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Plasma melatonin profiles in copper mahseer (*Neolissochilus hexagonolepis*) kept under natural and manipulated photoperiods

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Acknowledgement

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Trondheim, October 2011

Ane Maria Guttu

Abstract

In many countries around the world, health problems related to food access are an increasing threat, and many people today still don't have access to sufficient protein and energy in their diet. In poor countries with growing populations fisheries will become important because of lower levels of investments and running costs. Nepal, a country rich in freshwater and high fish diversity should be able to develop the aquaculture sector, but the major bottleneck blocking fish availability seems to be an inadequate fingerling supply.

Photoperiod is the main cue synchronizing the reproduction and the pineal organ is the mediator that converts the information of photoperiod into melatonin. Melatonin is secreted during dark, and because of this rhythmic manner it is found to be a time-keeping hormone, giving information about day length and season. Seasonally changing photoperiod is found to be an important cue initiating reproduction in temperate species. The understanding of the circadian axis and photoperiodic entrainment of reproduction have been used with success to manipulate the natural rhythm of spawning in many temperate species. Little is known about such biorhythm control in low latitude species, but a few recent studies have shown that tropical fish also can respond to photoperiodic changes by the use of the pineal system. This master thesis was initiated to investigate the plasma melatonin profile in copper mahseer (*Neolissochilus hexagonolepis*).

Fish kept under natural photoperiod in the present study showed a rapid rise in melatonin at beginning of the dark period, after which the values gradually drop and reach low daytime levels at the beginning of light phase. For fish exposed to extended dark period, the melatonin levels showed a significant increase at the time for natural dark and a significant decrease at the time for natural light. A shift was observed in the melatonin pattern, with a peak in the melatonin level four hours after the peak was seen under natural photoperiod. The results presented in this study might indicate an intra-pineal oscillator capable of self sustain the melatonin pattern. Melatonin half- life was estimated to 10 minutes. Plasma cortisol levels were high during the experiments, but it is thought not to have had an influence on the plasma melatonin levels.

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Introduction

In 2000 the United Nations presented the eight Millennium Development Goals (MDG) [1] with fight against poverty and hunger being the prime target (UN 2010). Since then some progress has been made, however the world still has almost one billion undernourished people (PRB 2010). In many countries around the world, health problems related to food access are an increasing threat, and many people today still don't have access to sufficient protein and energy in their diet (FAO 2010). More than 60 % of these people are living in the Asian and Pacific area.

Nepal is a landlocked country in South Asia, strategically placed between the two countries in the world with over 30 % of the population, China in the north, and India in the west, east and south. It is one of the least developed countries in the world, and 30 % of the population lives under the poverty line. Only 18 % of the land area in Nepal is cultivated, but still agriculture is the backbone of the economy (Maharjan and Joshi 2011). More frequent natural hazards is imposing a treath on the livelihood for many people (Pant 2011), and according to FAO, the country will not reach the MDG goal 1, to end poverty and hunger, if prevailing trends persist (FAO 2011).

Regarding freshwater resources Nepal is one of the richest countries in the world, holding more than 2,7 % of the world´s freshwater (Sharma *et al.* 2005). There is also a high diversity of fish, with 191 indigenous and nine exotic species, which can contribute to inland fisheries and aquaculture (Sharma 2008). Aquaculture may have a potential to meet the increasing demands for food security, livelihoods and nutritions (Subasinghe 2005), but so far the development of these resources has been slow (Sharma 2008; FAO 2010).

In poor countries with growing populations, fisheries will become important because of lower levels of investments and running costs (Dugan *et al.* 2007). Nepal, a country rich in freshwater and high fish diversity should be able to develop the aquaculture sector. The major

[1] The eight MDG´s are: (1) End poverty and hunger, (2) Achieve universal education, (3) Equality between gender, (4) Reduce child mortality, (5) Improve maternal health, (6) Combat HIV/AIDS, malaria and other diseases, (7) Ensure environmental sustainability, (8) Develop a global partnership for development (UN (2010). The Millenium development Goals Report. New York**:** 76.

bottleneck blocking fish availability seems to be an inadequate fingerling supply due to reproductive activity limited to one time a year (SPRN 2008; Gurung *et al.* 2010).
The NTNU headed SPRN (Sustainable Poverty Reduction in Nepal) programme was developed to investigate the fingerling shortage. As a part of the SPRN program this master thesis was initiated to shed light on the regulation of the reproduction in a local carp species, copper mahseer (*Neolissochilus hexagonolepis*).

Teleost reproduction

Reproduction in fish is known to be seasonal (Bromage *et al.* 2001). Gonadal development, gamete and embryo formation takes a long time to complete, and the development of the gonads must have been initiated many months earlier so that the production of fry is synchronized with seasonal improvements in climate and nutrient availability (Brooks *et al.* 1997; Maitra *et al.* 2001; Migaud *et al.* 2010).

The reproductive cycle is under endocrine control by hormones from the hypothalamus and other glands like the pituitary and gonads (Mylonas *et al.* 2010). Photoperiod is the main cue synchronizing the reproduction with the environmental, and the pineal organ is the mediator that converts the information of photoperiod into melatonin (Maitra *et al.* 2006; Paul *et al.* 2008). Melatonin is thought to act on the hypothalamus pituitary gonadal (HPG) axis, which in turn controls reproduction timing (Balik *et al.* 2004; Bayarri *et al.* 2004).

Teleost pineal and melatonin production

In teleost fish the pineal gland generally appears as an end vesicle connected to the diencephalon by a stalk, located below a window where light enters (Ekström and Meissl 1997; Dey *et al.* 2003). This area is often thinner, and the skin covering it is less pigmented (Boeuf and Le Bail 1999; Falcón *et al.* 2010; Kulczykowska *et al.* 2010). The epithelium of the pineal is made of true cone like photoreceptor cells that resembles the retina in function and structure (Falcón *et al.* 2010). When stimulated by light the photoreceptor cells are hyperpolarized, wich results in a inhibition of an exitatory neurotransmitter, wich is connected directly to the brain via axons (Ekström and Meissl 2003).

The pineal gland in teleosts is a lumunance detector that convey information about the light dark cycle and transforms it into a message in the form of melatonin (Ekström and Meissl 1997). Melatonin is secreted from the pineal gland and released quickly into the blood and cerebro-spinal fluid during dark (Reiter 1993). Because of this rhythmic manner melatonin is found to be a time-keeping hormone, giving information about day length and season (Paul *et al.* 2008). Seasonally changing photoperiods is an important cue initiating reproduction in temperate species (Bromage *et al.* 2001). However, animals living closer to equator are thought to depend more on non-photoperiodic cues (e.g. temperature, monsoon) for seasonal timing given the minimal changes in day length (Susilo *et al.* 2009).

Melatonin may function as a signal molecule for several prosesses in the fish body including osmoregulation, growth, reproduction, migration and feeding. Melatonin is found to act on its target cell trough G- protein-coupled receptors (MT1, MT2,MT3) (Falcón *et al.* 2010), and these receptors have been found in the retina (Falcón *et al.* 2003) and the gastrointestinal tract (Bubenik 2008). This may suggest a local sythesis in other places than the pineal.

Biosynthesis of melatonin

The biosynthesis of melatonin occurs in the pineal photoreceptor cells, and involves four enzymatic steps (Fig. 1). First, tryptophan is taken up from the circulation and catalyzed to 5-hydroxytryptophan by tryptophan hydroxylase (TpOH). 5-hydroxytryptophan is then decarboxylated to serotonin by aromatic amino acid decarboxylase (AAAD), which is then catalyzed to N-acetylseretonin by arylalkylamine N-acetyltransferase (AANAT). In the last step hydroxyindole-O-methyltransferase (HIOMT) converts N-acetylseretonin to melatonin (Ekström and Meissl 1997; Klein *et al.* 1997; Falcón *et al.* 2010).

The rhythm in melatonin production is driven by the activation and inactivation of AANAT, which is the rate-limiting enzyme. In darkness the pineal photoreceptor cells are depolarized, and lead to a influx of intracellular messengers (Ca^{2+}, cAMP) which are thought to be involved in the synthesis of AANAT (Falcón *et al.* 2001). In light the photoreceptor cells are hyperpolarized, AANAT is degraded and the melatonin production is inhibited (Klein *et al.* 1997).

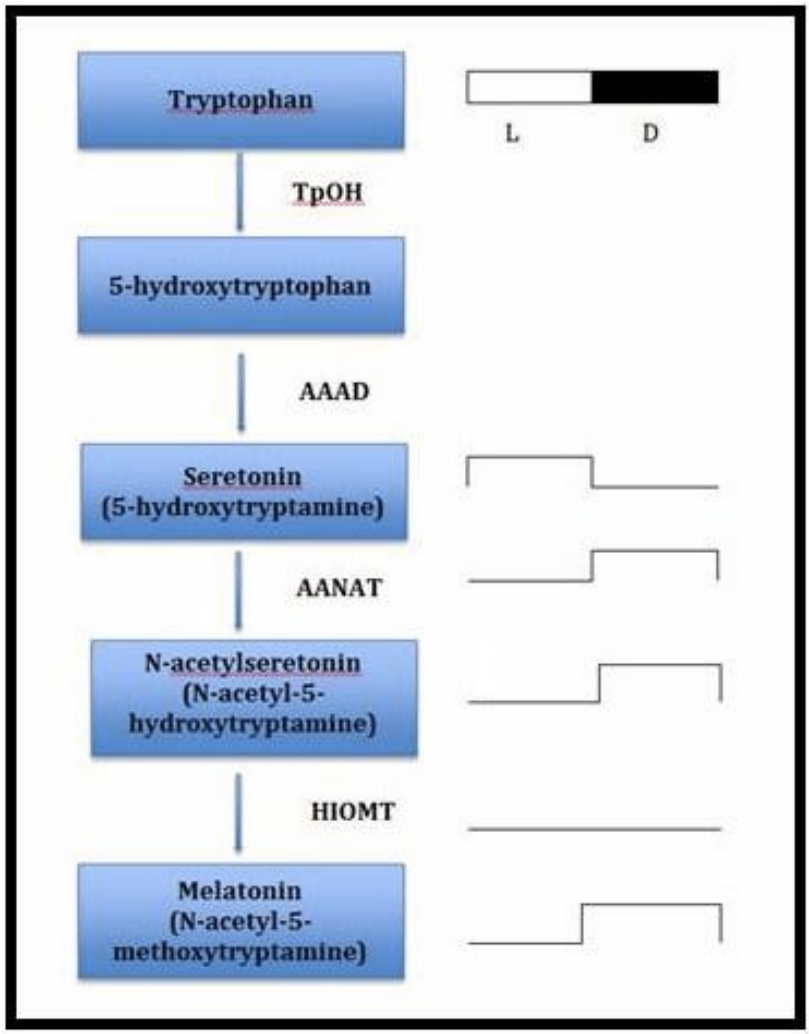


Figure 1: Melatonin biosynthesis pathway. A schematic indication of the variations in the corresponding compound or enzymatic activity following the light (L) dark (D) cycle (Modulated from Falcón et al. 2010)

Temperature

Temperature is found to have direct effect on melatonin production through the AANAT enzymatic activity (Falcón 1999). Like photoperiod, temperature will also influence the pineal melatonin production. Photoperiod determines the duration of the melatonin signal and temperature is thought to determine the seasonal variations in amplitude of melatonin rhythm. Together photoperiod and temperature is thought to provide accurate definitions of the daily and annual changes (Falcón *et al.* 2010).

Biorythms

Melatonin secretion from the pineal gland is a product of the light-dark cycle (Ekström and Meissl 1997), but most teleost species investigated have shown to have an intra-pineal oscillator that can self-sustain the melatonin rhythm without signal about the photoperiod (Bolliet *et al.* 1996; Ekström and Meissl 1997; Coon *et al.* 1998). Conversely, several salmonid teleost have shown a lack of circadian regulation of melatonin. The pineal is directly influenced by light and melatonin is regulated in an on/off manner (Iigo *et al.* 1998; Masuda *et al.* 2003; Oliveira *et al.* 2009).

Three patterns (Fig. 2) of nocturnal melatonin release have been identified between species (Reiter 1987; Reiter 1991). Animals with the type A profile are characterized by a delay after the start of the dark phase before melatonin rise to its peak at the end of the dark phase. In type B, plasma melatonin shows a gradual rise in the first half of dark period with a peak near mid-dark and gradually decreases to reach daytime level when light period starts. In animals with a type C profile there is a rapid rise in plasma melatonin right after onset of the dark and remain at a high plateau until the time of light period. These profiles might indicate if the species have an intra-pineal oscillator capable of self-sustaining the melatonin pattern.

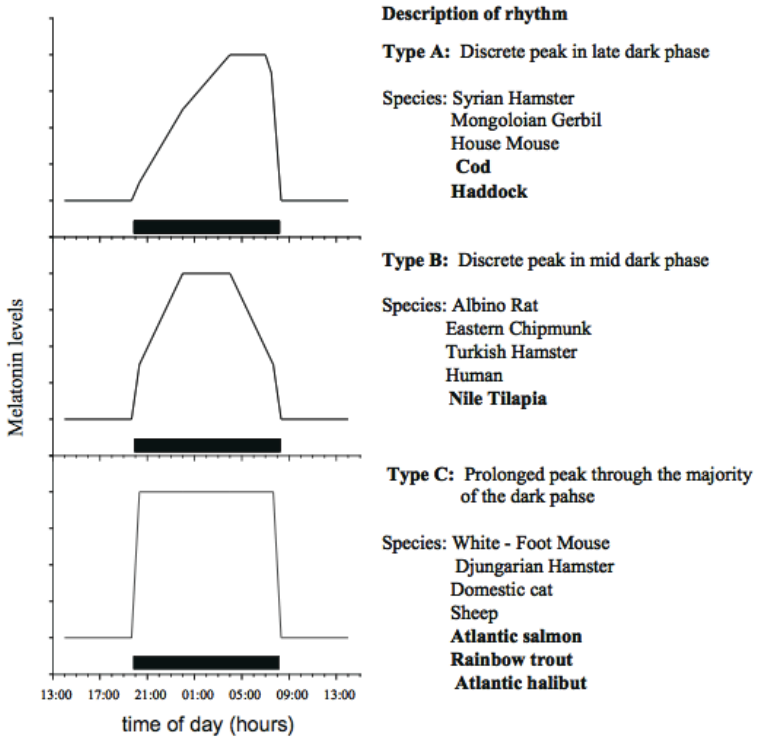


Figure 2: Representation of the different melatonin profiles in vertebrates. Species wich express A, B or C pattern of plasma melatonin are listed. Horizontal black bar denotes dark period (Falcón *et al.* 2010).

The stress response of teleost fish

Stress is considered an adaptive mechanism that allows the fish to cope with real or perceived stressors in order to maintain its homeostatic state (Barton and Iwama 1991). The acute response is beneficial and provokes physiological changes that optimize its biological performance when the exposure is brifely (Bonga 1997). If the intensity of the stressor is severe or long-lasting, the physiological response mechanisms may be compromised leading to adverse affects such as lower the immune capasity (Barton 2002; Ashley 2006). Stress has been reported to increase the plasma cortisol level in fish (Einarsdóttir and Nilssen 1996; Trushenski *et al.* 2010) and cortisol is often used as an indicator to evaluate the health and stress of the animal (Martinez- Porchas *et al.* 2009). Cortisol has also been found to have an effect on other hormones, including melatonin. For example in the pineal organ of tilapia (*Oreochromis mossambicus*), cortisol was found to lower melatonin synthesis (Nikado *et al.* 2010).

Physiological responses to a stressor have been grouped as primary and secondary (Pankhurst 2011). The primary response involves the activation of the hypoyhalamic-symphatetic-chromafin axis which leads to the relase of catecholamines (adrenalin and noradrenalin), and of the hypothalamic-pituitary-interrenal (HPI) axis which leads to the release of corticosteroids (cortisol) (Charmandari *et al.* 2005). The catecholamines level rises immediately after stress, but the increase of cortisol is a more delayed prosess. Secondary responses occur as a consequence of the released hormones and are related to physiological adjustments as increased metabolism, blood flow and oxygen uptake (Barton and Iwama 1991). If the stress becomes chronic it may become maldaptive for the animal as the physiological changes will remain elevated over a longer period, and this is known as the tertiary respons, which may supress non vital prosesses as growth, reproduction and the immune system (Pankhurst 2011).

Aim of study

There is a huge potential for aquaculture in Nepal, and by strenghtening this sector the quality of life for many poor people may improve. To do this one has to solve the problem with a insufficient supply of fry due to reproductive activity to only a limited time of year. The understanding of the circadian axis and photoperiodic entrainment of reproduction have been used with success to manipulate the natural rhythm of spawning in many temperate species (Bromage *et al.* 2001). A focus on gaining knowledge on the reproduction cycle and the possibility that a photoperiodic component can control reproduction in local carp species is needed.

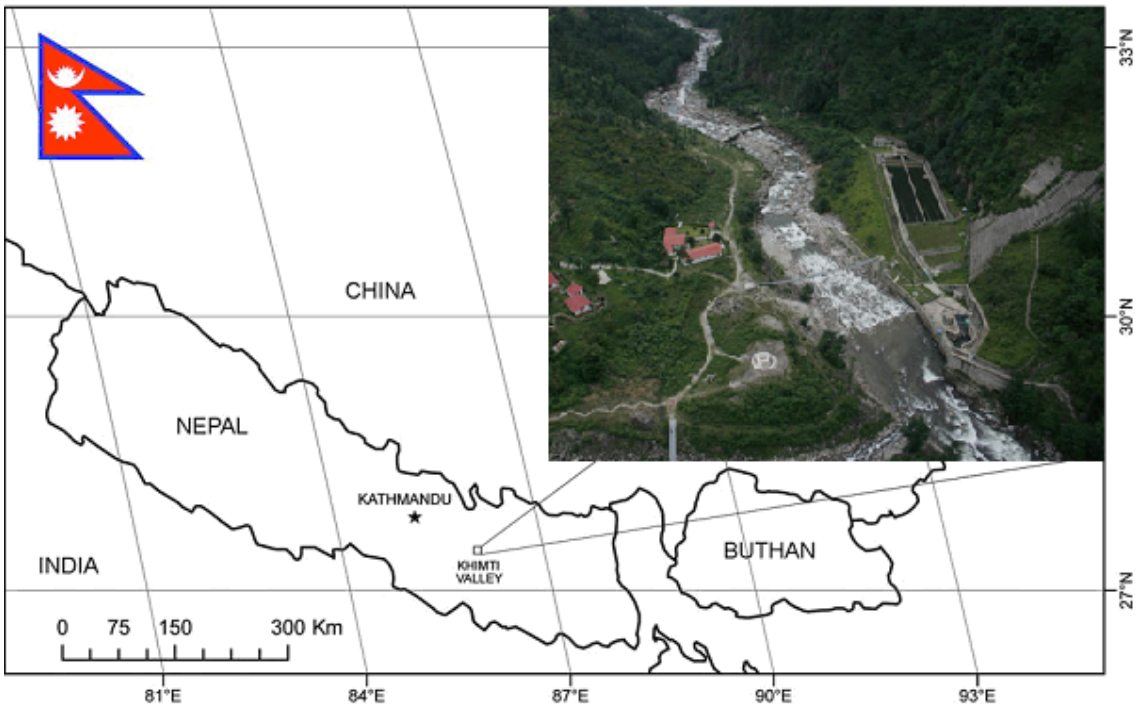
Based on this, the following questions to be adressed in this investigation were:

- How does plasma melatonin levels in copper mahseer change during natural photoperiod outdoor in Nepal?
- How does extended dark period influence on blood plasma melatonin levels in copper mahseer kept outdoor in Nepal?
- What is melatonin half-life in copper mahseer?
- Is there a relation between blood cortisol and melatonin levels in copper mahseer?

Materials and methods

Study site

Nepal has a land area of 147 181 km^2, and is divided into three geographic areas from south to north: The Terai plain, with an elevation ranging from 60-300 meters, the mid hills that range from 300-2000 meters, and the Himalayas that occurs at an elevation above 2000 meter. This leads to a considerable variation in the climate, and it varies from cool summers and severe winters in the north to subtropical summers and mild winters in the south (CIA 2011). Khimti Valley is located in the eastern mid-hills, about 100 km east of Kathmandu (Map 1). Khimti Khola River divides the valley, and forms the boundary between Ramechhap and Dolakha district where the Khimti hydropower project site is located (Map 1, top right; HPL 2010).



Map 1: Location of the study site. Khimti valley is located in the eastern mid-hills east of Khatmandu. The map is modified from Hveding 2008. Top right: The Khimti hydropower project site (HPL 2010).

Experimental animals and conditions

Fingerling (0+) copper mahseer (*Neolissochilus hexagonolepis*) caught in the Khimti Khola River during April 2008 had been fed a pelleted diet (NARC) while kept in two ponds on the hydropower plant premises. The ponds were supplied with water from a tributary from Khimti Khola river, and animals were adapted to environmental temperature fluctuating seasonally between 9 and 19^{o}C (Hveding 2008).

Prior to the experiments the pond water levels were lowered and the fish were captured and collected in one pond, and later transferred to the experimental tanks kept outdoor. During the experiments fish were not fed. The fish were allowed 24 hours of rest before blood sampling started. Environmental light (Fig. 3) were measured using a lux meter (INS DX-200, Digital illumination). Water temperature and oxygen were measured using a hand held optical meter (YSI ProODO).

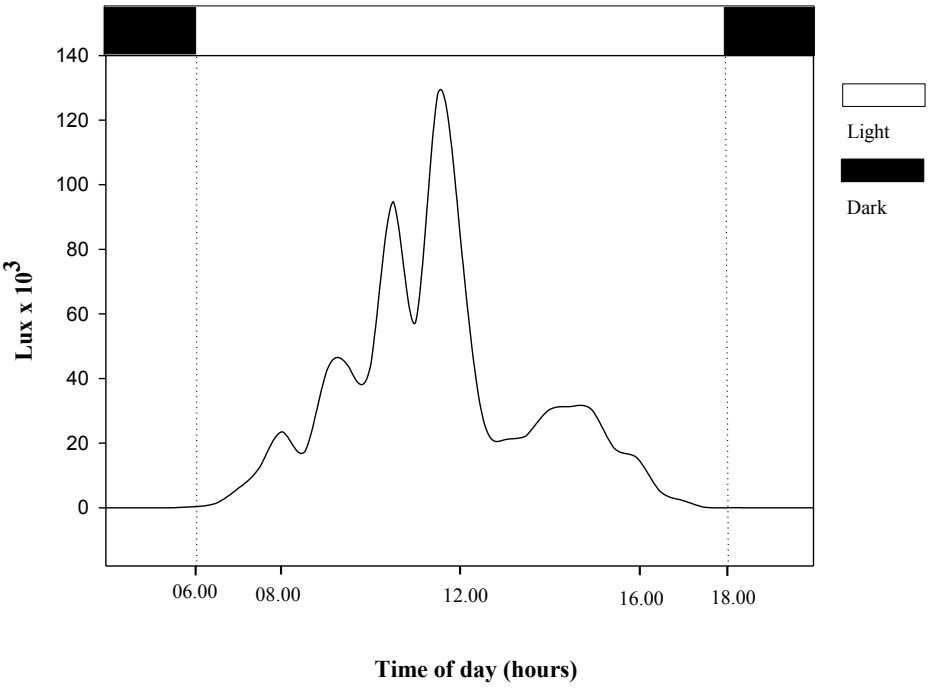


Figure 3: A section of a 24- hour illumination (lux*10^3) period measured (hours) outdoor at the experimental site (October 20 to October 21, 2010)

Experimental design

In October 2010 a setup for the experiment was established in the field. Ten tanks of 500 liter were modified for experimental use (Fig 4). They were painted white to reflect the sunlight, the top was cut off and an inlet and outlet were constructed for each tank. A pipe stock system was constructed, and connected to an inlet from a tributary of Khimti Khola River, which in turn was connected with the line of experimental tanks. The water delivery to each tank was ≥ 4 L/min, sufficient to keep O_2 level ≥ 6mg/L.

Figure 4: Adapting roof water tanks for fish experiment (a) The tanks before modification (b) modified tank for experimental use (c) the line of experimental tanks.

Experiment 1: 24 hour (LD-12: 12) melatonin levels in copper mahseer kept under natural photoperiod (outdoor).

Fish were netted and distributed randomly into 10 tanks. Blood samples of 6 fish were collected at: 12.00, 16.00, 18.00, 20.00, 23.00, 02.00, 04.00, 06.00, 08.00, 10.00 and 12.00 hours. After blood sampling body length (21±3 cm), and weight (84±39 g) were measured. The tank water temperatures ranged from 19-20 °C, while oxygen varied between 6-7 mg/L**.**

Experiment 2: 24 hour (LD-5: 19) melatonin levels in copper mahseer kept under extended dark period (outdoor).

Darkness was introduced at 15.00, three hours prior to natural nighttime, and removed at 10.00, four hours after occurrence of natural daylight. Fish were netted and distributed randomly into 10 tanks. Blood samples of 6 fish were collected at: 12.00, 14.00, 16.00, 18.00, 22.00, 02.00, 06.00, 08.00, 11.00, and 12.00. After blood sampling, body length (21±2cm) and body (85±31 g) were measured. Tank water temperatures ranged from 15-18 °C, while oxygen varied from 7-8 mg/L.

Experiment 3: Half-life ($t_{1/2}$) of melatonin in copper mahseer kept outdoor.

Calculations of half-life of plasma melatonin were based on blood sampling after onset of light in the dark phase. Fish were netted and distributed randomly into one tank. At 21.00 a lamp (2140 lux) was turned on, and single blood samples from 17 fish were taken during 25 minutes. After blood sampling body length (23±2 cm) and weight (103±34 g) were measured. The tank water temperature had a temperature of 16 °C, and an oxygen concentration of 6 mg/L.

Collection of blood samples

The fish were anesthetized using 50mgL^{-1} MS-222 (tricaine-sulphonate, PHARMAQ Ltd.Fordingbride), whereafter blood samples were collected from the caudal vein. The samples were obtained by use of heparinized syringes (Heparin, Leo pharma a/s, 5000IE/a.e. /ml). For blood sampling at nighttime red light was used (Petzl E99 PG, Headlamp). The fish

was killed by a blow to the head immediately after sampling. All fish were body measured (length, weight) and checked for gender and stage of maturity.

The blood samples were kept under cover on ice water until centrifugation (4500 rpm for 5 minutes, Heraeus Biofuge ® pic). The samples were stored in a freezer (-20 °C) at site, and later transferred to Norway (in icebox) for analysing, which were performed in the RIA-lab at Institute of Biology, Realfagsbygget, NTNU, Trondheim.

Hormone measurements

Principle

Blood plasma melatonin and cortisol concentrations were determined by the use of radioimmunoassay. The basic principle of radioimmunoassay is summarized in the following reaction:

$$Ag + Ag^* + Ab \leftrightarrow Ag\text{-}Ab + Ag^*\text{-}Ab + Ag + Ag^* \quad (1)$$

It is competition between a radioactive (Ag*) and a non-radioactive antigen (Ag), with the same binding affinity for the binding site of a specific antibody (Ab). The antibody is normally calculated to bind 50% of the "hot" ligand thus the two antigens will compete for antibody binding sites. As a result of the competition the ratio of Ab bound to Ag* is diminished as the concentration of Ag increases. To quantify the concentration of unknown samples the observed inhibition is compared with a reference curve with known standards (Berson and Yalow 1968)

Melatonin

The melatonin concentration was quantified by use of a commercial kit (Melatonin Research RIA, Labor Diagnostica Nord GmbH & Co. KG) .The assay follows the basic principle for radioimmunoassay. When the system is in equilibrium the Ag*-Ab is precipitated with a second antibody in polyethylene glycol. Quantification of the unknown samples (50 μl) is achieved by comparing their activity against a response curve of known standards (0,12,40,120,400,1200,4000 pg/mL). The precipitate was counted in a gamma counter (Packard COBRA ^{TM}II Auto gamma) for one minute.

A problem in the direct determination of melatonin is the difference in composition between the sample and the sample matrix (Welp *et al.* 2010). This was reduced by the use of a matrix-specific calibrator (equalizing reagent). The reagent was produced by the use of plasma from copper mahseer.

For validation of the RIA melatonin assay sensitivity, precision and accuracy (parallelism) were defined. The lower detection limit (Sensitivity) was determined as the minimum concentration that did not overlap with zero concentration (-2SD). Plasma samples below detection limit were set to 20,89 pg/mL. Precision was defined by calculating the coefficient of variation from two control samples of low (L) and high (H) concentration.

Inter- assay CV was calculated to 16 % (L) and 20 % (H). Intra- assay CV was calculated to 21 % (L) and 12 % (H). Parallelism was tested by serial dilutions (100 µl plasma: 100 µl equalizing reagent) of pooled plasma taken at nighttime (Fig 5).

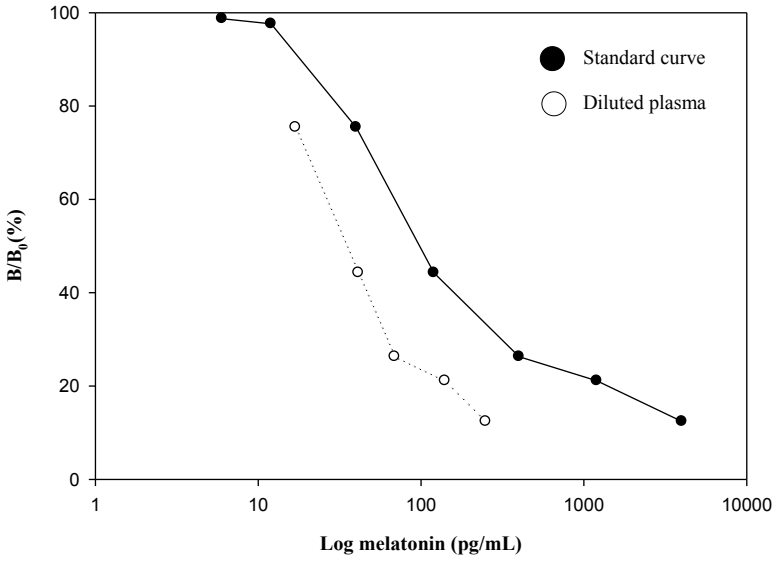


Figure 5: Parallelism between serial diluted copper mahseer (*Neolissochilus hexagonolepis)* plasma and the standard curve.

Conversion factor:
Melatonin concentration given in pmol/L in other published papers was converted to pg/mL.

pg/mL x4.3 = pmol/L (2)

Cortisol

The cortisol concentration was determined by the use of a Radio Immunoassay kit (Coat-A-Count, Siemens). 125-I labeled cortisol competes with cortisol in the sample for antibody sites. The antibody is immobilized to the wall of the tubes, so by decanting the supernatant the antibody-bound fraction is isolated. A gamma counter (Packard COBRA ^{TM}II Auto gamma) calculated the cortisol levels of the samples by comparing their activity against a response curve of known standards. Tests run of the assay revealed high cortisol concentrations. Consequently, the plasma samples were diluted (1:2) so the concentrations would fall within the standard curve (Fig 6)

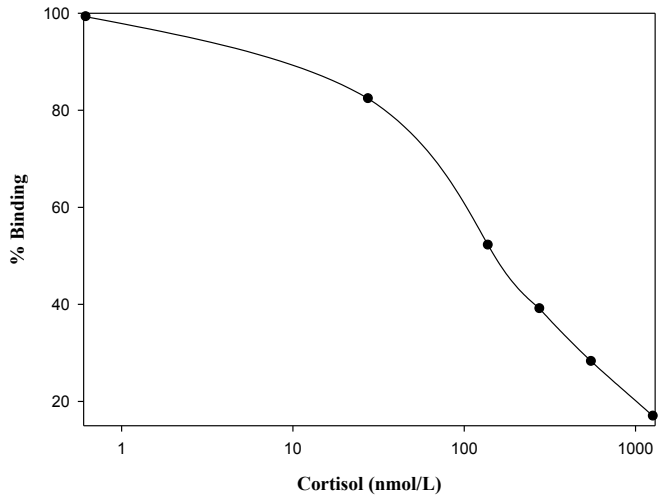


Figure 6: Standard curve for cortisol.

To evaluate the quality of the assay, precision and sensitivity were defined. Lower detection limit was calculated to 19,48 nM. Precision was tested with the use of control samples with low (L) and high (H) concentration. Intra- assay CV was calculated to 38,4 % (L), and 19,2 % (H).

Conversion factor:

Cortisol concentration given in ng/mL or μg/dl in other published papers was converted to nM.

ng/mL /10 = μg/dl (3)

μg/dl =nM/27,95 (4)

Statistics and graphics

For graphic presentation Sigma Plot 11.0 for Microsoft Windows was used. The values are presented as mean ± standard deviation (SD). SPSS 18.0 for Mac OS X was used for the statistical analysis.

Because the data was non- normalized, the non- parametric test Mann-Whitney U-test was used for statistical analysis for comparison between melatonin levels and cortisol levels at the different timepoints tested. The non - parametric correlation (r) test Spearmans rho was used to test for correlation between melatonin and cortisol. The significance level was set to $p < 0,05$.

Results

Experiment 1: Melatonin profile under natural photoperiod

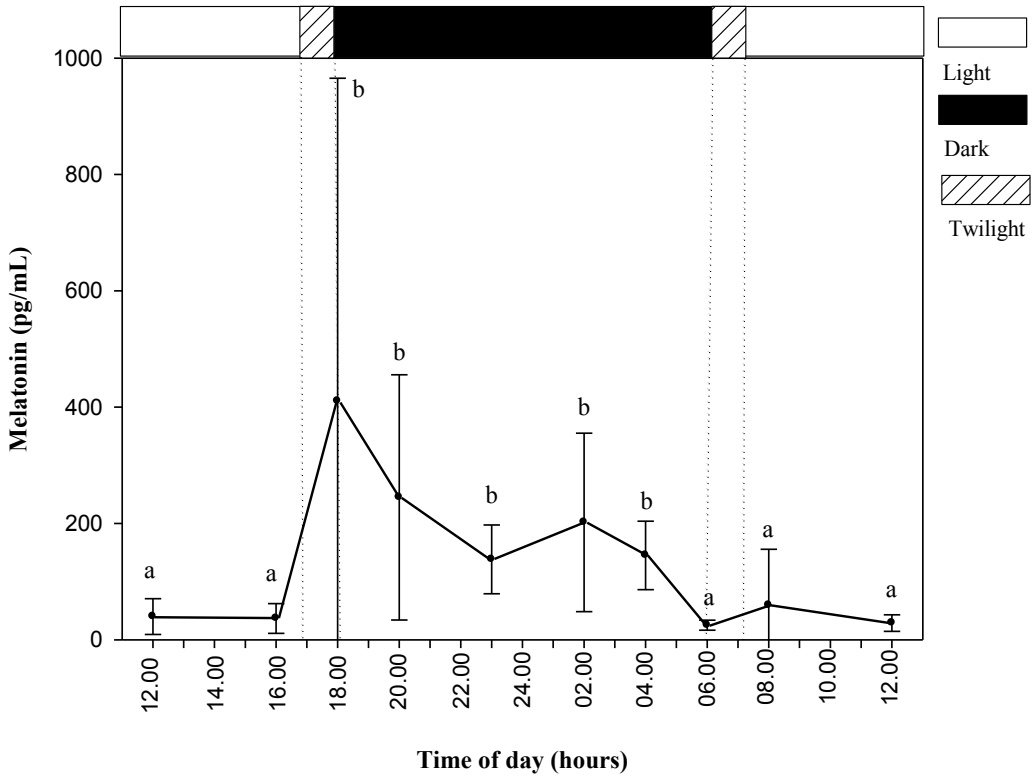


Figure 7: Plasma melatonin levels in copper mahseer (*Neolissochilus hexagonolepis*) kept in tanks out door under natural photoperiod (12L:12D). The values are given as mean ± SD (N = 6). Letters indicate statistically significant difference ($p < 0,05$).

Figure 7 shows melatonin levels in fish kept under natural photoperiod (12L:12D). A clear pattern was observed with low levels (< 60 pg/mL and < 30 pg/mL, respectively) during day, and higher melatonin levels (> 100 pg/mL and > 300 pg/mL, respectively) during night. The low melatonin levels during day was followed by a peak in the beginnig of the dark phase (18.00). Therafter the hormon levels gradually decreases to low levels at the beginning og light phase (06.00). All plasma levels measured during the dark phase were significant higher ($p < 0,05$) than the levels measured during light phase (indicated by letters).

Experiment 2: Melatonin profile under extended dark period

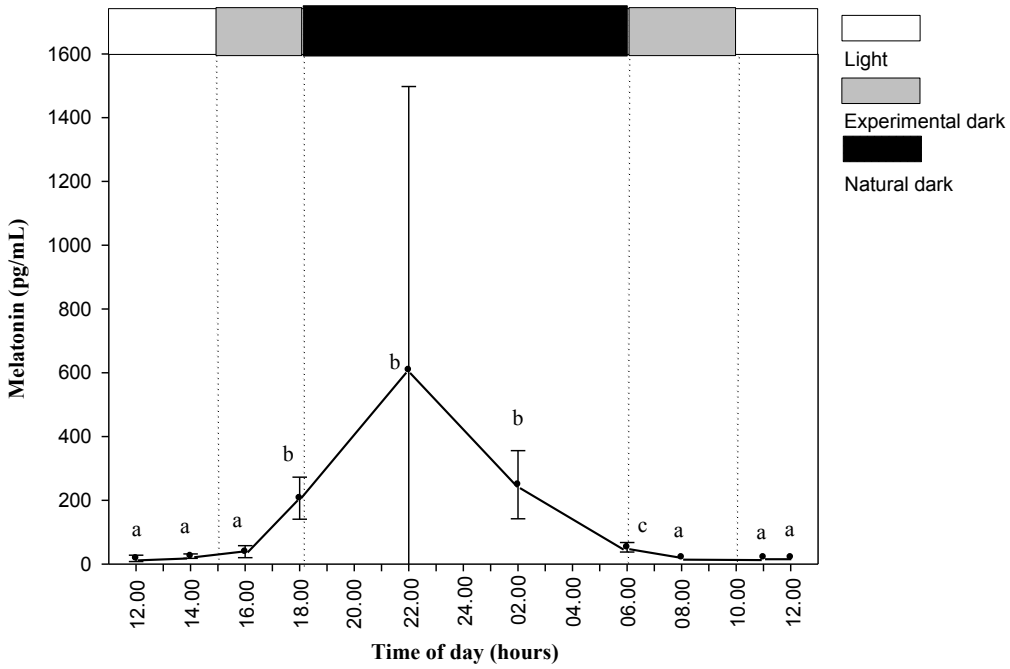


Figure 8: Plasma melatonin levels in copper mahseer (*Neolissochilus hexagonolepis*) kept outdoor under extended dark period (5L: 19D). The values are given as mean ± SD (N=6). Letters indicate statistically significant difference ($p < 0,05$).

Figure 8 shows melatonin levels in fish kept under extended dark period (5L:19D). The melatonin levels show low levels (< 60 pg/mL and < 30 pg/mL, respectively) during the light and experimental dark phase. The hormone levels showed a significant increase at the beginning of natural dark period, at 18.00 (> 200 pg/mL). A peak was seen at 22.00 (> 600 pg/mL), seven hours after dark were initiated, after which the concentrations decreased and reach low levels at the beginning of natural photoperiod. A big variation is seen between individuals measured at 22.00. Plasma levels measured under natural dark period were significant higher (< 0,05) from the other sampling points (indicated by letters).

Experiment 3: Melatonin Half-life ($t_{1/2}$)

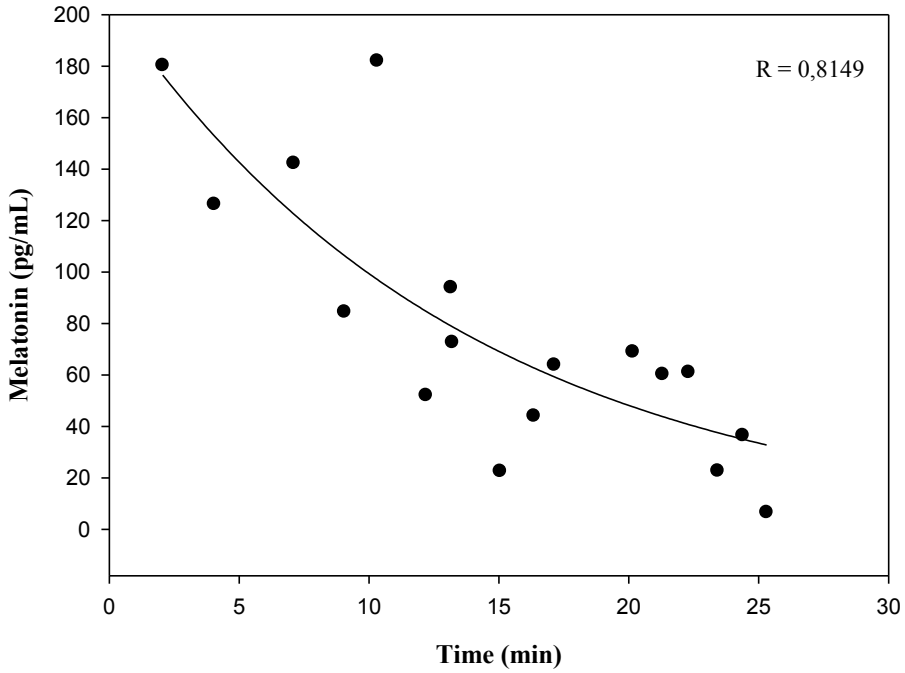


Figure 9: Plasma melatonin levels in copper mahseer (*Neolissochilus hexagonolepis*) after onset of light (at 21.00) during a natural dark period (kept outdoor). Function for the line: $204{,}95 * e^{-0{,}0724*x}$

Figure 9 shows melatonin levels in fish exposed to bright light (at 21.00) during natural dark period. A two parametric exponential function (5) was fitted to the data. Half-life for melatonin was calculated to be 10 minutes, respectively. The calculation was based on the function fitted to the line (6).

$$y = a*e^{-b*x} \quad (5)$$

$$y = 204{,}95 * e^{-0{,}0724*x} \quad (6)$$

Variations in plasma cortisol levels under photoperiod experiments

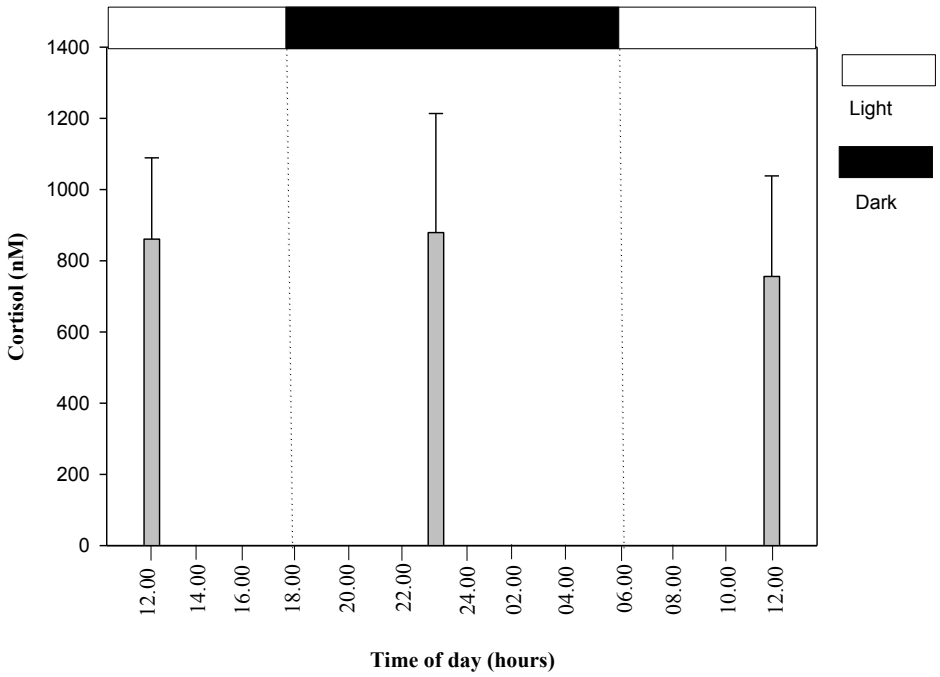


Figure 10: Plasma cortisol levels in copper mahseer (*Neolissochilus hexagonolepis*) kept outdoor under natural photoperiod (12L:12 D). Values are given as mean ± SD (N = 6). No statistical difference were seen ($p > 0,05$).

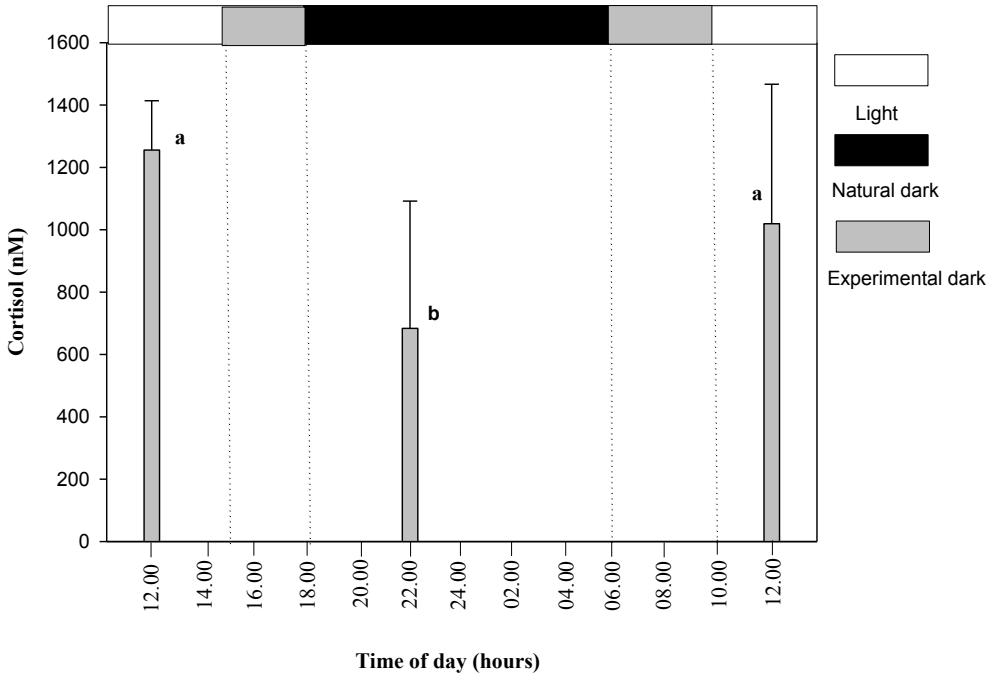


Figure 11: Plasma cortisol levels in copper mahseer (*Neolissochilus hexagonolepis*) kept outdoor under extended dark period (5L: 19D). Values are given as mean ± SD (N = 6). Letters indicate statistically significant difference ($p < 0,05$)

Figure 10 shows cortisol plasma levels in fish kept under natural photoperiod. According to the three timeponts tested the cortisol levels were relatively high both before, during and after darkness (861 ± 208 nM, 879 ± 305 nM and 756 ± 258 nM, respectively). No significant difference was found between the timepoints ($p > 0,05$).

Figure 11 shows cortisol levels in fish exposed to extended dark period. Plasma cortisol levels in fish at 22.00 (683 ± 373 nM) appear to be significant lower ($p < 0,05$) from fish tested at 12.00 (1256 ± 144 nM) at the beginning of the experiment, and 12.00 (1019 ± 408 nM) at the end of the experiment.

Correlation between melatonin and cortisol

Table 1: The relationship between plasma melatonin and cortisol consentration in copper mahseer (*Neolissochilus hexagonolepis)* sampled at three timepoints (12.00, 23.00,12.00) in a 24 hour period. Spearmans rho (r) and p values are displayed. D=dark L=light

Time	Melatonin (pg/mL)	Cortisol (nM)	r	p
12.00 (L)	40 ± 28	860 ± 208	-0,30	0,57
23.00 (D)	138 ± 58	879 ± 305	0,20	0,70
12.00 (L)	29 ± 13	756 ± 258	0,08	0,88

Table 2: The relationship between plasma melatonin and cortisol consentration in copper mahseer (*Neolissochilus hexagonolepis*) exposed to extended dark period sampled at two different time points (12.00 and 22.00) in a 24 hour period. Spearmans rho (r) and p values are displayed. D=dark L=light

Time	Melatonin (pg/mL)	Cortisol (nM)	r	p
12.00 (L)	18 ± 10	1256 ± 144	0,36	0,48
22.00 (D)	608 ± 889	683 ± 373	0,15	0,78

There was not found any statistically significant correlation ($p >> 0,05$) between melatonin and cortisol for fish held under natural photoperiod or extended dark period. For extended dark period melatonin samples at 12.00 (at the end of the experiment) was set to detection limit, and could not be tested for correlation.

Discussion

Seasonal changes in photoperiod has proven to be the most important single factor driving the sexual periodicity in mid- and high-latitude fish (Bromage *et al.* 2001). In temperate areas, the aquaculture industry has managed to develop control of fish reproductive physiology by manipulating photoperiod (Bromage *et al.* 2001; Hansen *et al.* 2001). Little is known about such biorhythm control in low-latitude species, but a few recent studies have shown that tropical fish also can respond to photoperiodic changes by the use of the pineal system (Maitra *et al.* 2005; Holtan 2011). Accordingly, it is possible that indoor photoregimes also can time reproduction in low latitude fish species, making it possible to produce fish fries all year round. As a possible step towards such control this thesis has studied the day-night melatonin pattern in copper mahseer (*Neolissochilus hexagonolepis*), an endemic carp species within Nepal.

Natural Photoperiod

Fish kept under natural photoperiod in the present study showed a rapid rise in plasma melatonin levels at the beginning of the dark period, after which the values gradually drop and reach low daytime levels at the beginning of light phase (Fig. 7). The low levels at day (< 60 pg/mL and < 30 pg/mL) and high levels (>100 pg/mL and >300 pg/mL) at night is in accordance with findings in other vertebrates (Paul *et al.* 2008). All samples taken at night showed a significant difference from samples taken at daytime.

There has not been any documented melatonin measurements for copper mahseer, but a few studies on low latitude cyprinides have been done. A former study on common carp (*Cyprinus carpio*) found similar results as presented in this study, where plasma melatonin showed a rapid increase at light off, and decreased through the darkphase until it returned to the low daytime levels at the beginning of light period (Kezuka *et al.* 1988). On the contrary, a study on common carp from Nepal with similar methodology as present study, plasma melatonin levels increased to a moderate plateau until a peak in the second half of the darkphase (Holtan 2011). Similar results was also shown in a *in vitro* study of the melatonin release from the pineal gland of common carp (Bolliet *et al.* 1996). Considering the somewhat inconsistant results on melatonin patterns in common carp, further *in vivo* studies should be conducted on copper mahseer to find out more about the melatonin profile for this species.

The plasma melatonin consentration showed a gradual drop in melatonin level from 18.00 (410 pg/mL), and decreased throughtout the dark period (< 300 pg/mL), and reached low levels at the beginning of the light period (Fig. 7). Resembling results have been seen in some tropical species, e.g Nile tilapia (*Oreocrhomis niloticus*), African catfish (*Clarias gariepinus*) (Martinez-Chavez *et al.* 2008) and gold fish (*Carassius aurtaus*) (Iigo *et al.* 1994). This might indicate a type B profile, and the decrease in melatonin levels seen during the dark in the present study might indicate a clock-controlled system that is capable of anticipating the next photoperiod.

There has been argued that strong endogenous rhythms (melatonin) in low-latitude species may reflect an adaption to photic environment they inhabit, as compared to strong seasonal variations experienced by temperate species (Martinez- Chavez *et al.* 2008). It might be that the circadian clockwork require a minimal time of integration of environmental signals to ultimately entrain the melatonin synthesis of the animal. The present study was conducted outdoor in Nepal where the photoperiod is almost constant with 12 light and 12 hour dark all year round. The fish were kept under extended dark period 24 hours before blood sampling startet. If the melatonin rhythm is a reflection of the photic environment they inhabit, and the circadian clock needs time to begin the melatonin sythesis, this might explain why the plasma melatonin profile shown under extended dark is similar to the profile under natural photoperiod with an increase and decrease at the time for natural dark.

Extended dark period

In order to further investigate the the plasma melatonin levels of copper mahseer, fish where exposed to extended dark period (Fig. 8). The melatonin pattern observed here was similar to that obtained in fish from natural photoperiod, with low levels (< 60 pg/mL and < 30 pg/mL) during natural light and experimental dark and high levels (> 200 pg/mL) during natural dark. At 18.00 (the time for natural dark) a significant increase was seen in melatonin levels even though dark was initiated three hours before. Furthermore, the melatonin level then decreased and reached low levels at 06.00 (the end of natural dark period), even though dark remained for four hours. The significant increase found at 18.00 and the significant decrease seen at 06.00 may indicate that the pineal have not been influenced by the new light regime,

indicating a pineal clock system in this species wich may be capable of self-sustaing the melatonin rhythm. The low melatonin levels seen during experimental dark can not be explained by possible light stimuli on the pineal gland, as the experimental tanks were covered. By comparing to a similar study as present on common carp the melatonin level showed a peak in the late dark phase (02.00) both under simulated extended dark period and simulated natural photoperiod, indicating a clock system in this species. However, when comapring the melatonin profile for copper mahseer and common carp differences is seen. Copper mahseer show a peak in melatonin levels in the beginning of the dark phase, while common carp shows a peak in the late dark phase. Higher melatonin levels is also seen in copper mahseer during dark compared to common carp (< 300 pg/mL). However it must be noted that the present study on copper mahseer was performed outside, unlike the study on common carp which was conducted inside with artificial light. Therfore, further studies on copper mahseer should investigate the melatonin profile inside under artifical light and see if this might have an affect on the melatonin profile.

Many studies suggests that intrapineal oscillators exists in fish (Bolliet *et al.* 1996; Okimoto and Stetson 1999), with the exception of salmonides (Iigo *et al.* 2007). Salmonids show a passive response to the light dark signal wich results in a suppression of pineal melatonin during constant light and high melatonin levels during constant dark (Randall *et al.* 1995; Masuda *et al.* 2003; Strand *et al.* 2008). Fish with a intra-pineal clock show a melatonin rhythm under a constant dark regime, and is found to free run with its own endogenous periodicity (Bolliet, et al. 1996). A shift in the melatonin peak was observed; from 18.00 in the natural photoperiod (Fig. 7) to 22.00 during the extended dark period (Fig. 8). The shifted melatonin peak seen during extended dark might indicate the beginning of a free run of the melatonin pattern, further indicating the presens of an intra-pineal clock. In further studies it might be interesting to expose copper mahseer to continous dark over a longer period to investigate if the pineal gland will self sustain the melatonin rhythm in the absense of the light-dark signal.

The observed individual variation in plasma melatonin consentration is especially profound during dark and may be related to individual differences in melatonin production. The melatonin synthesis may not be synchronised in between individuals, and at blood sampling the melatonin concentration might have reached its peak in some of the fish tested, while in others the peak is not reached at the time of blood sampling. Individual variations in plasma

melatonin during dark have also been found in other species such as common carp and Atlantic salmon (*Salmo salar*) (Randall *et al.* 1995; Holtan 2011).

Melatonin half-life

A rise in plasma melatonin levels is most likely the result of the production and release from the pineal, and the decrease in plasma levels is most likely a result of degradation in the liver and melatonin attached to its receptors. In this study half-life ($t_{1/2}$) of melatonin was calculated to be 10 minutes (Fig 9). This is in accordance with a former study conducted on Atlantic salmon, where $t_{1/2}$ was calculated to be 10 minutes (Skjulsvik 2005). Further studies should investigate if the liver metabolism will change during season and if this will have an affect on the plasma melatonin level in copper mahseer.

Possible influence on plasma melatonin

Temperature

As the relationship between melatonin and light has been examined carefully, other factors that might influence the melatonin production have come into focus. Temperature is known to show seasonal variations. *In vitro* studies have shown that temperature influence melatonin levels through the regulation of AANAT kinetic in trout (Benyassi *et al.* 2000) pike (Falcón et al., 1994) and carp (Seth & Maitra, 2010). Night time melatonin has shown to be lower at reduced temperature and elevated at increased temperature (Bromage *et al.* 2001). However, research have found that maximum AANAT activity reflects the preferred temperature for the fish and their adaption to the environment (trout, *Oncorhyncus mykiss*: 12^{o}C, pike, *Esox lucius*: 20 ^{o}C, seabream, *Sparus aurata*: 27 ^{o}C, zebrafish, *Danio rerio*: 30 ^{o}C) (Falcón *et al.* 2010).

In this study the experimental fish were adapted to the natural water temperature from the river fluctuating between 15- 20 ^{o}C. In the wild, it inhabit rivers and lakes of mid-hills and mountains, and can tolerate a wide range of water temperature (9 and 19 ^{o}C) (Hveding 2008). During the experiments the temperature were relatively constant, and it is belived that the temperature did not have an affect on the melatonin levels in this study. However, considering

the large variations in tempearture at this location, further studies should investigate if temperature have an effect on the plasma melatonin levels in copper mahseer.

Reproduction

The pineal organ translates information about the photoperiod into melatonin, and one of the main functions for this message is the control of reproduction (Ekström and Meissl 1997; Falcón *et al.* 2007). This might allso be the case for low latitude species (Bhattacharaya *et al.* 2005; Dey *et al.* 2005).

A recent study on common carp found changes in the concentration of melatonin during a 24 hour period in each of the reproductive phases (preparatory, pre-spawning, spawning, and post- spawning phases), however the patterns were inconsistent. The circulating level reached its peak during mid-night in each of the phases, with an exeption in the preparatory phase wich reached its peak in the late dark phase. It was also observed that melatonin reached its highest consentration during post spawning phase, and the lowest levels was seen during the spawning phase (Maitra *et al.* 2005). Furthermore, a maximum of AANAT activity during post-spawning phase have been reported to coincide with the peak of the melatonin concentration (Seth and Maitra 2010). In the present study, a few fish was found to vary in the degree of maturation. This might have influenced the plasma melatonin pattern, and could provide a possible explanation for the observed individual variations in melatonin levels. To investigate this, further studies on cooper mahseer should look at the plasma melatonin pattern during the different reproductive phases and see if any differences in plasma melatonin levels is detected.

Melatonin and stress

Stress has been reported to increase the plasma cortisol level in fish, and this hormone is often used as an indicator to evaluate the health and stress of the animal (Martinez- Porchas *et al.* 2009). If a stressor persist and the cortisol levels remain high it will lead to maladaptive consequences (Ashley 2006), emphasizing the importance of monitoring stress levels in research animals.

Cortisol levels detected for fish in this study were high in both experiments (Fig 10,11) with mean cortisol levels ranging between 680-1250 nM. In common carp, a resting level for cortisol has been found to be about 80 nM and raised to about 800 nM one hour after stress exposure (Ruane *et al.* 2001). Another study on commen carp found resting plasma cortisol levels of 7 nM and when exposed to stress the levels increased to 390 nM (Pottinger 2010). In a study on Atlantic salmon the resting level was found to be 23 nM and reached a maximum level of 535 nM (Einarsdóttir and Nilssen 1996). Similar studies as the present study have also detected high cortisol levels for carp species in Nepal. Holtan (2011) measured cortisol levels for common carp between 850-890 nM. A study on rohu (*Labeo rohita*) revealed cortisol levels ranging between 376-1094 nM (Bunes 2010). However, the high cortisol levels detected in this study compared to the resting levels found in other studies indicates that copper mahseer was stressed during the experiments.

In the present study the blood sampling did not take longer than 5-10 minutes, indicating that this is not the reason for the high cortisol levels, and that the stress response started before the blood sampling. One explanation for the high cortisol levels might be the transportation of the fish into the experimental tanks. Although the fish was allowed 24 hours of rest, this may not have been sufficient to overcome the stress induced from transportation and handling.

Several studies have found a relationship between melatonin and cortisol levels in fish. A recent study have shown that cortisol lowers melatonin synthesis in pineal organ of tilapia (*Oreochromis mossambiques*) (Nikado *et al.* 2010). Recently a study showed that glucocorticoid receptors (GR) are expressed in trout pineal, and that glucocorticoids may inhibit AANAT activity (Benyassi *et al.* 2001). In a study on rainbow trout there was not detected any relationship between melatonin and cortisol in unstressed fish, but a possitive correlation was seen in stressed fish during dark (Larson *et al.* 2004). In this study the cortisol levels remained high throughout the experiments, but there was still observed a fluctuations in the melatonin levels. There was also not found any significant correlation between these two hormones during day time or during night. This indicates that cortisol did not have an affect on the melatonin levels in copper mahseer in this study.

Extra-pineal melatonin production

Pinealectomy studies have shown that the pineal gland is the main source of melatonin in the blood at nigh (Iigo *et al.* 1995; Mayer 2000). However, extrapineal sources of melatonin have been reported (Lerner *et al.* 1958; Cahill and Besharse 1995), and it is important to know the mechanism for these production sites when measuring plasma melatonin levels.

The GIT is found to be the most abundant extra-pineal source of melatonin (Bubenik 2008). Melatonin has been found in the stomach, proximal gut, and distal gut in some species of fish (rainbow trout, carp) (Bubenik and Pang 1997). An increase of GIT melatonin seems to be related to food intake, but it has also been found to increase with starvation (Bubenik *et al.* 1992; Bubenik 2002).

If the high melatonin concentration detected in the GIT is released into the circulation it could destroy the calendar function of the pineal (Bubenik 2002). However, in sea bass (*Dicentrarchus labrax*) plasma melatonin levels were found to change relatively little with feeding (Herrero *et al.* 2006). In trout bile there has also been found a 10 fold the values of plasma melatonin during daytime (Jose *et al.* 2009). The reason for this high levels are not known, but a possible explenation could be that melatonin from GIT goes to the liver, where melatonin is degraded, and then through the bile back to intestines. And this might prevent the GIT melatonin from destroing the pineal time- keeping system. The fish were not fed during the experiment, and by this the possible influence from GIT melatonin is thought to be avoided.

Retina is also found capable of melatonin production, and the production takes place in the same way as in the pineal (Falcón *et al.* 2003). In most vertebrates so far investigated retinal melatonin is produced in parallel with that of the pineal gland (Falcón and Collin 1991; Iigo *et al.* 1997). However in sea bass melatonin has showed a peak during daytime (IIgo *et al.* 1997; Garcia- Allegue *et al.* 2001). An atypical AANAT pattern is also found in trout retina, where AANAT is active in the light period and low during dark (Besseau *et al.* 2006). Previous findings of deacetylase activity indicates that melatonin produced in retina is not secreted into the blood, and might have local functions (Grace *et al.* 1991).

Furthermore, low daytime levels indicates that no extrapineal melatonin contributed to the plasma melatonin consentrations in the present study.

Conclusion

- Blood plasma melatonin levels in copper mahseer show a clear pattern, with high levels during night and low levels during day.
- Blood plasma melatonin during extended dark period exhibit a similar melatonin profile as under natural photoperiod with higher levels during natural dark, but a shift in the melatonin peak is observed later in the dark phase. This might indicate an intra-pineal oscillator.
- Melatonin half- life was estimated to 10 minutes.
- It does not seem to be a relation between high cortisol levels and variations in melatonin levels.

Perspectives

As highly emphasised in the introduction food security is not something all people experience. The aquaculture sector is pointed out to have a big advantage in the fight against hunger, and Nepal with its many rivers and fish diversity has a potential for developing the aquaculture. The result presented in this thesis can contribute to the understanding of pineal melatonin, and this hormones role for reproduction in low latitude species.

In further studies it might be interesting to expose copper mahseer to continous dark over a longer period to investigate if the meltonin rhythm will self sustain in the absense of the light dark signal.

In the future studies on temperature and its effect on plasma melatonin levels should also be investigated, as this can have big consequences for the time- keeping system in fish, especially in the aquaculture industry. One should also investigate if there is a difference trough a 24 hour period and also trough seasons.

Comparative studies between different carp species should also be conducted, to see if melatonin patterns may vary between species. In the future the main goal is to use indoor photoregimes to time reproduction in low latitude fish species making it possible to produce fish fries all year round further studies should also investigate the plasma melatonin pattern for copper mahseer under artificial light.

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