Clock Controlled Endogenous Melatonin Rhythms in Nile Tilapia (*Oreochromis niloticus niloticus*) and African Catfish (*Clarias gariepinus*)

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Endogenous melatonin rhythms

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Abstract

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

Introduction

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

systems at work in fish which might have evolved due to the multitude of environments they inhabit (Hardeland *et al*. 2006; Migaud *et al*. 2007).

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

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

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

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

Materials and Methods

Mixed sex red Nile tilapia (*O. niloticus niloticus*) and African catfish (*C. gariepinus*) (mean weight ranging from 150-200 g) were obtained from the tropical aquarium facilities at the Institute of Aquaculture, University of Stirling. All fish were raised from first feeding under 12L:12D conditions and were acclimated for two to three weeks in the experimental rearing systems prior to the start of the experiments to 12L:12D or 6L:6D photoperiod. In all experiments, to exclude feed as an environmental input variable fish were fed to satiation with commercial trout pellets

(Standard Expanded, Skretting, Cheshire, UK) delivered continuously throughout the 24 h period with automatic feeders (Fish-mate F14 Pet-Mate, Surrey, UK). In all experiments under a 12L:12D photoperiod, lights were switched on at 08:00 h and off at 20:00 h. Similarly, lights for 6L:6D where switched on at 08:00 h and then switched off and on at 6 hour intervals. For each species experiments were done in closed water recirculation systems (27 ± 1 ^{o}C) as previously described (Campos-Mendoza *et al.* 2004) unless stated otherwise. Nitrate, nitrite, ammonia and pH were monitored throughout the experiments with aquarium water quality kits (C-Test kits, New Aquarium Systems, Mentor, USA) and remained within safe limits. In all experiments, fish were either anesthetized (0.1-0.15 g/l) or killed by a lethal dose (0.5-0.8 g/l) of benzocaine solution (SIGMA, Poole, UK) and blood sampled by venipuncture of the caudal vein using heparinised syringes. Rearing tanks were lit using standard 60 W GLS bulbs (CPC, Leeds, UK) providing a light intensity of approximately 0.75 Wm^{-2} at the water surface (measured by a single channel light sensor, Skye instruments, Powys, UK). Extreme care was made to the experimental lighting regimes and sampling to avoid potential light pollution. In all experiments light meter readings showed no detectable penetration of external light into the tank system. However, during all experiments the main laboratory lights were left on constantly to prevent the possibility of any background photoperiod affecting the system (except during night time sampling when lights were switched off to have access to the fish under DD and sampling was performed using a red dim light). Furthermore the rearing systems for tilapia and catfish include 4 and 8 light-proof individual compartments with two tanks in each, respectively. Tank size is 40x120x40 cm (200 L) and 46x46x41 cm (86 L) for tilapia and catfish, respectively. Fish under a given experimental photoperiodic treatment can therefore not be entrained to the

regime of the other compartments. All trials were carried out according to international ethical standards (Touitou *et al*. 2006).

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

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

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

The effects of both LL and DD regimes on plasma melatonin levels were studied firstly to confirm the inhibitory effect of LL on melatonin production and determine the profile of return to normal melatonin levels and secondly to establish whether or not circadian rhythmic melatonin production remained in fish exposed to DD for 18 days. For the LL trial, a total of 32 mixed sex tilapia were placed in 8 tanks (4 fish/tank, isolated by Perspex sheets with flow through holes). Fish from each tank were blood sampled every 12 hours (mid-photophase or subjective photophase at 14.00 h and mid-scotophase or subjective scotophase at 02:00 h). Fish from the three

first tanks were randomly selected and first blood sampled under anaesthesia during the 12L:12D ambient photoperiodic regime (day-night-day) after which continuous light treatment began in all tanks (day two of the trial). Fish were returned to a 12L:12D photoperiod on day nine. The sampling regime consisted in blood sampling fish under anaesthesia from all tanks once (corresponding to 8 sampling points, randomised design, fish were then allowed to recover in aerated water and returned to their tanks) and then sacrificing fish from each tank (same order) for a second blood sampling (sampling 9 to 16 from days 8 to 12). For the DD trial, a similar approach was taken although in this case, 44 fish in total (n=4) were exposed to DD for 18 consecutive days.

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

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

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

The aim of this experiment was to determine whether the endogenous melatonin rhythms previously observed in experiment 2 and 3 under DD (for tilapia and catfish, respectively) was circadian in nature. To do so, fish of both species (previously acclimated to 12L:12D) were subjected to DD for three consecutive days before performing a 24 h sampling. Four fish of each species were blood sampled every 4 hours during the subjective photophase at 14:00, 18:00 h (first day) and 10:00, 14:00 (second day) and subjective scotophase at 22:00, 02:00, 06:00 h.

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

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

Melatonin assay

Blood samples were centrifuged at 1200 x g for 15 min at 4^0C (Jouan CT422, Buckinghamshire, UK) and plasma stored at -70 ^{0}C until analysed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). Prior to the analyses, the kit has been validated by confirming the parallelism between serial dilutions of night-time pooled plasma from both species to the standard curve (data not presented). All standards and samples were assayed in duplicate. The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the

zero standard, was 3 $pgml^{-1}$. Intra-assay coefficient of variation was 5.5% (n=4) and inter-assay coefficient of variation was 9.4% (n=3). Pooled rainbow trout plasma with a melatonin content of 211.6 ± 2.3 $pgml^{-1}$, sampled during the night, was used to check the reproducibility of measurements between assays, i.e. for quality control.

Statistical analysis

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

Results

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

significantly decreased at the last scotophase sampling point (05:30 h) before returning to basal photophase levels after the light was switched on (08:30 and 11:30 h). Scotophase melatonin levels (sampling points from 02:30 h to 07:45 h) in Nile tilapia were significantly reduced although basal levels were only reached after the onset of photophase at 08:30 h and 14:30 h (Fig. 2a). On the other hand, catfish melatonin levels were shown to decrease significantly during the scotophase 1 hour prior to the start of the photophase (07:00 h) and reached basal levels shortly before the start of the photophase at 08:00 h (Fig. 2b).

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

In catfish, normal day and night melatonin fluctuations were observed in fish exposed to a control 12L:12D photoperiod (Fig 4a). When LL was applied, melatonin levels remained basal with no significant differences until a photo-cycle cycle was restored at which point melatonin significantly increased (Fig. 4b). However, when DD was

applied, a robust daily rhythm of significantly lower amplitude (mean subjective scotophase levels of 26.0 ± 0.6 pg ml^{-1}) to that observed under a control 12L:12D regime (mean subjective scotophase levels of 48.8 ± 3.2 pg ml^{-1}) was maintained (Fig. 4c). When fish were then exposed to a control photo-cycle (day 5-6), melatonin levels significantly increased and returned to normal scotophase levels (as control).

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

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

Discussion

Seasonal breeders rely on environmental factors such as photoperiod to synchronize their physiology (Arendt 1998; Pevet 2003). In teleosts, this has been thoroughly documented in temperate species which are exposed to marked seasonal changes of day-length and temperature (Falcon *et al.* 2007). Recently, a number of reproductive and growth performance studies have shown that tropical teleosts such as Nile tilapia and African catfish can also be responsive to photoperiodic changes (Campos-Mendoza *et al.* 2004; Almazan-Rueda *et al.* 2004; Biswas *et al.* 2005; Rad *et al.* 2006). However, importantly, the role of melatonin and circadian endogenous rhythms in these tropical species had not been previously studied.

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

*Per*2) and photoreceptor conserved elements (PCEs) that are capable of regulating E-box and promoter regions of genes such as AANAT which will ultimately affect the rate and production of melatonin synthesis (Klein *et al.* 1997; Zordan *et al.* 2001; Pando *et al.* 2001; Appelbaum and Gothilf 2006).

In order to further characterise the circadian control of melatonin production in tilapia, melatonin rhythms under constant photic conditions and its entrainment were investigated. Constant photoperiod (i.e. LL/DD) has commonly been used to describe rhythmic melatonin production in many vertebrates species including teleosts (Gern and Greenhouse 1988; Falcon *et al.* 1989; Kezuka *et al.* 1989; Okimoto and Stetson 1999b; Migaud *et al.* 2006; Takemura *et al.* 2006). In the current study constant LL regimes resulted in a clear suppression of day-night plasma melatonin rhythms in both species, as already reported in many other fish species (Falcon *et al.* 2007). Interestingly, following 7-8 days under LL, normal rhythmic melatonin production was restored during the second night period in fish exposed to day-night cycles. The initial surge (50% of full night-time melatonin) might be explained by a desensitisation of the melatonin production system after having been suppressed for seven complete days under LL. On the other hand, when exposed to DD, a strong endogenous melatonin rhythm was maintained for 18 days (duration of DD exposure) in tilapia previously acclimatised to a 12L:12D photoperiod. A similar endogenous rhythm was also able to sustain itself for at least 4 days (duration of DD exposure) in African catfish. Importantly, results also demonstrated that melatonin rhythm in both species exposed to DD for three days was circadian (i.e. cycling over approximately 24 hours). However, from these trials it is not possible to determine whether there was a phase-shift in the circadian rhythm later on as a single sampling point was performed in the middle of the subjective photophase/scotophase throughout the DD

trials. Furthermore, if full amplitude of melatonin oscillations were shown in tilapia under DD, melatonin levels during mid subjective scotophase (02:00 h) were only 50-60 % of normal scotophase levels under 12L:12D in catfish. It is difficult at this stage to explain these findings however, it is possible that the oscillator driving the endogenous melatonin production rhythm might be 'desenzitised' during prolonged exposure to DD resulting in a near 50% output signal. These results raise interesting questions as to whether these robust clock controlled melatonin rhythms may eventually dampen and/or free run or continue.

In support to the present results, many *in vitro* studies suggested that intrapineal oscillators exist in fish (Bolliet *et al.* 1996) with the exception of salmonids (Iigo *et al.* 2007) in which the pineal gland would either not contain such oscillators or these would not control melatonin production (still to be demonstrated). However, *in vivo* data on endogenous melatonin rhythms are clearly lacking in fish with only very few species studied (Randall *et al.* 1995; Pavlidis *et al.* 1999; Kazimi and Cahill 1999; Bayarri *et al.* 2004b; Migaud *et al.* 2006; Vera *et al.* 2007; Oliveira *et al.* 2007). A recent *in vivo* study performed on temperate fish species (sea bass, Atlantic salmon and Atlantic cod) has shown that when acclimatised to a 12L:12D photoperiod at two different temperatures (10 and 18^0C) and thereafter exposed to DD, no circadian endogenous melatonin rhythm was maintained, with levels remaining as high as during night-time (Migaud *et al.* unpublished). These results obtained in sea bass and Atlantic cod appear to not support previous *in vitro* findings obtained in the same species (Bolliet *et al.* 1996; Ron 2004) in which endogenous melatonin rhythms from isolated pineal glands were reported although rearing temperatures were different than in the *in vivo* trials (24 and 20-22^{0}C for sea bass and cod respectively). This could therefore highlight the importance of studying the circadian system as a whole (*in*

vivo). Finally, it could be hypothesized that such strong endogenous rhythms in tropical species may reflect an adaptation to the rather steady photic environment they inhabit as compared to the strong seasonal variations experienced by temperate species. Two main differences were observed between tilapia and catfish; a clearer melatonin anticipation to photic changes and a reduced melatonin amplitude under DD as compared to 12L:12D in catfish. It is difficult at this stage to determine whether these differences are related to specific behavioural adaptations.

The present studies also reported interesting results on the entrainment of the endogenous system to short photo-cycles. Indeed, when acclimated to a short 6L:6D photoperiod, no melatonin rhythms were observed under DD in tilapia with levels remaining high although only two subjective photo-cycles have been studied. This suggests that, irrespective of its location (pineal, retina and/or brain), the endogenous melatonin oscillator was either a) not able to entrain to such short photo-cycles or b) able to entrain but the coupling with the output (melatonin) became dissociated resulting in constant high plasma melatonin levels when subsequently exposed to DD. Further studies with longer exposure to DD are needed to determine if free running melatonin rhythms occur as observed in humans (Foster and Kreitzman 2005).

In the last decade or so understanding of the molecular bases of circadian clock mechanisms has substantially progressed and has been shown to involve transcriptional and translational feedback loops involving a highly conserved set of "clock genes" across vertebrates (Iuvone *et al.* 2005). It could thus be hypothesised that this circadian clockwork requires a minimal time/lapse of integration of environmental signals for the gene expression of the positive and negative components (transcriptional/translational factors) and the synthesis and activation of the final products (protein, metabolite and molecular signals) to ultimately entrain the

physiology of the animal. Thus, according to this hypothesis, the 6L:6D photoperiod under which fish were acclimatized in this study could be too short for the circadian clock to entrain an endogenous melatonin rhythm output that would continue under DD conditions, as observed in fish previously acclimatised to a 12L:12D photoperiod. These results could thus explain why eggs from broodstock subjected to the same 6L:6D photoperiod were shown to be not viable as compared to control 12L:12D (Biswas *et al.* 2005). However, these preliminary results can only suggest that the entrainment of the melatonin rhythm may have been affected by the previous acclimation to 6L:6D as only four samplings over two subjective 6L:6D photo-cycles were performed. In order to conclude that the entrainment is truly disturbed, further studies in a range of teleosts species raised under various short photo-cycles and sampled over longer periods under DD are needed to confirm such a hypothesis and determine the critical minimal period required for the system to be entrained.

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

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Figure Legends

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

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

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

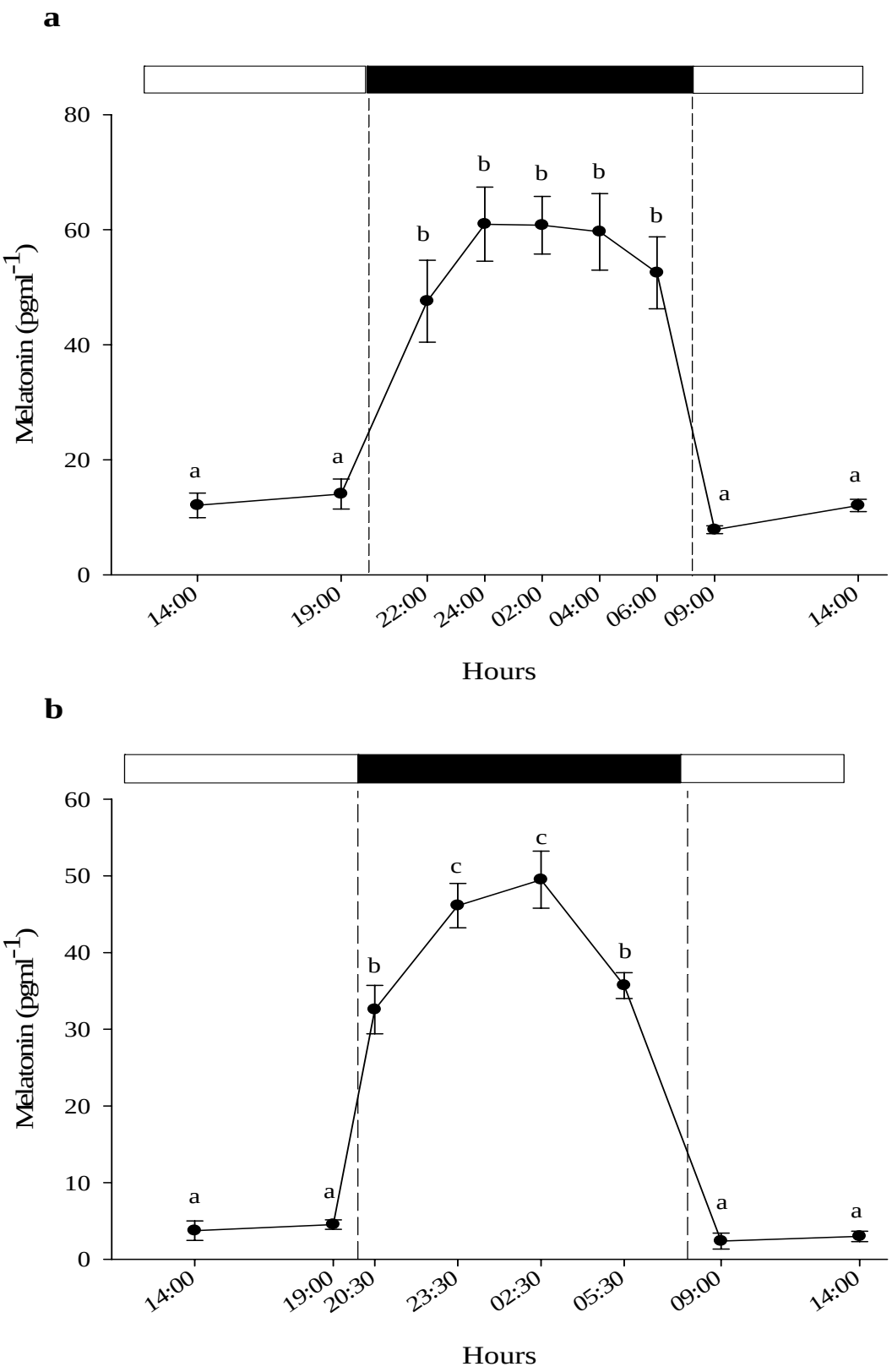
Figure 4. Plasma melatonin profile of African catfish subjected to a) 12L:12D, b) LL and c) DD regimes. Values shown are mean ± SEM (n= 6). Letters indicate significant (p<0.05) difference between sampling points. Open and filled boxes indicate photophase and scotophase respectively.

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

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

($p<0.05$) differences between sampling points. Open and filled boxes indicate photophase and scotophase respectively.

Figure 1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Melatonin (pgml-1)
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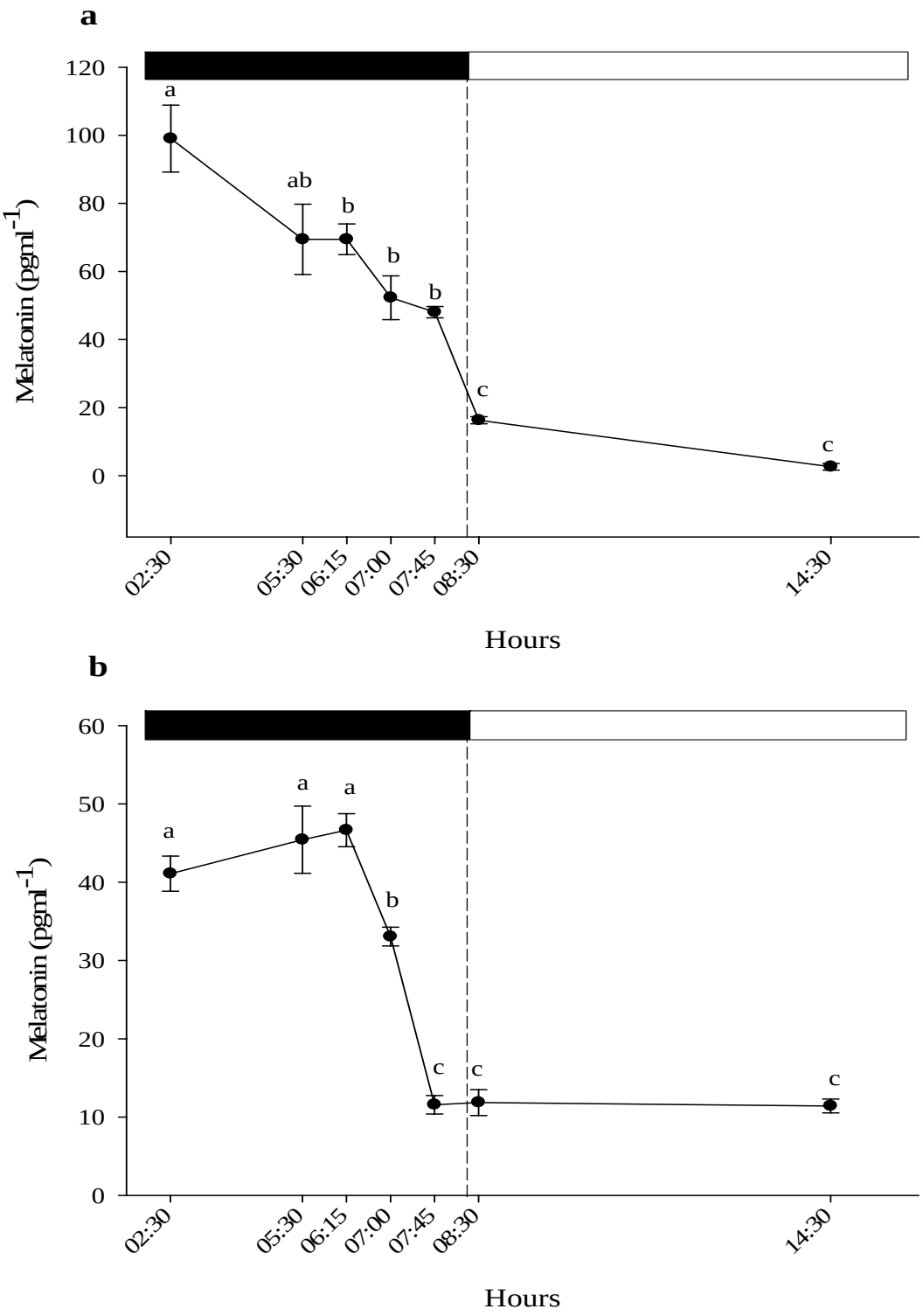
Figure 2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Melatonin (pgml-1)
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Melatonin (pgml-1)
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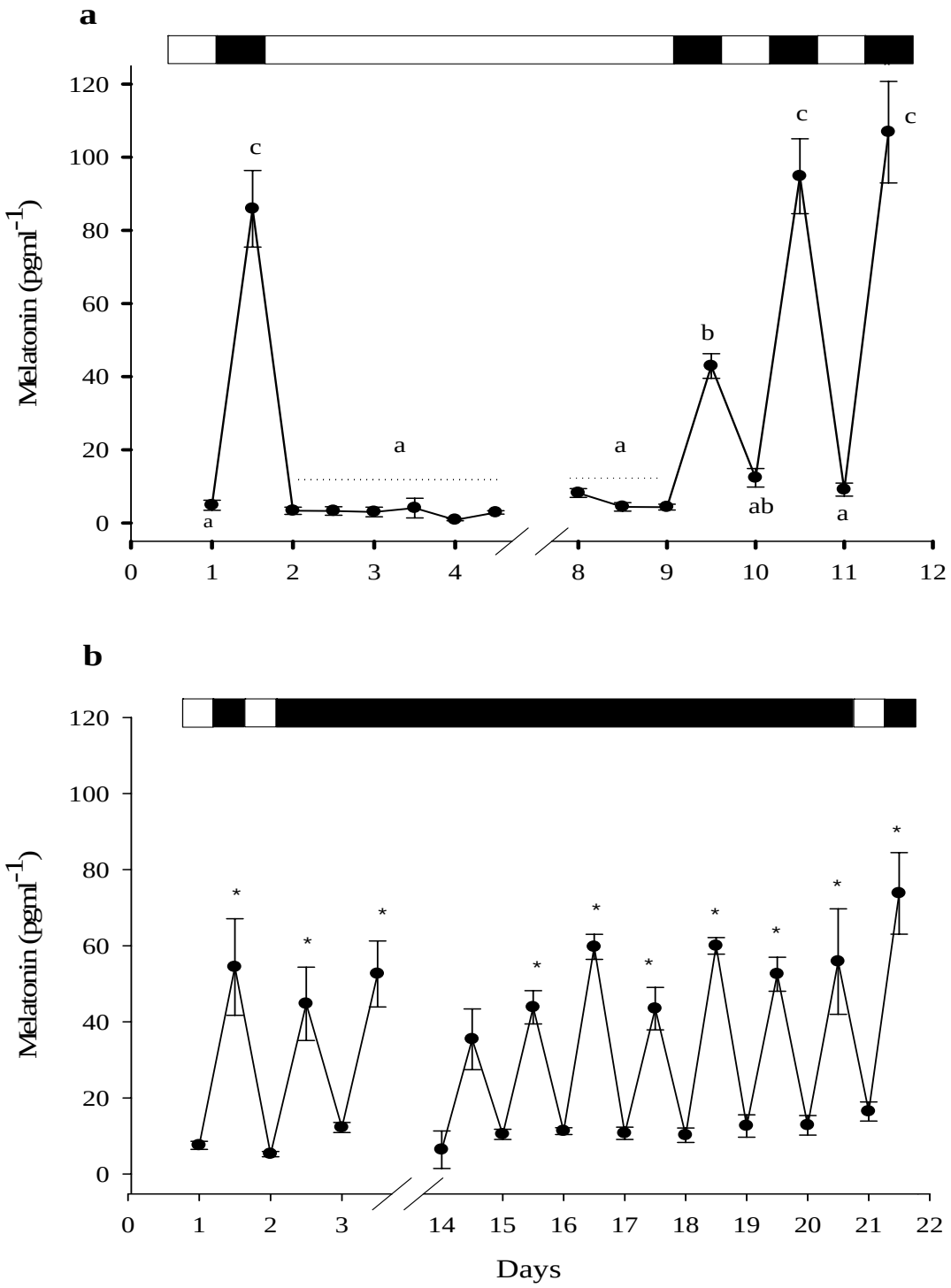
Figure 3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b
Melatonin (pgml-1)
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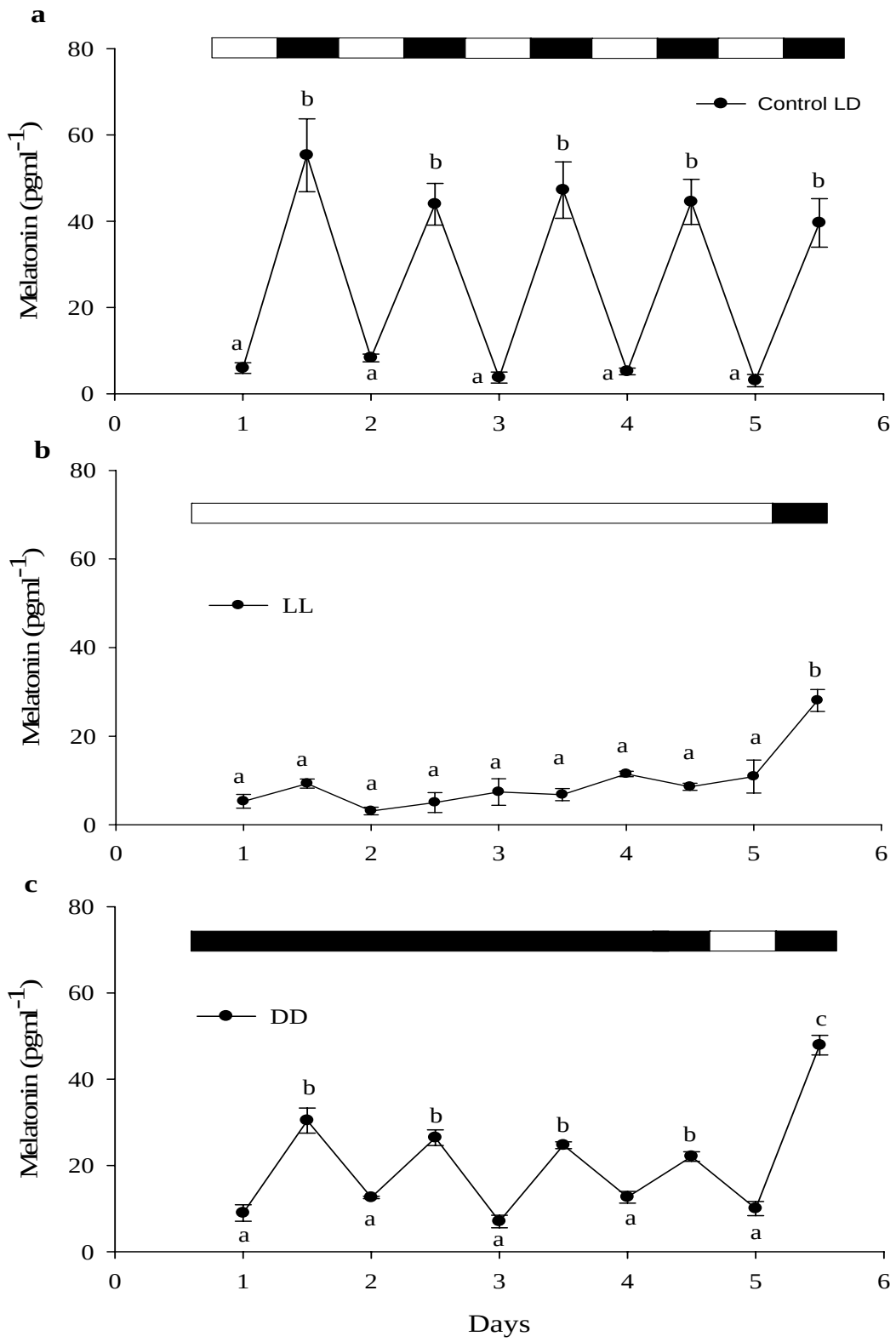
Figure 4



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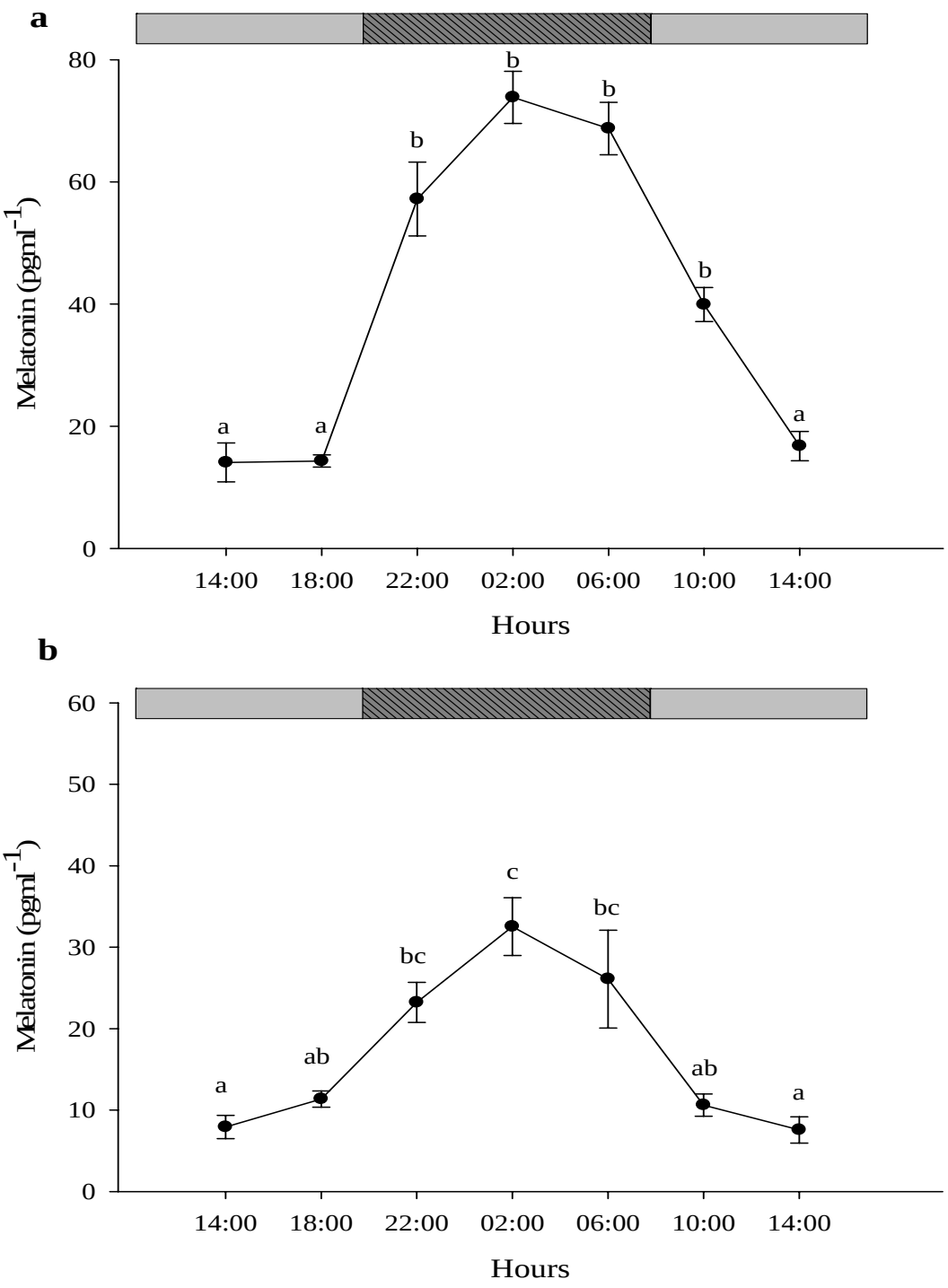
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Melatonin (pgml-1)
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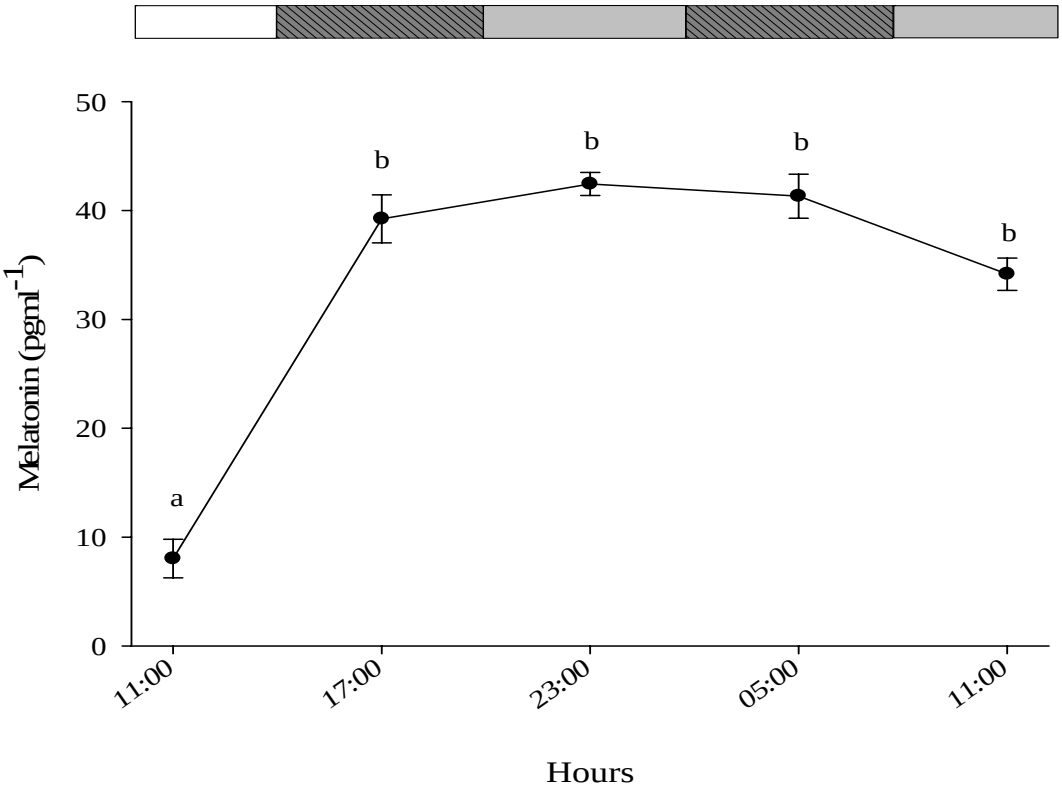
Figure 6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