Cahill, G.M. (**1998**). Circadian rhythms of locomotor activity in zebrafish. *Physiology and Behavior* 65**,** 465-472.

Iigo, M., Sanchez-Vasquez, F.J., Madrid, J.A., Zamora, S. and Tabata, M. (**1997**). Unusual responses to light and darkness of ocular melatonin in European seabass. *Neuroreport* 8**,** 1631-1635.

Iigo, M., Furukawa, K., Tabata, M. and Aida, K. (**2003a**). Circadian variations of melatonin binding sites in the goldfish brain. *Neuroscience Letters* 347**,** 49-52.

Iigo, M., Mizusawa, K.Y.Ma., Hara, M., Ohtani-Kaneko, R., Tabata, M., Aida, K. and Hirata, K. (**2003b**). *In vitro* photic entrainment of the circadian rhythm in melatonin release from the pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-through culture. *Brain Research* 982**,** 131-135.

Iigo, M., Fujimoto, Y., Gunji-Suzuki, M., Yokosuka, M., Hara, M., Ohtani-Kaneko, R., Tabata, M., Aida, K. and Hirata, K. (**2004**). Circadian rhythm of melatonin release from the photoreceptive pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-thorough culture. *Journal of Neuroendocrinology* 16**,** 45-51.

Iigo, M., Abe, T., Kambayashi, S., Oikawa, K., Masuda, T., Mizusawa, K., Kitamura, S., Azuma, T., Takagi, Y., Aida, K. and Yanagisawa, T. (**2007**). Lack of circadian regulation of *in vitro* melatonin release from the pineal organ of salmonid teleosts. *General and Comparative Endocrinology* 154**,** 91-97.

Inouye, S. (**1982**). Restricted daily feeding does not entrain circadian rhythms of the suprachiasmatic nucleus in the rat. *Brain Research* 232**,** 194-199.

Iuvone, P.M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D.C. and Chaurasia, S.S. (**2005**). Circadian clocks, clock networks, Arylalkylamine N-Acetyltransferase, and melatonin in the retina. *Progress in Retinal and Eye Research* 24**,** 433-456.

Jauncey, K. (**1998**). Feed and feedstuff quality control, storage and tilapia feeding husbandry. In: *Tilapias feed and feeding*,Pisces Press LTD. Stirling, UK. pp.175-188.

Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., De Vries, G.J. and Reppert, S.M. (**1999**). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 96**,** 57-68.

Josupeit, H. (**2007**). World tilapia trade., INFOFISH Tilapia Conference., Kuala Lumpur, Malaysia. (www.globefish.org/index.php?id=4278)

Kah, O. (**1986**). Central regulation of reproduction in teleosts. *Fish Physiology and Biochemistry* 2**,** 25-34.

Kang, S.W., Thayananuphat, A., Bakken, T. and El Halawani, M.E. (**2007**). Dopamine-melatonin neurons in the avian hypothalamus controlling seasonal reproduction. *Neuroscience* 150**,** 223-233.

Kardong K.V., (**2002**). Vertebrates: comparative anatomy, function, evolution. 3rd, Boston MA. pp.

Kauffman, A.S. and Rissman, E.F. (**2004a**). A critical role for the evolutionarily conserved gonadotropin-releasing hormone II: mediation of energy status and female sexual behavior. *Endocrinology* 145**,** 3639-3646.

Kauffman, A.S. and Rissman, E.F. (**2004b**). The evolutionarily conserved gonadotropin-releasing hormone II modifies food intake. *Endocrinology* 145**,** 686-691.

Kauffman, A.S., Wills, A., Millar, R.P. and Rissman, E.F. (**2005**). Evidence that the type-2 gonadotrophin-releasing hormone (GnRH) receptor mediates the behavioural effects of GnRH-II on feeding and reproduction in musk shrews. *Journal of Neuroendocrinology* 17**,** 489-497.

Kauffman, A.S., Park, J.H., Phie-Lalmansingh, A.A., Gottsch, M.L., Bodo, C., Hohmann, J.G., Pavlova, M.N., Rohde, A.D., Clifton, D.K., Steiner, R.A. and Rissman, E.F. (**2007**). The Kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. *Journal of Neuroscience* 27**,** 8826-8835.

Kazimi, N. and Cahill, G.M. (**1999**). Development of a circadian melatonin rhythm in embryonic zebrafish. *Developmental Brain Research* 117**,** 47-52.

Kitahashi, T., Sato, H., Sakuma, Y. and Parhar, I.S. (**2005**). Cloning and functional analysis of promoters of three GnRH genes in a cichlid. *Biochemical and Biophysical Research Communications* 336**,** 536-543.

Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Begay, V., Falcon, J., Cahill, G.M., Cassone, V.M. and Baler, R. (**1997**). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-Acetyltransferase in the pineal gland. *Recent Program in Hormone Research* 52**,** 307-356.

Klein, D.C., Ganguly, S., Coon, S., Weller, J.L., Obsil, T., Hickman, A. and Dyda, F. (**2002**). 14-3-3 Proteins and photoneuroendocrine transduction: role in controlling the daily rhythm in melatonin. *Biochemical Society Transactions* 30**,** 365-373.

Klein, D.C. (**2004**). The 2004 Aschoff/Pittendrigh Lecture: theory of the origin of the pineal gland-A tale of conflict and resolution. *Journal of Biological Rhythms* 19**,** 264-279.

Klein, D.C. (**2006**). Evolution of the vertebrate pineal gland: the AANAT hypothesis. *Chronobiology International* 23**,** 5-20.

Kohbara, J., Hidaka, I., Kuriyama, I., Yamashita, M., Ichikawa, M., Furukawa, K., Aida, K., Sanchez-Vazquez, F.J. and Tabata, M. (**2000**). Nocturnal/diurnal demand-feeding pattern of yellowtail *Seriola quinqueradiata* under different keeping conditions. *Fisheries Science* 66**,** 955-962.

Kohbara, J., Hidaka, I., Matsuoka, F., Osada, T., Furukawa, K., Yamashita, M. and Tabata, M. (**2003**). Self-feeding behavior of yellowtail, *Seriola quinqueradiata*, in net cages: diel and seasonal patterns and influences of environmental factors. *Aquaculture* 220**,** 581-594.

Kohsaka, A. and Bass, J. (**2007**). A sense of time: how molecular clocks organize metabolism. *Trends in Endocrinology and Metabolism* 18**,** 4-11.

Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.-M., Le Poul, E., Brezillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schiffmann, S.N., Vassart, G. and Parmentier, M. (**2001**). The Metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *Journal of Biological Chemistry* 276**,** 34631-34636.

Kotrschal, K., Van Staaden, M.J. and Huber, R. (**1998**). Fish brains: evolution and enviromental relationships. *Reviews in Fish Biology and Fisheries* 8**,** 373-408.

Kuohung, W. and Kaiser, U.B. (**2006**). GPR54 and KISS-1: role in the regulation of puberty and reproduction. *Reviews in Endocrine Metabolic Disorders* 7**,** 257-263.

Kusmic, C. and Gualtieri, P. (**2000**). Morphology and spectral sensitivities of retinal and extraretinal photoreceptors in freshwater teleosts. *Micron* 31**,** 183-200.

Langmesser, S. and Albrecht, U. (**2006**). Life time-circadian clocks, mitochondria and metabolism. *Chronobiology International* 23**,** 151-157.

Lauffenburger D.A. and Linderman J.J., (**1993**). Receptors. Oxford, UK. pp.365.

Lethimonier, C., Madigou, T., Munoz-Cueto, J.A., Lareyre, J.J. and Kah, O. (**2004**). Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. *General and Comparative Endocrinology* 135**,** 1-16.

Levavi-Sivan, B., Safarian, H., Rosenfeld, H., Elizur, A. and Avitan, A. (**2004**). Regulation of gonadotropin-releasing hormone (GnRH)-receptor gene expression in tilapia: effect of GnRH and dopamine. *Biology of Reproduction* 70**,** 1545-1551.

Levavi-Sivan, B. and Avitan, A. (**2005**). Sequence analysis, endocrine regulation, and signal transduction of GnRH receptors in teleost fish. *General and Comparative Endocrinology* 142**,** 67-73.

Little, D.C. and Hulata, G. (**2000**). Strategies for tilapia seed production. In: *Tilapias: Biology and Exploitation*, Eds. Beveridge, M.C.M. and McAndrew, B., Kluwer Academic Publishers. pp.267-326.

Liu, T. and Borjigin, J. (**2005**). N-Acetyltransferase is not the rate-limiting enzyme of melatonin synthesis at night. *Journal of Pineal Research* 39**,** 91-96.

Lobban, C.S. and Harrison, P.J. (**1994**). Light and photosynthesis. In: *Seaweed Ecology and Physiology*,Cambridge University Press. Cambridge. pp.123-162.

Lopez-Olmeda, J.F., Madrid, J.A. and Sanchez-Vasquez, F.J. (**2006**). Light and temperature cycles as zeitgebers of zebrafish (*Danio rerio*) circadian activity rhythms. *Chronobiology International* 23**,** 537-550.

Lorenzen, K. (**2000**). Population dinamics and management. In: *Tilapias: Biology and Explotation*, Eds. Beveridge, M.C.M. and McAndrew, B., Kluwer Academic Publishers. London, UK.

Macintosh, D.J. and Little, D.C. (**1995**). Nile tilapia (*Oreochromis niloticus*). In: *Broodstock Management and Egg and Larval Quality*, Eds. Bromage, N.R. and Roberts, J.R., Blackwell Sciences. Oxford.424.

Malpaux, B., Daveau, A., Maurice-Mandon, F., Duarte, G. and Chemineau, P. (**1998**). Evidence that melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe: presence of binding sites and stimulation of luteinizing hormone secretion by *in situ* microimplant delivery. *Endocrinology* 139**,** 1508-1516.

Malpaux, B., Migaud, M., Tricoire, H. and Chemineau, P. (**2001**). Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. *Journal of Biological Rhythms* 16**,** 336-347.

Masuda, T., Iigo, M. and Aida, K. (**2005**). Existence of an extra-retinal and extra-pineal photoreceptive organ that regulates photoperiodism in gonadal development of an osmerid teleost, ayu (*Plecoglossus altivelis*). *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology* 140**,** 414-422.

Mateos, J., Mananos, E., Carrillo, M. and Zanuy, S. (**2002**). Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean seabass. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 132**,** 75-86.

Matsumoto, T. and Kawamura, G. (**2005**). The eyes of the common carp and Nile tilapia are sensitive to near-infrared. *Fisheries Science* 71**,** 350-355.

Mayer, I., Bornestaf, C. and Borg, B. (**1997**). Melatonin in non-mammalian vertebrates: physiological role in reproduction? *Comparative Biochemistry and Physiology Part A: Physiology* 118**,** 515-531.

McAndrew, B.J., Roubal, F.R., Roberts, R.J., Bullock, A.M. and McEwen, I.M. (**1988**). The genetics and histology of red, blond and associated colour variants in *Oreochromis niloticus*. *Genetica* 76**,** 127-137.

Meier, A.H. (**1992**). Circadian basis for neuroendocrine regulation. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Publishing Corporation. pp.109-126.

Meijer, J.H. and Rietveld, W.J. (**1989**). Neurophysiology of the SCN circadian pacemaker in rodents. *Physiol Rev* 69**,** 671-707.

Melamed, P., Gur, G., Elizur, A., Rosenfeld, H., Levavi-Sivan, B., Rentier-Delrue, F. and Yaron, Z. (**1996**). Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II beta subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology* 64**,** 320-328.

Menaker, M., Moreira, L.F. and Tosini, G. (**1997**). Evolution of circadian organization in vertebrates. *Brazilian Journal of Medical and Biological Research* 30**,** 305-313.

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R., Malinge, I., Lomet, D., Carlton, M.B.L., Colledge, W.H., Caraty, A. and Aparicio, S.A.J.R. (**2005**). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *PNAS* 102**,** 1761-1766.

Migaud, H., Taylor, J.F., Taranger, G.L., Davie, A., Cerda-Reverter, J.M., Carrillo, M., Hansen, T. and Bromage, N.R. (**2006**). A comparative *ex vivo* and *in vivo* study of day and night perception in teleosts species using the melatonin rhythm. *Journal of Pineal Research* 41**,** 42-52.

Migaud, H., Davie, A., Martinez Chavez, C.C. and Al-Khamees, S. (**2007a**). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *Journal of Pineal Research* 43**,** 327-335.

Migaud, H., Cowan, M., Taylor, J. and Ferguson, H.W. (**2007b**). The effect of spectral composition and light intensity on melatonin, stress and retinal damage in post-smolt Atlantic salmon, *Salmo salar*. *Aquaculture* 270**,** 390-404.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K. and Maudsley, S.R. (**2004**). Gonadotropin-releasing hormone receptors. *Endocrine Reviews* 25**,** 235-275.

Millar, R.P., Pawson, A.J., Morgan, K., Rissman, E.F. and Lu, Z.L. (**2007**). Diversity of actions of GnRHs mediated by ligand-induced selective signaling. *Frontiers in Neuroendocrinology* 29**,** 17-35.

Mohamed, J.S., Benninghoff, A.D., Holt, G.J. and Khan, I.A. (**2007**). Developmental expression of the G protein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. *Journal of Molecular Endocrinology* 38**,** 235-244.

Montero, M., Le Belle, N., Vidal, B. and Dufour, S. (**1996**). Primary cultures of dispersed pituitary cells from estradiol-pretreated female silver eels (*Anguilla anguilla* L.): immunocytochemical characterization of gonadotropic cells and stimulation of gonadotropin release. *General and Comparative Endocrinology* 104**,** 103-115.

Morrison, C.M., Miyake, T. and Wright, J.J.R. (**2001**). Histological study of the development of the embryo and early larva of *Oreochromis niloticus* (Pisces: Cichlidae). *Journal of Morphology* 247**,** 172-195.

Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekeres, P.G., Sarau, H.M., Chambers, J.K., Murdock, P., Steplewski, K., Shabon, U., Miller, J.E., Middleton, S.E., Darker, J.G., Larminie, C.G.C., Wilson, S., Bergsma, D.J., Emson, P., Faull, R., Philpott, K.L. and Harrison, D.C. (**2001**). AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *Journal of Biological Chemistry* 276**,** 28969-28975.

Njiru, M., Ojuok, J.E., Okeyo-Owuour, J.B., Muchiri, M., Ntiba, M.J. and Cowx, I.G. (**2006**). Some biological aspects and life history strategies of Nile tilapia *Oreochromis niloticus* (L.) in Lake Victoria, Kenya. *African Journal of Ecology* 44**,** 30-37.

Noble, C., Kadri, S., Mitchell, D.F. and Huntingford, F.A. (**2007**). The impact of environmental variables on the feeding rhythms and daily feed intake of cage-held 1+ Atlantic salmon parr (*Salmo salar* L.). *Aquaculture* 269**,** 290-298.

Nocillado, J.N., Levavi-Sivan, B., Carrick, F. and Elizur, A. (**2007**). Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, *Mugil cephalus*. *General and Comparative Endocrinology* 150**,** 278-287.

North, B.P., Turnbull, J.F., Ellis, T., Porter, M.J., Migaud, H., Bron, J. and Bromage, N.R. (**2006**). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 255**,** 466-479.

Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O. and Fujino, M. (**2001**). Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 411**,** 613-617.

Okimoto, D.K. and Stetson, M.H. (**1999**). Presence of an intrapineal circadian oscillator in the teleostean family poeciliidae. *General and Comparative Endocrinology* 114**,** 304-312.

Ooka-Souda, S., Kadota, T. and Kabasawa, H. (**1993**). The preoptic nucleus: the probable location of the circadian pacemaker of the hagfish, *Eptatretus burgeri*. *Neuroscience Letters* 164**,** 33-36.

Pankhurst, N.W. and Porter, M. (**2003**). Cold and dark or warm and light: variations on the theme of environmental control of reproduction. *Fish Physiology and Biochemistry* 28**,** 385-389.

Parhar, I.S., Pfaff, D.W. and Schwanzel-Fukuda, M. (**1996a**). Gonadotropin-releasing hormone gene expression in teleosts. *Molecular Brain Research* 41**,** 216-227.

Parhar, I.S., Soga, T. and Sakuma, Y. (**1996b**). *In situ* hybridization for two differentially expressed GnRH genes following estrogen and triiodothyronine treatment in the brains of juvenile tilapia (cichlid). *Neuroscience Letters* 218**,** 135-138.

Parhar, I.S. (**2003**). Gonadotropin-releasing hormone receptors: neuroendocrine regulators and neuromodulators. *Fish Physiology and Biochemistry* 28**,** 13-18.

Parhar, I.S., Ogawa, S., Hamada, T. and Sakuma, Y. (**2003a**). Single-cell real-time quantitative polymerase chain reaction of immunofluorescently identified neurons of gonadotropin-releasing hormone subtypes in cichlid fish. *Endocrinology* 144**,** 3297-3300.

Parhar, I.S., Soga, T., Ogawa, S. and Sakuma, Y. (**2003b**). FSH and LH-β subunits in the preoptic nucleus: ontogenic expression in teleost. *General and Comparative Endocrinology* 132**,** 369-378.

Parhar, I.S., Ogawa, S. and Sakuma, Y. (**2004**). Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology* 145**,** 3613-3618.

Parhar, I.S. (**2005**). GnRH and gpcr: laser-captured single cell gene profiling. *Fish Physiology and Biochemistry* 31**,** 153-156.

Parhar, I.S., Ogawa, S. and Sakuma, Y. (**2005**). Three GnRH receptor types in laser-captured single cells of the cichlid pituitary display cellular and functional heterogeneity. *PNAS* 102**,** 2204-2209.

Pawson, A.J. and McNeilly, A.S. (**2005**). The pituitary effects of GnRH. *Animal Reproduction Science* 88**,** 75-94.

Pawson, A., Morgan, K., Maudsley, S. and Millar, R. (**2003**). Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology. *Reproduction* 126**,** 271-278.

Peters, R.C. and Veeneklaas, R.J. (**1992**). Ultradian rhythms in fishes. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Publishing Corporation. New York, USA. pp.51-82.

Pevet, P. (**2003**). Melatonin: from seasonal to circadian signal. *Journal of Neuroendocrinology* 15**,** 422-426.

Popa, S.M., Clifton, D.K. and Steiner, R.A. (**2005**). A KiSS to remember. *Trends in Endocrinology and Metabolism* 16**,** 249-250.

Porter, M.J.R., Randall, C.F. and Bromage, N.R. (**1996**). The effect of pineal removal on circulating melatonin levels in Atlantic salmon parr. *Journal of Fish Biology* 48**,** 1011-1013.

Porter, M.J.R., Duncan, N., Handeland, S.O., Stefansson, S.O. and Bromage, N.R. (**2001**). Temperature, light intensity and plasma melatonin levels in juvenile Atlantic salmon. *Journal of Fish Biology* 58**,** 431-438.

Priede, I.G., Williams, L.M., Wagner, H., Thom.A., Brierley, I., Collins, M.A., Collin, S.P., Merrett, N.R. and Yau, C. (**1999**). Implication of the visual system in the regulation of activity cycles in the abscence of solar light: 2-[123I]-iodomelatonin binding sites and melatonin receptor gene expression in the brains of demersal deep-sea gadiform fish. *Proceedings of the Royal Society of London B.* 266**,** 2295-2302.

Pullin, R.S.V. and Lowe-Mconnell, R.H. (**1982**). The biology and culture of tilapias., International Center for Living Aquatic Resources Management (ICLARM) 7., Bellagio, Italy.

Rad, F., Bozaoglu, S., Ergene Gozukara, S., Karahan, A. and Kurt, G. (**2006**). Effects of different long-day photoperiods on somatic growth and gonadal development in Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture* 255**,** 292-300.

Rajendran, R.R., Van Niel, E.E., Stenkamp, D.L., Cunningham, L.L., Raymond, P.A. and Gonzalez-Fernandez, F. (**1996**). Zebrafish interphotoreceptor retinoid-binding protein: differential circadian expression among cone subtypes. *Journal of Experimental Biology* 199**,** 2775-2787.

Rana, K.J. (**1988**). Reproductive biology and the hatchery rearing of tilapia eggs and fry. In: *Recent Advances in Aquaculture*, Eds. Muir, J.F. and Roberts, J.R., Croom Helm Ltd. London. pp.343-406.

Reebs, S.G. (**2002**). Plasticity of diel and circadian activity rhythms in fishes. *Reviews in Fish Biology and Fisheries* 12**,** 349-371.

Reiter, R.J., Richardson, B.A., Matthews, S.A., Lane, S.J. and Ferguson, B.N. (**1983**). Rhythms in immunoreactive melatonin in the retina and harderian gland of rats: persistence after pinealectomy. *Life Sciences* 32**,** 1229-1236.

Reiter, R.J. (**1993**). The melatonin rhythm: both a clock and a calendar. *Experientia* 49**,** 654-664.

Reiter R.J. and Robinson J., (**1995**). Melatonin. New York, USA. pp.416.

Reppert, S.M. (**1997**). Melatonin receptors: molecular biology of a new family of G protein-coupled receptors. *Journal of Biological Rhythms* 12**,** 528-531.

Reppert, S.M., Weaver, D.R. and Godson, C. (**1996**). Melatonin receptors step into the light: cloning and classification of subtypes. *Trends in Pharmacological Sciences* 17**,** 100-102.

Ridha, M.T. and Cruz, E.M. (**2000**). Effect of light intensity and photoperiod on Nile tilapia *Oreochromis niloticus* L. seed production. *Aquaculture Research* 31**,** 609-617.

Rispoli, L.A. and Nett, T.M. (**2005**). Pituitary gonadotropin-releasing hormone (GnRH) receptor: Structure, distribution and regulation of expression. *Animal Reproduction Science* 88**,** 57-74.

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L. and Tena-Sempere, M. (**2008**). New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. *Frontiers in Neuroendocrinology* 29**,** 48-69.

Ron, B. (**2004**). *In vitro* melatonin rhythm reveals a clock controlled pineal in the European seabass, *Dicentrarchus Labrax*. *The Israeli Journal of Aquaculture-Bamidgeh* 56**,** 281-285.

Ross, L.G. (**2000**). Enviromental physiology and energetics. In: *Tilapias: Biology and Exploitation*, Eds. Beveridge, M.C.M. and McAndrew, B., Kluwer Academic Publishers. London, UK. pp.89-128.

Safarian, H., Gur, G., Rosenfeld, H. and Levavi-Sivan, B. (**2001**). Regulation of GnRH receptors in tilapia pituitary. In: *Perspective in Comparative Endocrinology: Unit and Diversity*, Eds. Goos, H.J.Th., Rastogi, R.K., Vaudry, H. and Pierantoni, R., Monduzzi Editore. Bologna. pp.639-645.

Sanchez-Vazquez, F.J., Madrid, J.A. and Zamora, S. (**1995**). Circadian rhythms of feeding activity in seabass, *Dicentrarchus labrax* L.: dual phasing capacity of diel demand-feeding pattern. *Journal of Biological Rhythms* 10**,** 256-266.

Sanchez-Vazquez, F.J., Madrid, J.A., Zamora, S., Iigo, M. and Tabata, M. (**1996**). Demand feeding and locomotor circadian rhythms in the goldfish, *Carassius auratus*: dual and independent phasing. *Physiology and Behavior* 60**,** 665-674.

Sanchez-Vasquez, F.J., Azzaydi, M., Martinez, F.J., Zamora, S. and Madrid, J.A. (**1998**). Annual rhythms of demand-feeding activity in seabass: evidence of a seasonal phase inversion of the diel feeding pattern. *Chronobiology International* 15**,** 607-622.

Sanchez-Vazquez, F.J., Aranda, A. and Madrid, J.A. (**2001**). Differential effects of meal size and food energy density on feeding entrainment in goldfish. *Journal of Biological Rhythms* 16**,** 58-65.

Schomerus, C. and Korf, H.W. (**2005**). Mechanisms regulating melatonin synthesis in the mammalian pineal organ. *Annals of the New York Academy of Sciences* 1057**,** 372-383.

Scott, A.G., Mair, G.C., Skibinski, D.O.F. and Beardmore, J.A. (**1987**). "Blonde": A useful new genetic marker in the tilapia, *Oreochromis niloticus* (L.). *Aquaculture and Fisheries Management* 18**,** 159-165.

Scott, A.P., Baynes, S.M., Skarphedinsson, O. and Bye, V.J. (**1984**). Control of spawning time in rainbow trout, *Salmo gairdneri*, using constant long daylengths. *Aquaculture* 43**,** 225-233.

Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S.Jr., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B.L., Crowley, W.F., Jr., Aparicio, S.A.J.R. and Colledge, W.H. (**2003**). The GPR54 gene as a regulator of puberty. *The New England Journal of Medicine* 349**,** 1614-1627.

Senthilkumaran, B., Okuzawa, K., Gen, K., Ookura, T. and Kagawa, H. (**1999**). Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. *Journal of Neuroendocrinology* 11**,** 181-186.

Serino, I., D'Istria, M. and Monteleone, P. (**1993**). A comparative study of melatonin production in the retina, pineal gland and harderian gland of *Bufo viridis* and *Rana esculenta*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology* 106**,** 189-193.

Shand, J. and Foster, R.G. (**1999**). The extraretinal photoreceptors of non-mammalian vertebrates. In: *Adaptative Mechanisms in the Ecology of Vision*, Eds. Archer, S., Djamgoz, M.B., Loew, E., Partridge, J.C. and Vallerga, S., Kluwer Academic. Cornwall, UK. pp.197-222.

Sherwood, N.M. and Adams, B.A. (**2005**). Gonadotropin-releasing hormone in fish: evolution, expression and regulation of the GnRH gene. In: *Hormones and their Receptors in Fish Reproduction*, Eds. Melamed, P. and Sherwood, N.M., World Scientific Publishing Co. London, UK. pp.1-39.

Simonneaux, V. and Ribelayga, C. (**2003**). Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacology Reviews* 55**,** 325-395.

Siu, A.W., Maldonado, M., Sanchez-Hidalgo, M., Tan, D.X. and Reiter, R.J. (**2006**). Protective effects of melatonin in experimental free radical-related ocular diseases. *Journal of Pineal Research* 40**,** 101-109.

Smith, J.T., Clifton, D.K. and Steiner, R.A. (**2006**). Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. *Reproduction* 131**,** 623-630.

Soga, T., Ogawa, S., Millar, R.P., Sakuma, Y. and Parhar, I.S. (**2005**). Localization of the three GnRH types and GnRH receptors in the brain of a cichlid fish: Insights into their neuroendocrine and neuromodulator functions. *Journal of Comparative Neurology* 487**,** 28-41.

Spieler, R.E. (**1992**). Feeding-entrained circadian rhythms in fishes. In: *Rhythms in fishes*, Ed. Ali, M.A., Plenum Press. New York, USA. pp.137-147.

Srisakultiew, P. (**1993**). Studies on the reproductive biology of *O. niloticus*. PhD thesis, Institute of Aquaculture, University of Stirling,

Stehle, J.H., von Gall, C. and Korf, H.-W. (**2003**). Melatonin: a clock-output, a clock-input. *Journal of Neuroendocrinology* 15**,** 383-389.

Sugden, D., Pickering, H., Teh, M. and Garratt, P.J. (**1997**). Melatonin receptor pharmacology: toward subtype specificity. *Biology of the Cell* 89**,** 531-537.

Swanson, P., Dickey, J.T. and Campbell, B. (**2003**). Biochemistry and physiology of fish gonadotropins. *Fish Physiology and Biochemistry* 28**,** 53-59.

Tacon, P., Ndiaye, P., Cauty, C., Le Menn, F. and Jalabert, B. (**1996**). Relationships between the expression of maternal behaviour and ovarian development in the mouthbrooding cichlid fish *Oreochromis niloticus*. *Aquaculture* 146**,** 261-275.

Taghert, P.H. (**2001**). How does the circadian clock send timing information to the brain? *Seminars in Cell and Developmental Biology* 12**,** 329-342.

Tan, D.X., Manchester, L.C., Hardeland, R., Lopez-Burillo, S., Mayo, J.C., Sainz, R.M. and Reiter, R.J. (**2003**). Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *Journal of Pineal Research* 34**,** 75-78.

Taylor, J.F., North, B.P., Porter, M.J.R., Bromage, N.R. and Migaud, H. (**2006**). Photoperiod can be used to enhance growth and improve feeding efficiency in farmed rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 256**,** 216-234.

Tilgner, S., Lehmann, L. and Westphal, U.I. (**1990**). Retinohypothalamic connections in vertebrates. *Klin Mbl Augenheilkd* 197**,** 295-301.

Trewavas E., (**1983**). Tilapiine fishes of the genera *Sarotherodon, Oreochromis*, and *Danakilia*. London, UK. pp.583.

Tucker, B.J., Booth, M.A., Allan, G.L., Booth, D. and Fielder, D.S. (**2006**). Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper *Pagrus auratus*. *Aquaculture* 258**,** 514-520.

Turner, G.F. and Robinson, R.L. (**2000**). Reproductive biology, mating systems and parental care. In: *Tilapias: Biology and Exploitation*, Eds. Beveridge, M.C.M. and McAndrew, B., Kluwer Academic Publishers. London, UK. pp.33-58.

Vallone, D., Lahiri, K., Dickmeis, T. and Foulkes, N.S. (**2005**). Zebrafish cell clocks feel the heat and see the light! *Zebrafish* 2**,** 171-187.

van Aerle, R., Kille, P., Lange, A. and Tyler, C.R. (**2008**). Evidence for the existence of a functional Kiss1/Kiss1 receptor pathway in fish. *Peptides* 29**,** 57-64.

Vanecek, J. (**1998**). Cellular mechanisms of melatonin action. *Physiology Reviews* 78**,** 687-721.

Vanecek, J. (**1999**). Inhibitory effect of melatonin on GnRH-induced LH release. *Reviewes of Reproduction* 4**,** 67-72.

Vansteensel, M.J., Michel, S. and Meijer, J.H. (**2007**). Organization of cell and tissue circadian pacemakers: a comparison among species. *Brain Research Reviews* In Press, 10.1016/j.brainresrev.2007.10.009.

Vera, L.M., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2006**). Locomotor, feeding and melatonin daily rhythms in sharpsnout seabream (*Diplodus puntazzo*). *Physiology and Behavior* 88**,** 167-172.

Vera, L.M., De Pedro, N., Gomez-Milan, E., Delgado, M.J., Sanchez-Muros, M.J., Madrid, J.A. and Sanchez-Vazquez, F.J. (**2007**). Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. *Physiology and Behavior* 90**,** 518-524.

Vigh, B., Manzano, M.J., Rohlich, P. and Szel, A. (**2002**). Comparative fine structural organization and histochemistry of the pineal organ. In: *Treatise on Pineal Gland and Melatonin*, Eds. Haldar, Ch., Singaravel, M. and Kumar M, S., Science Publishers, Inc. Plymouth, UK. pp.17-50.

Vigh, B., Manzano, M.J., Zádori, A., Frank, C.L., Lukáts, A., Röhlich, P., Szél, A. and Dávid, C. (**2002**). Nonvisual photoreceptors of the deep brain, pineal organs and retina. *Histology and Histopathology* 17**,** 555-590.

Vijayan, M.M., Pereira, C., Grau, E.G. and Iwama, G.K. (**1997**). Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 116**,** 89-95.

Weaver, D.R., Liu, C. and Reppert, S.M. (**1996**). Nature's knockout: the mel1b receptor is not necesary for reproductive and circadian responses to melatonin in Siberian hamsters. *Molecular Endocrinology* 10**,** 1478-1487.

Weltzien, F.A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K. and Norberg, B. (**2004**). The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology* 137**,** 447-477.

Whitlock, K.E., Illing, N., Brideau, N.J., Smith, K.M. and Twomey, S. (**2006**). Development of GnRH cells: setting the stage for puberty: Puberty: a sensor of genetic and environmental interactions throughout development. *Molecular and Cellular Endocrinology* 254-255**,** 39-50.

Whitmore, D., Foulkes, N.S., Strahle, U. and Sassone-Corsi, P. (**1999**). Zebrafish clock rhythmic expression reveals independent peripheral. *Nature Neuroscience* 1**,** 701-707.

Wullimann, M.F. and Meyer, D.L. (**1990**). Phylogeny of putative cholinergic visual pathways through the pretectum to the hypothalamus in teleost fish. *Brain Behaviour and Evolution* 36**,** 14-29.

Yamamoto, N., Parhar, I.S., Sawai, N., Oka, Y. and Ito, H. (**1998**). Preoptic gonadotropin-releasing hormone (GnRH) neurons innervate the pituitary in teleosts. *Neuroscience Research* 31**,** 31-38.

Yamamoto, N. (**2003**). Three gonadotropin-releasing hormone neuronal groups with special reference to teleosts. *Anatomical Science International* 78**,** 139-155.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A. and Levavi-Sivan, B. (**2003**). Regulation of fish gonadotropins. *International Review of Cytology* 225**,** 131-185.

Yoshikawa, T. and Oishi, T. (**1998**). Extraretinal photoreception and circadian systems in nonmammalian vertebrates. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 119**,** 65-72.

Yoshikawa, T., Okano, T., Oishi, T., and Fukada, Y. (**1999**). Deep brain photoreceptors in lower vertebrates., Fifth International Congress of Comparative Physiology and Biochemistry., 124 pp.S129-

Young, G., Kusakabe, M. and Nakamura, I. (**2005**). Gonadal steroidogenesis in teleost fish. In: *Hormones and their receptors in fish reproduction*, Eds. Melamed, P. and Sherwood, N.M., World Scientific Publishing. London. pp.155-223.

Yu, H.S., Pang, S.F. and Tang, P.L. (**1981**). Increase in the level of retinal melatonin and persistence of its diurnal rhythm in rats after pinealectomy. *Journal of Endocrinology* 91**,** 477-481.

Zachmann, A., Ali, M.A. and Falcon, J. (**1992**). Melatonin and its effects in fishes: an overview. In: *Rhythms in Fishes*, Ed. Ali, M.A., Plenum Publishing Corporation. New York, USA. pp.149-165.

Zachmann, A., Falcon, J., KniJff, S.C.M., Bolliet, V. and Ali, M.A. (**1992**). Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the white sucker (*Catostomus commersoni*) *in vitro*. *General and Comparative Endocrinology* 86**,** 26-33.

Zilberman-Peled, B., Benhar, I., Coon, S.L., Ron, B. and Gothilf, Y. (**2004**). Duality of serotonin-N-Acetyltransferase in the gilthead seabream (*Sparus aurata*): molecular cloning and characterization of recombinant enzymes. *General and Comparative Endocrinology* 138**,** 139-147.

Zilberman-Peled, B., Ron, B., Gross, A., Finberg, J.P.M. and Gothilf, Y. (**2006**). A possible new role for fish retinal serotonin-N-Acetyltransferase-1 (AANAT1): dopamine metabolism. *Brain Research* 1073-1074**,** 220-228.

Ziv, L., Tovin, A., Strasser, D. and Gothilf, Y. (**2007**). Spectral sensitivity of melatonin suppression in the zebrafish pineal gland. *Experimental Eye Research* 84**,** 92-99.

Zohar, Y., Elizur, A., Sherwood, N.M., Powell, J.F.F., Rivier, J.E. and Zmora, N. (**1995**). Gonadotropin-releasing activities of the three native forms of gonadotropin-releasing hormone present in the brain of gilthead seabream, *Sparus aurata*. *General and Comparative Endocrinology* 97**,** 289-299.

Zordan, M.A., Rosato, E., Piccin, A. and Foster, R. (**2001**). Photic entrainment of the circadian clock: from *Drosophila* to mammals. *Seminars in Cell and Developmental Biology* 12**,** 317-328.

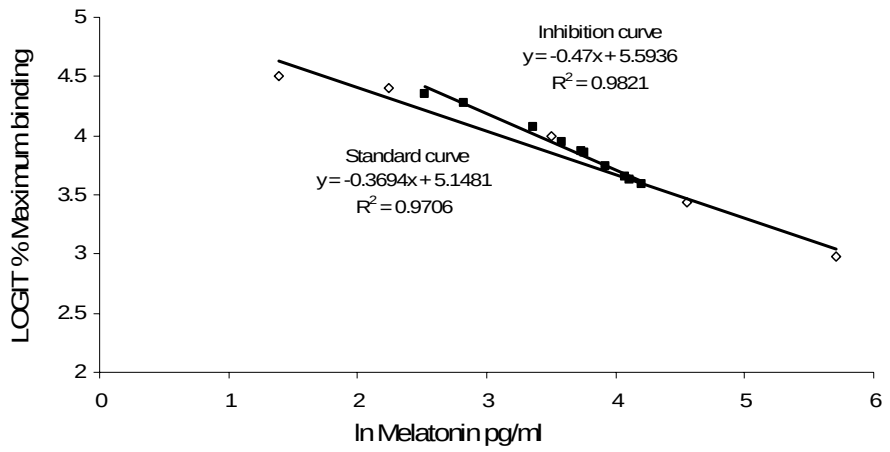
Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M. (**1998**). Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* 20**,** 1103-1110.

11 Appendix

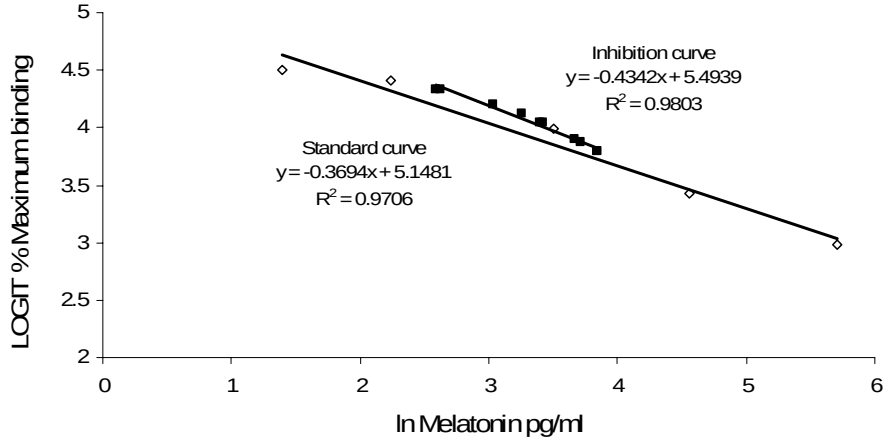
Appendix I

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.

a



b



Appendix II

Plasma cortisol of Nile tilapia graphed by order of sampling (a) 48 hours following ophthalmectomy and (b) a week later. Arrow shows cortisol tendency as sampling progresses. Data is expressed as mean ± SE (n=3). Superscripts indicate significant (p<0.05) differences (ANOVA) between treatments and sample points as analysed with Sigma stat (Ver. 3.1).