Research report

RETINAL LIGHT INPUT IS REQUIRED TO SUSTAIN PLASMA MELATONIN RHYTHMS IN NILE TILAPIA OREOCHROMIS NILOTICUS NILOTICUS

Martinez-Chavez C.C. and Migaud H.

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

Number of pages: 22

Number of figures-Tables: 4

Corresponding author:

Dr. Hervé Migaud

Institute of Aquaculture, University of Stirling

FK9 4LA, Stirling, UK

Tel: 0044 1786 467886

Fax: 0044 1786 472133E-mail: herve.migaud@stir.ac.uk

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 organisation. To do so, melatonin levels were firstly measured in eyecups and plasma to determine circadian patterns of melatonin production. Secondly, the effect of partial ophthalmectomy on the suppression of melatonin production was determined in vivo as well as ex vivo pineal light/dark sensitivity. Finally, to investigate whether such findings could be related to post-surgery stress, melatonin analyses were performed in the subsequent 24 h and 7 days post-ophthalmectomy with cortisol levels assessed as an indicator of stress. Our results showed an inverse pattern of melatonin production in the eye cups of tilapia compared to blood circulating levels, suggesting different roles played by melatonin in these two tissues. Results then demonstrated that total or partial ophthalmectomy resulted in the suppression of night-time melatonin production. Furthermore, although pineals in culture were shown to be photosensitive, night-time melatonin levels were much lower than seen in other species. 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 most probably due to social and handling stress. Taken together, these results provide further evidence of a new type of circadian organisation in a teleost species where the eyes are required to sustain night time melatonin levels.

Section: Regulatory systems

Keywords: Ophthalmectomy, Melatonin, Nile tilapia, pineal, retina, cortisol

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 gland and the retina. Melatonin produced by the pineal gland in response to the day-night rhythm is released into the bloodstream and cerebrospinal fluid (Tricoire *et al*., 2002) and to act as a "zeitgeber" (endocrine role) to other processes. In the retina, melatonin has been suggested to play a paracrine role such as protecting the tissue through detoxification (Falcon et al., 2003; Klein, 2004; Iuvone et al., 2005; Siu et al., 2006).

In a number of teleost species, rhythmic melatonin production by both tissues is in phase (low during day and high at night) while 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., 1997; Besseau et al., 2006; Iigo et al., 2007b). These phase shift patterns of secretion could be linked to differences within the melatonin biosynthesis pathway 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).

The photoperiodic and circadian organization (light perception and the melatonin entrainment pathway) in teleosts has, until recently, been believed to differ significantly from the centralised organisation of mammals where light information, perceived by retinal photoreceptors, is relayed via the retinohypothalamic tract (RHT) to the master clock in the SCN 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 and Meissl, 2003). In teleosts however, no homologous circadian master clock has been found to date. Importantly pineal glands, in all species studied, appeared to be reliant on autonomous photoreception and entrainment of melatonin synthesis which in some species was further supported by an endogenous clock mechanism (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 (Dicentrarchus labrax), Atlantic cod (Gadus morhua), Nile tilapia (Oreochromis niloticus niloticus) and African catfish (Clarias gariepinus) has suggested the existence of more than one model for light entrainment of melatonin synthesis in teleosts (Migaud et al., 2007). The most interesting organisation was found in Nile tilapia where the eyes appeared to be required to sustain rhythmic melatonin production. This was also recently suggested in another tilapia species, Oreochromis mossambicus (Nikaido et al., 2009). 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 light perception mechanisms in the tropical batch spawner Nile tilapia. To do so, a series of trials were performed to first compare day/night melatonin levels in eyecups and plasma. Secondly, the study aimed at confirming the suppression of melatonin production at night in ophthalmectomised fish in vivo and in vitro. Thirdly, the effects of post-surgery stress on melatonin secretion were investigated.

RESULTS

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

Significant day-night melatonin fluctuations were shown in both the eye cups and blood (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 were significantly higher at night (49.7 ± 6.7 pg ml^{-1}) in comparison to day levels (16.0 ± 1.9 pg ml^{-1}).

Experiment 2: Effects of total or partial ophthalmectomy.

No mortalities were observed following the surgery (as in exp. 4) and video recordings showed fish returned to normal feeding behaviour within 24hrs of the surgery and displayed normal social behaviour typical of the species (i.e. dominant display). 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 pg ml^{-1}) (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 (67.4 ± 5.4 pg ml^{-1}) than at night in control fish (95.3 ± 6.8 pg ml^{-1}). A normal day-night plasma melatonin rhythm was observed in control fish. Furthermore, video recordings showed a return to normal feeding and social dominant-submissive behaviour within 24 hrs post-surgery (data not shown).

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 minimum 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 significant differences between the two times post-surgery were observed with levels remaining basal as per during the day (<20 pg ml^{-1}). Mean (day-night) cortisol levels of ophthalmectomised (124.1 ± 31.5 and 115.3 ± 28.8 pg ml^{-1}) and control (169.0 ± 41.4 and 138.1 ± 41.0 pg ml^{-1}) fish were found to be higher than basal levels reported in the same species at both sampling points respectively.

DISCUSSION

As previously shown in several other species of vertebrates including some teleosts such as European sea bass (Iigo et al., 1997; Bayarri et al., 2002), rainbow trout, Oncorhynchus mykiss (Besseau et al., 2006) and Nile tilapia (Iigo et al., 2007), retinal melatonin rhythm in the present study was phase shifted (4 fold higher during the day than during the night) in comparison to the plasma rhythm. This would suggest that retinal melatonin in Nile tilapia is most likely having a different role than melatonin produced by the pineal gland and would not significantly contribute to night-time plasma melatonin levels. This is further supported by the strong melatonin deacetylase activity shown in the retina which might prevent melatonin from being released into the bloodstream (Grace *et al*., 1991). More robust conclusions could only be obtained

through pinealectomy although the species does not lend itself well to such a technique due to the physiology and location of the pineal gland in tilapia.

In rainbow trout, at least two different melatonin synthesis pathways appear to exist 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).

Irrespective of the function of retinal and pineal melatonin, the role of the eyes in the control of the pineal melatonin production (Migaud et al., 2007) is of great interest. This was the first time that a "mammal-like" circadian organisation had been suggested in teleosts without considering endogenous rhythmicity nor the existence or not of a SCN like structure in fish. In the current study, while control and partially ophthalmectomized fish continued 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. These results thus confirmed that the eyes are required by the pineal gland to produce and secrete melatonin in a normal manner into the blood circulation as suggested previously (Migaud et al., 2007). The fact that the tilapia pineal appears to be reliant on retinal photoreception could be due to the nature of the photoreceptors which are present in the tilapia pineal. In Lamprey and pike, three types of melatonin synthetising cells have been shown to co-exist in the pineal from true photosensitive photoreceptors to pinealocytes potentially depending on extra-pineal light perception (e.g. retina and/or deep brain)(Ekstrom and Meissl,

2003). This opens up an interesting hypothesis that the circadian organisation that one finds in a species might therefore depend to a large extent on the ratio of the different photoreceptor cell types present within the pineal.

In vitro results also confirmed that Nile tilapia pineal glands do not follow the general photo-responsiveness pattern observed in most teleosts studied so far. Indeed, numerous reports on a range of teleosts have shown that their 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 able to sustain these rhythms under constant darkness (Falcon et al., 1989; Bolliet et al., 1996; Ron, 2004; Migaud et al., 2006; Iigo et al., 2007a). Importantly, night time in vitro melatonin levels in all species studied excluding Nile and Mozambique tilapias 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; Nikaido et al., 2009). As recently shown in Mozambique tilapia (Nikaido et al., 2009), pineal sensitivity to day-night rhythm appeared to be far less pronounced in the Nile tilapia than in other teleosts with very low levels produced at night (<30 pg ml^{-1}, equivalent to plasma levels at night) even in the presence of L-tryptophan or serotonin. However. isolated pineal glands still appeared to be directly photosensitive by producing melatonin during the dark periods. As a comparison, levels >2-3 ng ml^{-1} have been reported in temperate species such as sea bass or Atlantic cod using the same in vitro set up (same flow rate), validated for each species (media, temperature, pH)(Migaud et al., 2006). It must, however, be acknowledged that although in vitro pineal culture is a standard procedure routinely used in our laboratory, with the technique including the pineal dissection being validated in the species, further optimisation may still be required.

Nonetheless, taken together, the in vivo and in vitro results confirm that photic stimuli from the eyes are required to sustain the rhythmic pineal melatonin production.

However, the possibility that melatonin suppression is due to post-surgery stress can not be overlooked. In the current study, night-time melatonin suppression was observed in both fish sampled within 24hrs of surgery or one week following ophthalmectomy. Plasma cortisol is known to be a good indicator of stress levels in fish (Wendelaar Bonga, 1986) and as such it was monitored in the present study. Results clearly indicated that sampling procedure was not appropriate. Indeed, high cortisol levels, comparable to those found in stress related studies in Nile tilapia (Vijayan et al., 1997; Barcellos et al., 1999; Barreto and Volpato, 2006) were recorded in ophthalmectomized fish as well as intact control fish. This could suggest that suppression of night melatonin in ophthalmectomized fish is not 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 (similar to that of mammals) in a teleost based on photo-entrainment of the pineal melatonin production by the eyes. Further ultra-structural, binding and gene expression studies are needed to determine the potential involvement of the brain in regulating melatonin synthesis through neural pathways such as the retinohypothalamic tract as shown in mammals (Herzog and Tosini, 2001; Lee et al., 2003). If demonstrated, then it would suggest a convergent evolution of this

system between species belonging to two distinct and divergent groups of vertebrates (teleosts and mammals).

EXPERIMENTAL PROCEDURE

Animals

Mixed sex red Nile tilapia (220.4 ± 5.5 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 for at least two weeks in the experimental tanks prior to the start of the trial. 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 was delivered at satiation every second day with commercial trout pellets (Standard Expanded, Skretting, Cheshire, UK). In all experiments lights were switched on at 08:00 and turned off at 20:00. 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 experiments were carried in accordance with the Animal (Scientific Procedures) Act 1986, UK. 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 sampled for blood and whole eye cups were removed in the middle of the day and night (i.e. 14:00 and 02:00). Whole eyecups 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 for melatonin. 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. An intact group of 16 fish was also used as controls. 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 was carried out in the afternoon (17:00) and sampling (n=4 / treatment / sampling point) commenced during the night of the same day and during two complete cycles (Fig. 2). Sampling was only carried out in the middle of the day and night to reduce the number of fish undergoing surgery as previous investigation confirmed the circadian nature of melatonin rhythmic

production when tilapia was exposed to continuous darkness (Martinez-Chavez et al., 2008).

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). Results presented correspond to a total of 6 Nile tilapia pineal glands which were dissected 2 hours before darkness (at 18:00) and placed individually in flow through chambers inside an in vitro cabinet (26 ^{0}C). Pineal glands were then exposed to a normal LD cycle and hourly samples were collected during two consecutive 12L: 12D cycles. During each 24 hrs cycle, two night (02:00 and 06:00) and two day (14:00 and 18:00) sample points were taken and melatonin analysed (Fig. 3). Optimization of the in vitro system was previously done by testing a range of temperatures (18-27 ºC) and supplementing the culture media with increasing concentrations of melatonin precursors (L-tryptophan and serotonin, SIGMA, Poole, UK) with no significant effects (data not shown). At the end of the culture period the cell viability of the pineal glands 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 4: Effects of post-surgery stress on melatonin rhythms in ophthalmectomised vs. intact Nile tilapia.

This preliminary trial was designed to investigate whether post-surgery stress caused by the ophtalmectomy, 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 was left intact as a control. Sampling occurred at two different times post-surgery (in the following 24 hrs and 7 days later), where 4 fish were sampled at each time point during two dark and one consecutive light periods (Fig. 4). Melatonin and cortisol analyses were performed on the same blood samples as described below.

Hormonal assays

Melatonin was analysed using a commercially available ELISA kit (IBL, Hamburg, Germany). 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 analyses. 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 standards, was 3 pg ml^{-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 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 %. Prior to the analyses, the RIAs have been validated by confirming the parallelism between serial dilutions of night-time pooled plasma from both species to the standard curve (data not presented).

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 were 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 $p \leq 0.05$.

ACKNOWLEDGEMENTS

This study was possible through the financial support from CONACyT (Consejo Nacional de Ciencia y Tecnologia, Mexico) and the University of Stirling. We would also like to specially thank Dr. Ian Berrill for his help with the cortisol assay and Dr. Andrew Davie for his assistance in editing the manuscript.

REFERENCES

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 Res. 30, 887-892.

Barrenetxe, J., Delagrange, P. and Martinez, J.A., 2004. Physiological and metabolic functions of melatonin. J. Physiol. Biochem. 60, 61-72.

Barreto, R.E. and Volpato, G.L., 2006. Stress responses of the fish Nile tilapia subjected to electroshock and social stressors. Braz. J. Med. Biol. Res. 39, 1605-1612.

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. J. Pineal Res. 32, 34-40.

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 Physiol. Biochem. 30, 87-89.

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. Exp. Eye Res. 82, 620-627.

Beyer, C.E., Steketee, J.D. and Saphier, D., 1998. Antioxidant properties of melatonin-an emerging mystery. Biochem. Pharmacol. 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. J. Comp. Physiol. B. 165, 677-683.

Cahill, G.M., 1996. Circadian regulation of melatonin production in cultured zebrafish pineal and retina. Brain Res. 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. and Klein, D.C., 2006. Evolution of Arylalkylamine N-Acetyltransferase: emergence and divergence. Mol. Cell. Endocrinol. 252, 2-10.

Ekstrom, P. and Meissl, H., 2003. Evolution of photosensory pineal organs in new light: the fate of neuroendocrine receptors. Philos. T. R. Soc. B. 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. J. Fish Biol. 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. J. Neurosci. 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. J. Neuroendocrinol. 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. J. Pineal Res. 31, 68-75.

Gern, W.A., Owens, D.W. and Ralph, CH.L., 1978. The synthesis of melatonin by the trout retina. J. Exp. Zool. 206, 263-270.

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

Hardeland, R., Pandi-Perumal, S.R. and Cardinali, D.P., 2006. Melatonin. Int. J. Biochem. Cell Biol. 38, 313-316.

Herzog, E.D. and Tosini, G., 2001. The mammalian circadian clock shop. Semin. Cell Dev. Biol. 12, 295-303.

Iigo, M., Kezuka, H., Aida, K. and 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., 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., 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 Res. 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. 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. and Yanagisawa, T., 2007a. Lack of circadian regulation of in vitro melatonin release from the pineal organ of salmonid teleosts. Gen. Comp. Endocrinol. 154, 91-97.

Iigo, M, Furukawa, K, Nishi, G, Tabat,a M, Aida, K,2007b. Ocular melatonin rhythms in teleost fish. Brain, Behav. Evol. 69, 114-121.

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. Prog. Retin. Eye Res. 24, 433-456.

Kezuka, H., Aida, K. and Hanyu, I., 1989. Melatonin secretion from golfish pineal gland in organ culture. Gen. Comp. Endocrinol. 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 Prog. Horm. Res. 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. J. Biol. 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. Gen. Comp. Endocrinol. 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. Nat. Neurosci. 6, 111-112.

Martinez-Chavez Carlos C., Al-Khamees S., Campos-Mendoza A., Migaud H., 2008. Clock Controlled Endogenous Melatonin Rhythms in Nile Tilapia (*O. niloticus niloticus*) and African Catfish (*C. gariepinus*). Chronobiology International 25:1, 31 – 49.

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. J. Pineal Res. 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. J. Pineal Res. 43, 327-335.

Nikaido Y., Ueda S., Takemura A., 2009. Photic and circadian regulation of melatonin production in the Mozambique tilapia Oreochromis mossambicus. Comp. Biochem. Physiol. 152, 77-82.

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. Gen. Comp. Endocrinol. 114, 304-312.

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 Sci. 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. Isr. J. Aquacult.-Bamid. 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. Comp. Biochem. Phys. C. 106, 189-193.

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. J. Pineal Res. 40, 101-109.

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. J. Pineal Res. 40, 236-241.

Tamai, T.K., Vardhanabhuti, V., Arthur, S., Foulkes, N.S. and Whitmore, D., 2003. Flies and fish: birds of a feather. J. Neuroendocrinol. 15, 344-349.

Tricoire, H., Locatelli, A., Chemineau, P. and Malpaux, B., 2002. Melatonin enters the cerebrospinal fluid through the pineal recess. Endocrinol. 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. Comp. Biochem. Physiol. Part C 116, 89-95.

Wendelaar Bonga, S.E., 1986. The stress response in fish. Physiol. Rev. 77, 591-625

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. J. Endocrinol. 91, 477-481.

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. Gen. Comp. Endocrinol. 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. Semin. Cell. Dev. Biol. 12, 317-328.

Figure legends

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

Figure 2. Mid-day and mid-night circulating plasma melatonin levels in fully ophthalmectomised (enucleated), partially ophthalmectomised (right/left) and non-ophthalmectomised (control) fish during 2 consecutive days, following surgical procedure. Data are presented as mean ± SE (n = 4 /treatment / sampling point). Superscripts above the bars indicate significant ($p<0.05$) difference between treatments at each time point (day or night). Open and filled boxes indicate photophase and scotophase, respectively.

Figure 3. Mean in 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 ophthalmectomised (enucleated) and intact (control) fish sampled during 36 hrs at night and day either following surgical ophthalmectomy or a week later. Data are presented as mean ± SE (n=4 /treatment / sampling point). Superscripts indicate

significant ($p<0.05$) difference between sample points and treatments. Open and filled boxes indicate photophase and scotophase, respectively.

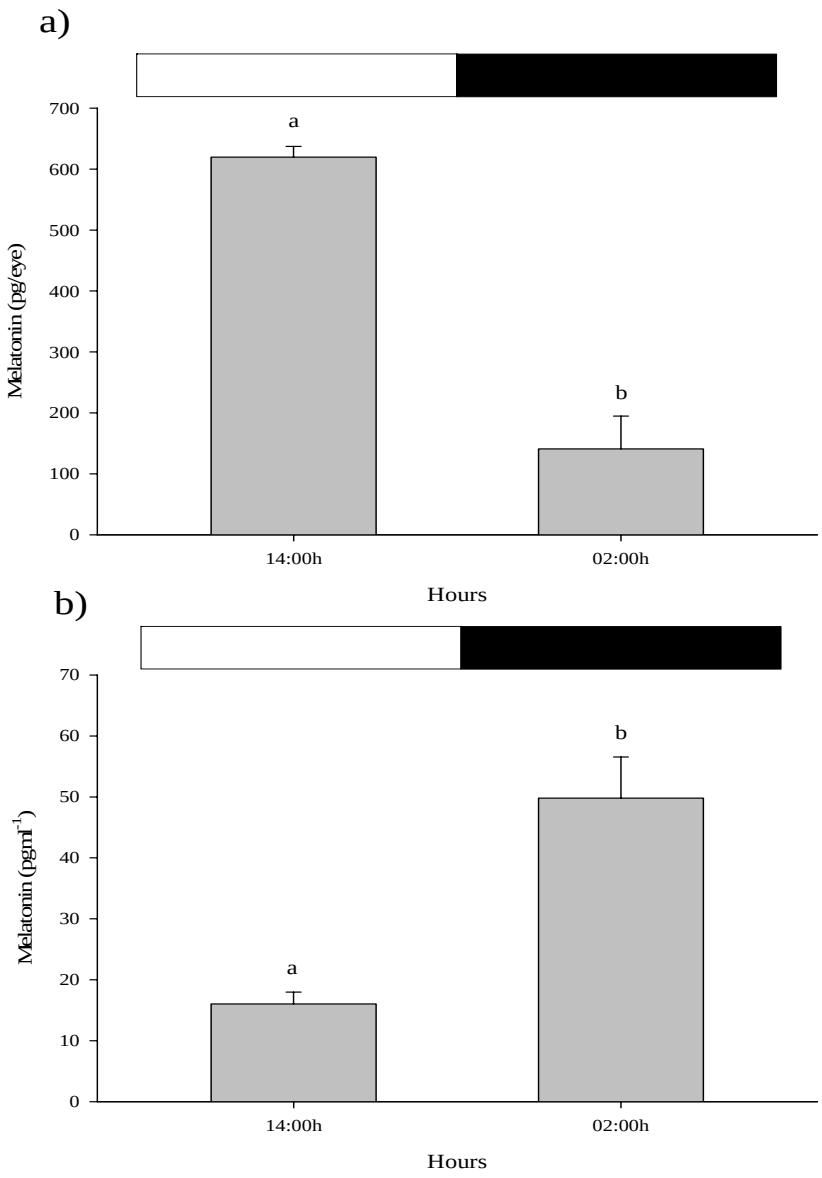
a)
700
600
500
400
300
200
100
0
Melatonin (pg/eye)
a
b
14:00h
02:00h
Hours
b)
70
60
50
40
30
20
10
0
Melatonin (pgml-1)
a
b
14:00h
02:00h
Hours

Figure 1.

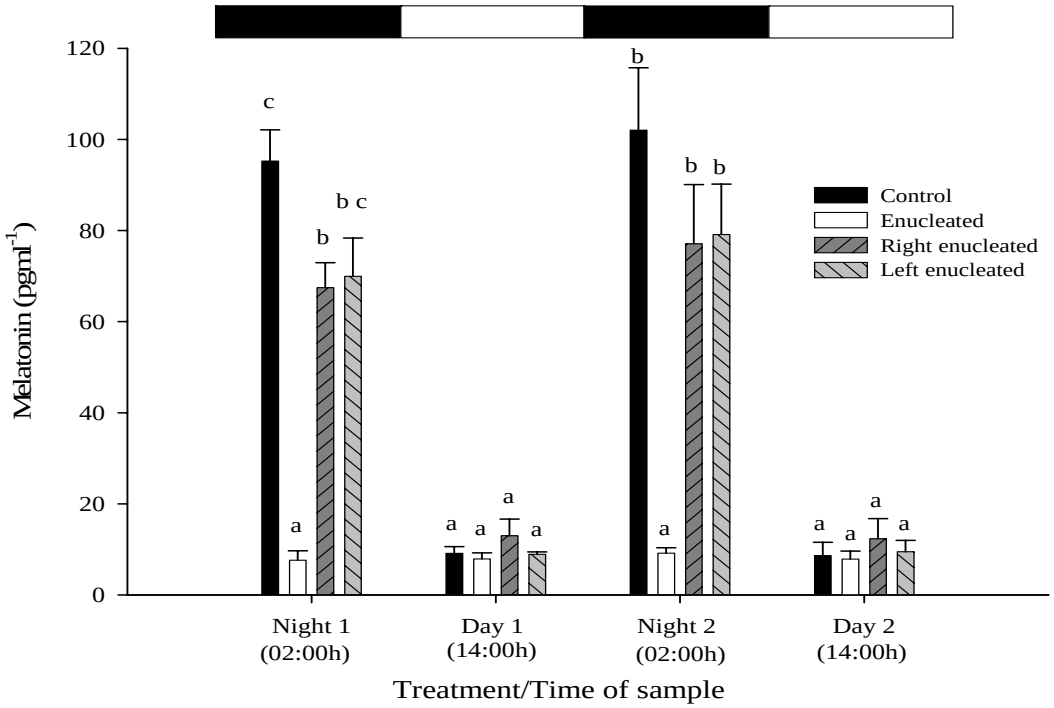
120
100
80
60
40
20
0
Melatonin (pgml-1)
c
a
b
b c
a
a
a
a
b
a
b
b
a
a
a
a
Control
Enucleated
Right enucleated
Left enucleated
Night 1
(02:00h)
Day 1
(14:00h)
Night 2
(02:00h)
Day 2
(14:00h)
Treatment/Time of sample

Figure 2.

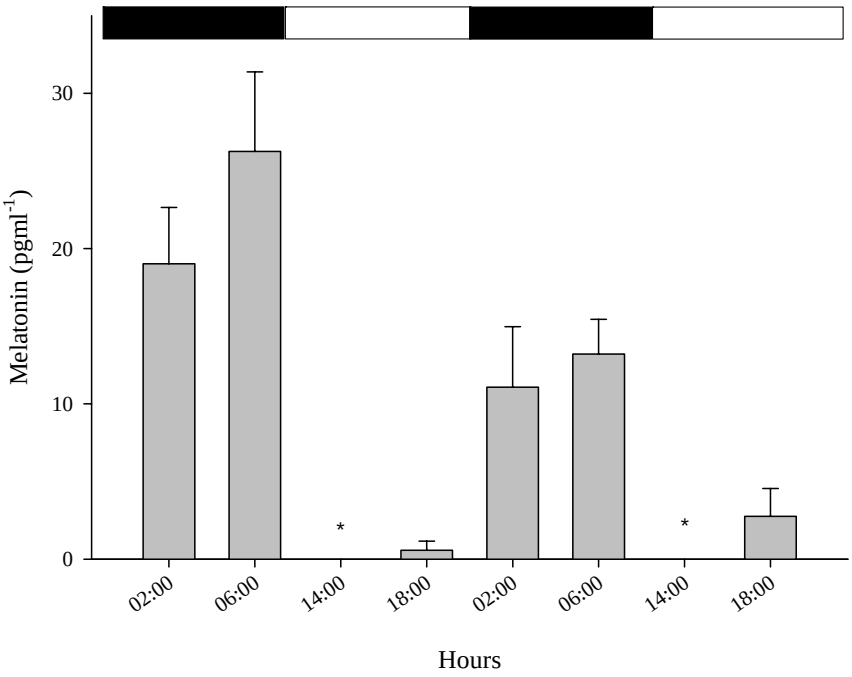
Melatonin (pgml-1)
30
20
10
0
*
*
02:00
06:00
14:00
18:00
02:00
06:00
14:00
18:00
Hours

Figure 3.

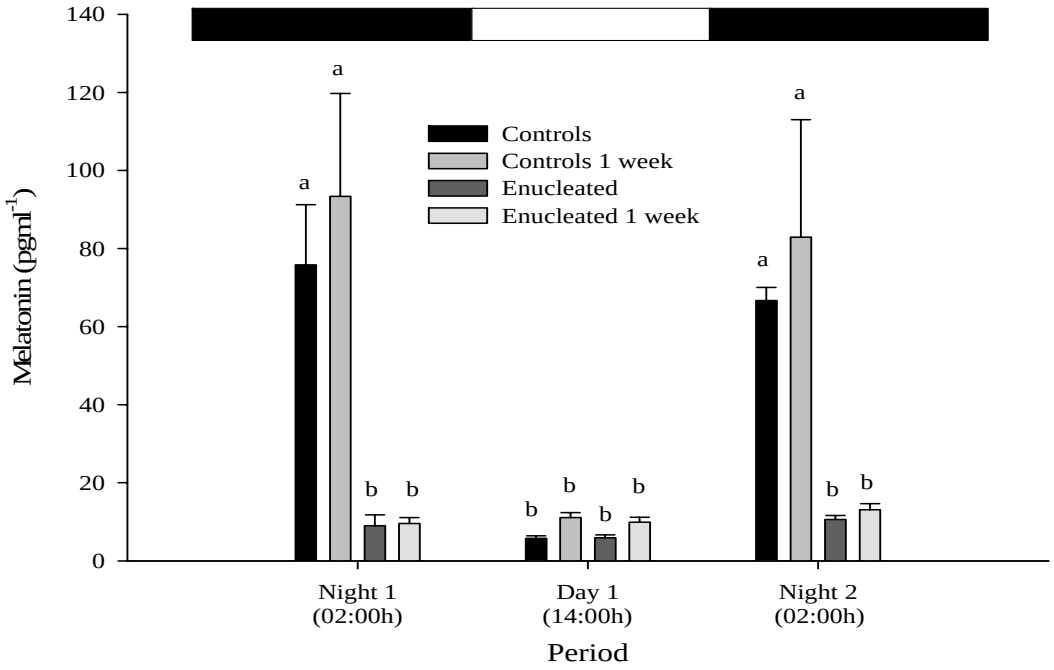
140
120
100
80
60
40
20
0
Melatonin (pgml-1)
Controls
Controls 1 week
Enucleated
Enucleated 1 week
a
a
b
b
b
b
b
b
a
a
b
b
Night 1
(02:00h)
Day 1
(14:00h)
Night 2
(02:00h)
Period

Figure 4.