NTNU – Trondheim
Norwegian University of
Science and Technology

Plasma melatonin profiles in mrigal carp *(Cirrhinus mrigala)* kept under natural and manipulated photoperiods

Ingun Næve

Biology

Supervisor: Kjell J. Nilssen, IBI

Norwegian University of Science and Technology
Department of Biology

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Trondheim, May 2012

Ingun Næve

Front page picture: The Fish tail mountain (Machhapuchhre) is holy to the local population in Nepal, and has never been climbed. Photo: Ingun Næve

Abstract

One major challenge in Asian aquaculture is the limited availability of carp fries. In order to establish out of season supply of fries, knowledge about the pineal melatonin clock and calendar function, and its influence on the reproductive system, is needed. Three experiments were carried out to examine pineal melatonin dynamics of mrigal carp (*Cirrhinus mrigala*), under natural outdoor, and manipulated photoperiods. Plasma melatonin profile of the mrigal carp kept under natural outdoor photoperiod reached a peak early in the dark phase, and then showed a tendency of falling plasma melatonin levels until daytime levels were reached at first light. The second experiment intended to induce production of melatonin at different times during the light phase, and gave production of melatonin only in late afternoon. This indicates a refractory period of the pineal gland during daytime, and that production of melatonin might be controlled by a circadian clock. In the third experiment, animals were exposed to continuous darkness. The resulting plasma melatonin profile was in accordance with subjective darkness and light, with a peak late in the dark phase. This further supports the assumption that a circadian clock controls pineal melatonin production and secretion in the mrigal carp.

Sammendrag

Den største flaskehalsen i asiatisk akvakultur er den sesongbegrensede tilgangen på karpeyngel. For å oppnå kontinuerlig tilgang på yngel fra karpene, kreves kunnskap om melatoninsystemets klokke- og kalenderfunksjon, samt hvordan dette påvirker reproduksjon. Det ble utført tre eksperimenter for å undersøke pineal melatoninutskillelse i karpearten mrigal (*Cirrhinus mrigala*) under naturlig utendørs, og manipulerte fotoperioder. Melatoninnivået i blodplasma fra mrigal under naturlig utendørs fotoperiode nådde en topp tidlig i mørkeperioden, og hadde deretter en synkende tendens til lyset kom tilbake og dagtid-verdier ble nådd. Et eksperiment hvor hensikten var å igangsette melatoninproduksjon på dagtid, gav kun produksjon av melatonin sent på ettermiddagen. Dette indikerer at pinealkjertlen har en refraktær periode hvor melatoninproduksjon ikke kan settes i gang, og at produksjonen er kontrollert av en circadiansk klokke. I det tredje eksperimentet ble forsøksdyrene utsatt for kontinuerlig mørke. Resultatet var en melatoninprofil i samsvar med subjektivt lys og mørke, hvor topp-punktet ble nådd sent i mørkeperioden. Dette støtter antakelsen om at en circadiansk klokke kontrollerer produksjon og sekresjon av melatonin fra pinealkjertlen hos mrigal.

Contents

Introduction

The Food and Agricultural Organization (FAO) of the United Nations emphasize aquaculture as being of major importance regarding future food security, employment and gender equality in developmental countries (FAO 2010). It is especially desirable to increase production of herbivorous and omnivorous species, which do not require a large amount of animal protein for growth, compared to carnivorous species (Tacon and Metian 2008, Benfey 2011). One major problem in expanding the aquaculture industry in developmental countries is a limited availability of carp fries (Sarkar *et al*. 2010). The goal is therefore to achieve out of season production of fries through manipulation of the carp reproductive system in low latitude countries. One main research strategy at the Norwegian University of Science and Technology is to be involved in studies related to globalization and climate change. Accordingly, the Sustainable Poverty Reduction in Nepal (SPRN) – project was developed to improve food security in Nepal through aquaculture (SPRN 2008). The present work reports results from studies of mrigal carp (*Cirrhinus mrigala*) response to environmental changes in photoperiod.

Biological rhythms and seasonal change

Behaviours and physiological processes in teleosts, such as feeding, rest and locomotion, shoaling behaviour, oxygen consumption, thermoregulation and skin pigmentation may be influenced and regulated by environmental cues (Ekström and Meissl 1997, Falcón *et al.* 2007, 2010, 2011). Survival of temperate species is dependent on seasonal events and processes (e.g. growth, migration, and reproduction) being performed at the most suitable time of year. Thus, spawning should take place when the environmental conditions ensure maximal survival of the offspring (Falcón *et al*. 2011). Since the maturation process may take several months, the reproductive event must be initiated under very different environmental conditions from those present when the offspring hatch (Bromage *et al.* 2001). Fish must therefore be able to anticipate the coming season in order to initiate appropriate physiological processes in time (Bye 1984, Reiter 1993).

Photoperiod is one of the factors involved in synchronizing daily, seasonal and annual events in temperate species, it is a zeitgeber (Aschoff 1965). Other environmental cues available to fish are changes in temperature (Glasser *et al.* 2004), seasonal rainfall and food availability (Bromage *et al*. 2001, Sarkar *et al*. 2010, Migaud *et al.* 2010, Bye 1984). However, photoperiod and temperature are assumed to be more important for temperate and high

latitude species, and photoperiod represents the most noise-free signal (Migaud *et al*. 2010). Animals inhabiting equatorial areas are not exposed to the same variations in photoperiod through the year as seen in temperate and high latitude regions. For these species other seasonal cues, such as temperature (García-López *et al.* 2006) and seasonal rainfall (Susilo *et al.* 2009) are assumed to be of greater importance.

Circadian clocks

I addition to environmental influence and regulation of physiological processes, fish also possess endogenous circadian clocks that organise and regulate their physiology (Zhang *et al.* 2011). A molecular circadian clock is a system of transcription factors that feedback on each other, and are transcribed and translated with oscillations of approximately 24 hours (Prasai *et al.* 2011). Pineal production of the hormone melatonin (N-acetyl-5-methoxytryptamine) is in most fish controlled by cellular circadian clocks in each photoreceptor cell. This circadian clock is suggested to consist of two sets of transcription factors, BMAL/CLOCK and PER/CRY, that influence each other in a cyclic manner (Fig. 1). BMAL/CLOCK acts positively on the transcription of PER/CRY, which again give negative feedback on BMAL/CLOCK. Per2 is in addition reset during photic entrainment of the circadian clock, and influence activity of the rate limiting enzyme for melatonin production, arylalkylamine N-acetyltransferase (AANAT), which is a clock controlled gene (Falcón *et al*. 2011, Zhang *et al*. 2011, Prasai *et al*. 2011). Because of this circadian clock, the pineal gland will still produce high levels of melatonin during the subjective night, and low levels of melatonin during the subjective day, when kept under constant dark conditions (Bolliet *et al.* 1996, Cahill 1996, Oliveira *et al.* 2009a). There are however some exceptions to this general rule, such as in salmonids. They do not have a circadian clock controlling production of melatonin in their pineal cells, and instead, melatonin production is controlled by changes in light and dark conditions directly (Max and Menaker 1992, Iigo *et al.* 2007a).

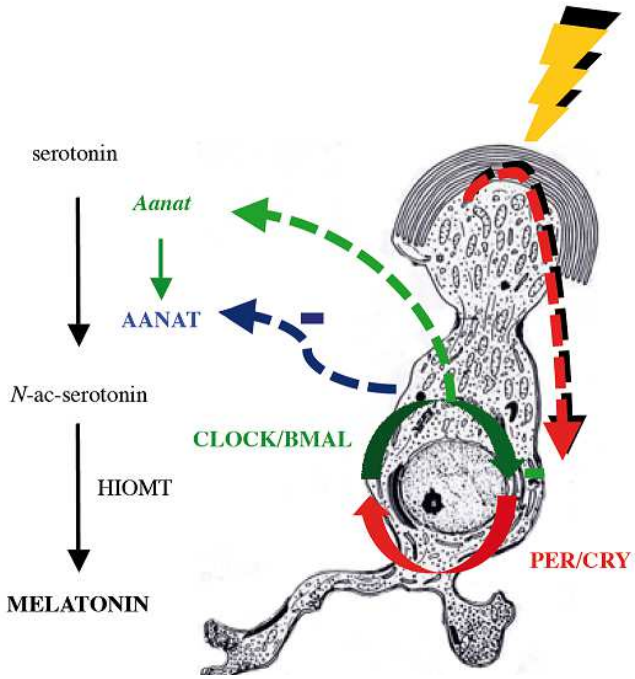

Figure 1: Control of melatonin production in a teleost pineal photoreceptor cell. The transcription factors BMAL/CLOCK and PER/CRY feedback on each other. Per2 regulates Aanat gene expression, and is in addition reset by morning light. For details, refer to the text. AANAT: arylalkylamine N-acetyltransferase; N-ac-serotonin: N-acetylserotonin; HIOMT: hydroxyindole-O-methyltransferase. Modulated from Falcón *et al*. (2011).

The pineal gland and melatonin production

Animals must be capable of registering changes in the light-dark cycle, and to display a physiological response to this. Different vertebrate groups register changes in light-dark conditions in different ways, but all have in common that the output signal from this system is the hormone melatonin (Falcón *et al.* 2009). Pineal melatonin is produced only in darkness, as its production is inhibited by light (Falcón *et al*. 2010). The pineal gland produces the melatonin which is secreted to the blood circulatory system of the animal (Falcón *et al*. 2009). This gland phylogenetically stems from a photoreceptor organ (Simonneaux and Ribelayga 2003), and the major difference in pineal function in higher and lower vertebrates is related to anatomy and location of the gland (Ekström and Meissl 1997).

In teleost fish, the pineal gland is a directly photosensitive gland located above the forebrain (Fig. 2), connected to the brain by the pineal stalk. In some species the lumen of the pineal gland is connected to the third ventricle, and is filled with cerebrospinal fluid (Omura and Oguri 1969, Ekström and Meissl 1997). Light reaches the pineal gland through a pineal window in the skull roof, and hits photoreceptor cells, whose melatonin production is inhibited by light (Ekström and Meissl 1997).

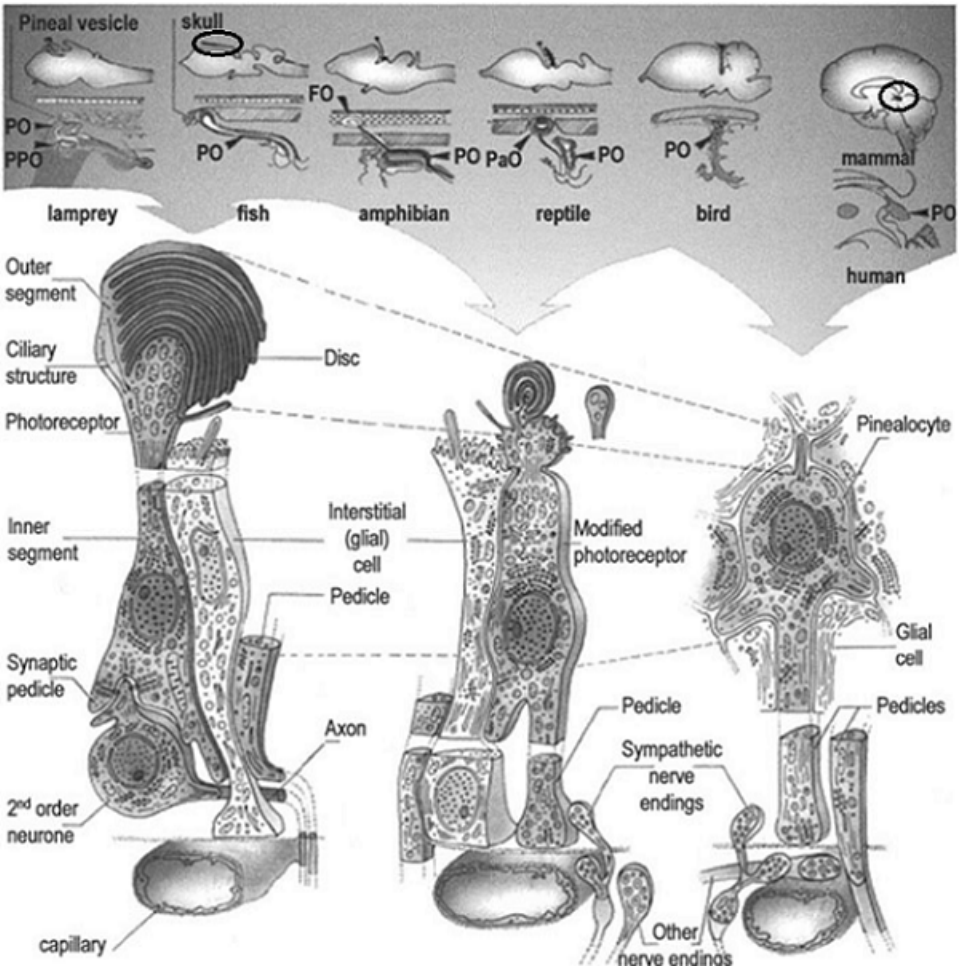

Figure 2: Evolution of the pineal gland in vertebrates. The pineal gland of teleosts (upper left, encircled) is located above the brain and is directly photosensitive. Through evolution there has been a development towards an internally located gland in mammals (upper right, encircled) that is not directly photosensitive. The loss of direct photosensitivity can also be seen in the cells represented in the lower panel, as they lose their photoreceptive part (outer segment). FO: frontal organ; PaO: parietal organ; PO: pineal organ; PPO: parapineal organ. Modified from Falcón *et al*. (2009).

Transmission of light information differs in mammals and teleosts. In mammals, light reaches the eyes of the animal, and the information is conveyed through several nervous pathways to the pineal gland (Fig. 2), where neuroendocrine cells produce melatonin in response to secreted noradrenalin (Simonneaux and Ribelayga 2003, Falcón *et al*. 2010, Walton *et al.* 2011).

Production of melatonin in the pineal gland is a four step process. The starting amino acid, tryptophan, is taken up from the circulation and converted to melatonin through several enzymatic steps (Fig. 3), that takes place in darkness, light, or both (Falcón *et al*. 2010, 2011). The rate limiting enzyme, AANAT, catalyse the penultimate step in this process (Falcón *et al.* 2001, Seth and Maitra 2010). AANAT is responsible for the typical differences in plasma melatonin concentration seen during one light-dark cycle, with high levels during the dark

phase and low levels in light (Falcón *et al*. 2001). A cellular circadian clock in each pineal photoreceptor cell, regulates amount and activity of AANAT (Fig. 1). In addition, melatonin production is inhibited by light (Falcón *et al*. 2009), as this degrades the enzyme (Falcón *et al*. 2001). In most vertebrates there is one AANAT gene, but because of a genome duplication in teleosts they have two; AANAT1 found in the retina, and AANAT2 found in the pineal gland (Coon *et al.* 1999).

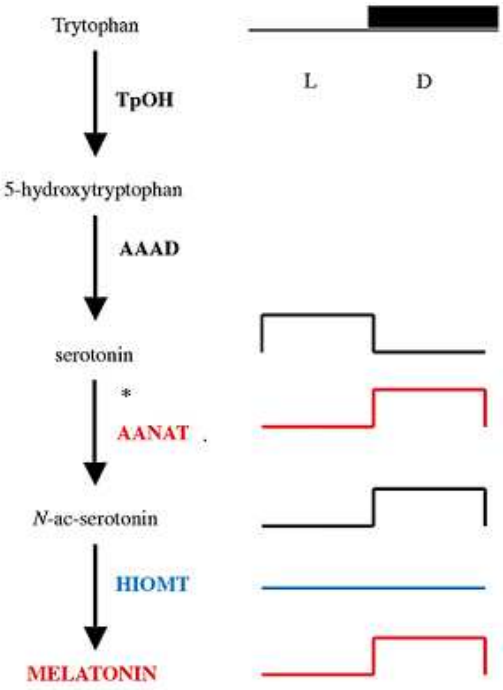

Figure 3: Production of melatonin is a four step process with enzymes that are active either in light, darkness or both. Bars at the top of the figure represent the light conditions. Asterisk marks the light sensitive step. AAAD: aromatic aminoacid decarboxylase; AANAT: arylalkylamin N-acetyltransferase; D: dark; HIOMT: hydroxyindole-O-methyltransferase; L: light; N-ac-serotonin: N-acetylserotonin; TpOH: tryptophane hydroxylase. Modified from Falcón *et al*. (2011).

Melatonin production and plasma levels are high during the dark phase, and inhibited by light, independent of the activity pattern of the animal. For diurnal species such as humans, rest and sleep will be associated with high plasma melatonin levels, while in nocturnal species such as some rodents, melatonin levels will reach a maximum when the animal is most active (Reiter 1993, Challet 2007). There are variations in both amplitude and duration of melatonin secretion, related to relative length of the light and dark periods (Ekström and Meissl 1997, Falcón *et al*. 2007). During summer in temperate regions, the photoperiod is long and the dark period is short, and melatonin secretion during night will be short in duration, but have high amplitude. The opposite is the case during winter, when days are short and the dark period is longer; there is a long duration of the melatonin production, but the amplitude is lower

(García-Allegue *et al.* 2001, Falcón *et al*. 2007). Based on the plasma melatonin profile through the light-dark cycle, and these differences through the year, melatonin levels can be used as basis for a clock and a calendar (Reiter 1993, García-Allegue *et al*. 2001). In this way, the animal can anticipate the conditions it will meet, both during the day and the shifting seasons of the year.

In general, blood plasma melatonin levels in vertebrates during one light-dark cycle are elevated at night, and suppressed during daytime. The specific melatonin secretory pattern may however vary between different vertebrate species. Thus, three different melatonin profiles (Fig. 4) have been documented (Reiter 1993, Falcón *et al*. 2010).

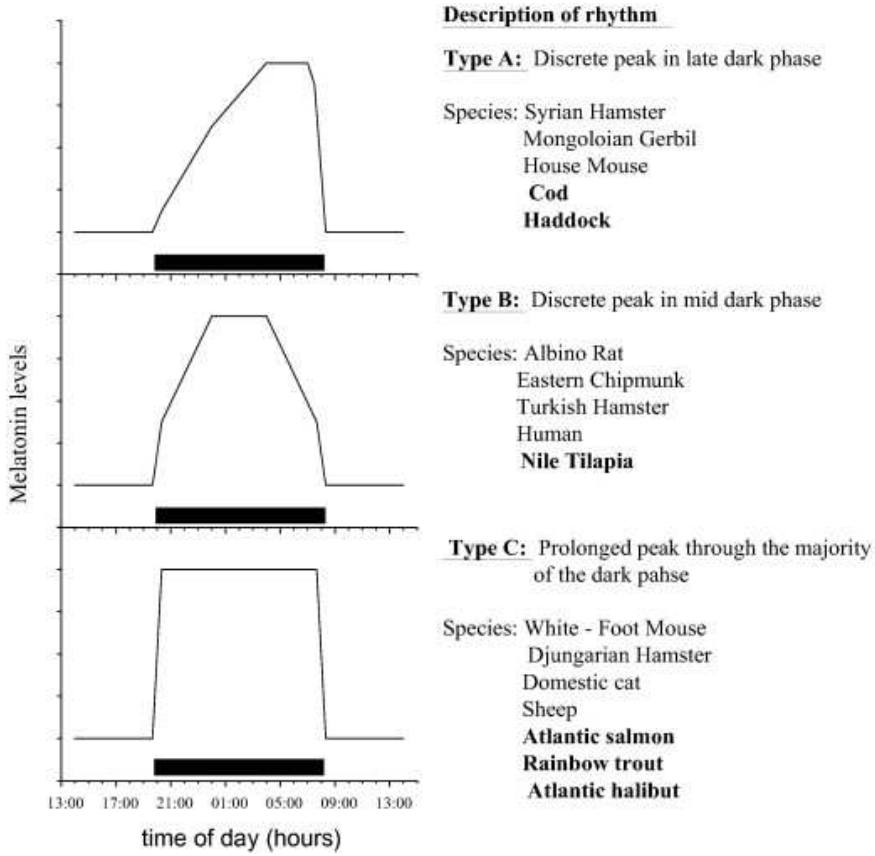

Figure 4: Patterns of melatonin secretion during the dark phase in different animals. There are three variations of plasma melatonin profiles seen in vertebrates; they differ in timing and duration of the peak in plasma melatonin. Bar at the x-axis represents darkness. From Falcón *et al*. (2010).

In type A (Fig. 4), there is a delay from the onset of darkness until peak level of melatonin is reached late in the dark phase, as seen in Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus)*. Type B profile exhibits a peak in plasma melatonin level during the middle part of the dark phase, as seen in Nile tilapia (*Oreochromis niloticus)*. Finally, type C plasma melatonin profile is seen in animals that do not have a circadian clock

controlling pineal melatonin production, and thus secretes melatonin passively in response to darkness. This gives a rapid increase in plasma melatonin immediately at the onset of darkness, and an evenly elevated plasma melatonin level through the dark phase, until light comes back. This profile type is mainly seen in salmonids. (Reiter 1993, Falcón *et al*. 2010).

Salmonids respond directly to changes in the light-dark conditions, and are not controlled by a circadian clock. During a period of extended darkness they will therefore secrete high levels of melatonin independent of the time of day (Gern and Greenhouse 1988, Max and Menaker 1992). In most other fish species the light sensitive pineal production of melatonin is under control of a circadian clock, which gives a rhythm in melatonin secretion based on subjective day and night, even if kept under constant dark conditions (Bolliet *et al*. 1996, Cahill 1996, Oliveira *et al*. 2009a). There are however exceptions to this rule, as seen in European sea bass (*Dicentrarchus labrax*) and Atlantic cod, where both the eyes and the pineal gland are required to sustain a rhythm of plasma melatonin (Bayarri *et al.* 2003). In Nile tilapia and African catfish (*Clarias gariepinus*), the pineal gland appears not to be light sensitive, and the eyes are more central to regulation of pineal melatonin production (Migaud *et al.* 2007).

Extrapineal production of melatonin

I addition to pineal melatonin, there are other sources of melatonin in the body, of which the retina and the gastrointestinal system are the most important (Velarde *et al.* 2010).

Enzymes that take part in the production of melatonin have been identified in several cell types in the retina, such as photoreceptors, and cells in the inner nuclear layer and ganglion cell layer (Iuvone *et al.* 2005, Besseau *et al.* 2006, Falcón *et al*. 2010). The pattern of retinal melatonin production is not as uniform as seen in the pineal gland (Falcón *et al*. 2010). For some species, such as zebra fish (*Danio rerio*) and goldfish (*Carassius auratus*), one has documented retinal melatonin release pattern mimicking that of the pineal gland, with stimulated melatonin production during the dark phase, and depressed production during daytime (Cahill *et al.* 1991, 1996, Iigo *et al.* 1997a). In European sea bass and rainbow trout (*Oncorhynchus mykiss*) there is an increase in retinal melatonin levels during light (Iigo *et al.* 1997b, García-Allegue *et al*. 2001, Besseau *et al*. 2006). In contrast, Japanese eel (*Anguilla japonica*) and Japanese seaperch (*Lateolabrax japonica*), do not exhibit a pattern in retinal melatonin release through the light-dark cycle (Iigo *et al.* 2007b). Melatonin produced in the retina is assumed to have paracrine and autocrine functions within the retina itself, and therefore, does not reach the general circulation or contribute to the blood plasma melatonin

levels (Falcón *et al*. 2010, 2011). Among the putative functions of melatonin in the retina, is a role in dark-adaption, and protection from oxidative stress (Iuvone *et al*. 2005, Falcón *et al*. 2010).

In gastrointestinal (gut) tissues of several vertebrates, levels of melatonin are documented to be 10 – 100 times higher than in plasma (Bubenik and Pang 1997). The enterochromaffin cells in gastrointestinal mucosa produce this gut melatonin (Huether 1993, Bubenik 2002). There has been much debate regarding whether gut melatonin can affect plasma levels of melatonin, because this would disturb the clock function of pineal plasma melatonin considerably. One proposed hypothesis is that gut melatonin undergoes enterohepatic cycling, meaning that melatonin produced in the gut is transported through the portal vein to the liver, where some is degraded, and the rest is recycled to the gallbladder (Herrero *et al.* 2007), before it re-enters the gut through the bile. Thus, gastrointestinal melatonin is suggested to take part in communication between gut and liver, and does not reach the general circulation (Messner *et al.* 2001). Other functions assigned to gut melatonin is protection of the mucosal cells, scavenging of free radicals, and a function as an antioxidant (Messner *et al*. 2001).

Reproduction of teleosts

Puberty is the process that makes animals able to reproduce for the first time. This takes place by activation of the hypothalamus – pituitary – gonad (HPG) axis, controlling maturation and reproduction (Okuzawa 2002). Gonadotropin releasing hormone (GnRH) secreted from the preoptic area (POA) of the hypothalamus is the main control hormone in the HPG-axis (Fig. 5). It stimulates the anterior part of the pituitary gland to produce the gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Kah and Dufour 2011). In male teleosts, FSH and LH stimulates spermatogenesis and production of the testicular hormones, such as testosterone, 11-ketotestosterone, and 11β-hydroxy-androstenedion (Knapp and Carlisle 2011). In female teleosts, growth and development of oocytes is under the control of FSH, which stimulates production of 17β-estradiol, while LH stimulates maturation and spawning by initiating production of 17α,20β-dihydroxy-4-pregnen-3-one, also called maturation inducing hormone (MIH) (Urbatzka *et al.* 2011).

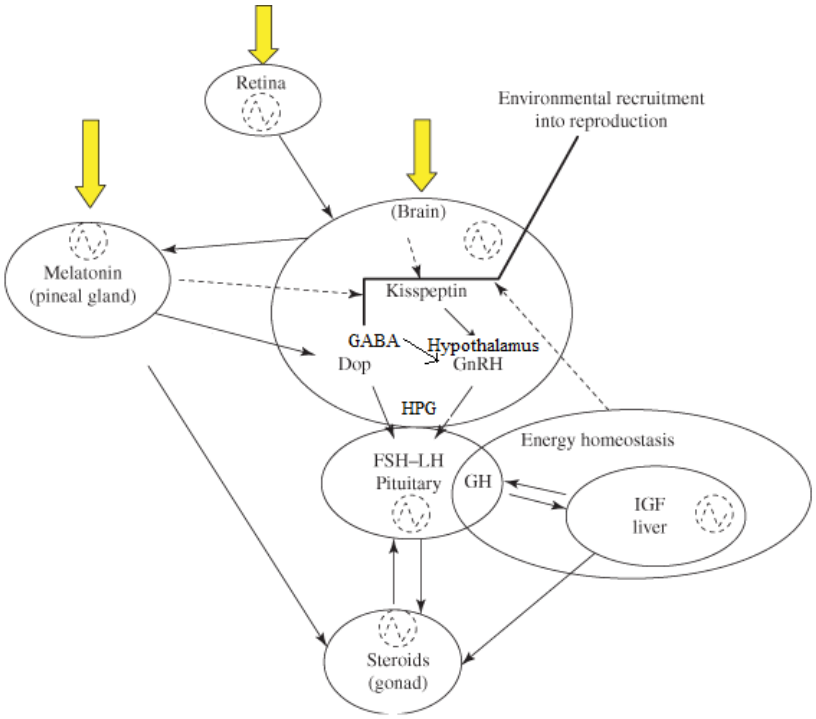

Figure 5: Photoneuroendocrine control of the hypothalamus-pituitary-gonad (HPG) axis in teleost. Photic information and melatonin is suggested to have a regulatory role at several levels in the control of maturation and reproduction. Dop: dopamine; FSH: follicle stimulating hormone; GABA: γ-aminobutyric acid; GH: growth hormone; GnRH: gonadotropin releasing hormone; HPG: hypothalamus – pituitary – gonad axis; IGF: insulin-like growth factor; LH: luteinizing hormone. Modified from Migaud *et al*. (2010).

In addition to GnRH, neuropeptid Y (NPY), γ-aminobutyric acid (GABA), gonadotropin inhibiting hormone (GnIH), and kisspeptins (Fig. 5) affects both GnRH and gonadotropin production and secretion (Kah and Dufour 2011). Gonadotrops are also regulated negatively by dopamine. In addition to all these factor, sex steroids such as 17β-estradiol, testosterone, and MIH exert feedback on the HPG-axis (Kah and Dufour 2011).

Timing of maturation and reproduction is of crucial importance to ensure maximal survival of the offspring. Environmental cues help animals anticipate the coming season, and to time the reproductive event (Bromage *et al*. 2001). Changes in photoperiod is regarded the most noise free environmental cue (Migaud *et al*. 2010), and melatonin is its most important physiological output. It is quite clear that photoperiod is an important factor in regulating the reproduction of temperate and high latitude teleost species (Fig. 5), but the precise mechanism for how photoperiod and melatonin can regulate and influence the HPG-axis is still unclear (Pankhurst and Porter 2003). It is known that melatonin together with information from the eyes is integrated in the preoptic area (POA) of the brain. This area also influences the pituitary and the releasing factors that affect the pituitary, such as GnRH, and the dopamine

system (Falcón *et al*. 2007, Dufour *et al.* 2010). Evidence also suggest that melatonin can affect other levels of the HPG-axis, and that a clock system could be controlling reproduction (Migaud *et al*. 2010). These possible effects of melatonin on maturation and timing of reproduction, makes knowledge about the melatonin system crucial to the commercial fish farming industry.

Control of maturation and reproduction in temperate species (salmonids)

In a commercial context, the goal is to produce a maximum number of offspring from the available broodstock at a desirable time of year (Bromage *et al.* 1992). This can be achieved by manipulating environmental cues registered by the animals. In salmonids and other temperate fish, manipulation of photoperiod is important in regulating timing of maturation and spawning (Taranger *et al.* 2010). For other farmed fish species, manipulation of temperature can be equally important (Taranger *et al*. 2010). In some species it can be difficult to mimic the correct environmental cues to start maturation in captivity, and in these cases, hormonal treatments may be an alternative (Mylonas *et al.* 2010).

Sexual maturation is a costly process, and resources used to complete maturation results in less resources available for somatic growth (Thorpe *et al.* 1990). In addition, fish held in culture do not meet the same challenges as in nature, and will have a strategy of growth and increased size only until a certain critical size required for maturation is reached. Sexual maturation then proceeds at the cost of further somatic growth (Thorpe 2004). The quality of the flesh will start to deteriorate when animals reach sexual maturity, and it is therefore desirable to avoid pre-harvest maturation (Aksnes *et al.* 1986). This can be achieved by controlling the photoperiod that the animals are exposed to. Exposing immature salmon to continuous light (LL) from winter solstice can reduce the percentage that reaches early maturity (Oppedal *et al.* 1997, Schulz *et al.* 2006, Migaud *et al*. 2010). The opposite effect can also be the intention of manipulating salmon, giving access to eggs and milt at all times of the year by changing the timing of spawning. Although these processes have different aims, they need to be initiated months in advance to achieve the best possible result (Bromage *et al*. 2001).

Control of maturation and reproduction in tropical and subtropical species (carps)

Fish inhabiting tropic and subtropical areas are exposed to minor or no seasonal photoperiodic change throughout the year. Seasonal cues such as temperature (García-López *et al*. 2006, Oliveira *et al.* 2009b), and seasonal rainfall (Susilo *et al*. 2009), has therefore been regarded

as more important in timing of reproduction in these species (Bye 1984, Bromage *et al*. 2001). Tropical and subtropical species does however have a functioning melatonin system as seen in all other teleosts (Bolliet *et al*. 1996, Oliveira *et al*. 2009a, Guttu 2011, Holtan 2011), and several studies have shown that changes in photoperiod and plasma melatonin levels can influence the reproductive status of these fish species (Davies *et al.* 1986, Campos-Mendoza *et al.* 2004).

Sexual maturation can be manipulated to occur during winter, several months before it naturally would by use of longer photoperiod and high water temperature (Sarkar *et al*. 2010). The effects of changing the length of the photoperiod, depends on what reproductive phase the animal is in. A longer photoperiod will in the preparatory and pre-spawning phases be stimulating to oocyte maturation and spawning, while a shorter photoperiod in the preparatory, pre-spawning and spawning phases will have an inhibitory effect (Dey *et al.* 2005, Maitra and Chattoraj 2007). Although there seems to be reason to assume that duration of, and changes in photoperiod, are important factors regulating maturation and spawning in tropical and subtropical species, other important factors, such as temperature, must still be kept in mind (Billard *et al.* 1978, Stacey 1984, Drori *et al.* 1994, Peter and Yu 1997, Glasser *et al*. 2004).

The hypothalamus-pituitary-interrenal (HPI) axis

According to Selye (1973), stress is "the nonspecific response of the body to any demand made upon it". A stressful stimulus that disturbs the internal dynamic equilibrium, induces a response to re-establish this homeostasis. During this response, catecholamins (adrenalin and noradrenalin), are immediately secreted from chromaffin cells in the head kidney, and the HPI-axis is activated, secreting the main teleost corticosteroid, cortisol, from interrenal cells in the head kidney with a short delay. This results in a suit of essential physiological responses that makes the animal capable of restoring the internal equilibrium (Wendelaar Bonga 1997, Barton 2002, Pankhurst 2011). These physiological changes affect the animal at many levels (Ashley 2007), and in research it is important to exclude the possibility of stress in experimental animals affecting the physiological parameter one is studying.

Aims of study

To develop a successful aquaculture in Nepal, and also establish a beneficial fish pond culture among poor people, one need to increase the supply of carp fries throughout the year. Such ambitions call for more knowledge about the carp melatonin system, and its possible mediation of photoperiodic control of maturation.

The aims of the present work were to answer following questions;

- Which plasma melatonin profile does mrigal carp exhibit if kept under natural outdoor photoperiod?
- Can melatonin production be induced during daytime if the mrigal carp is subjected to darkness?
- Does the plasma melatonin profile change if the mrigal carp is kept under continuous darkness?
- Does cortisol influence circulating plasma melatonin level in the mrigal carp?

Materials and methods

Working site and conditions

Experiments were conducted during March 2011, at the Mandal fish farm, located between Butwal and Bhairahawa cities in the Terai area of Nepal (Fig. 6). Nepal is a country that despite a small area (147 181 km^2), has very large variations in geography and climate, ranging from tropic lowland close to the Indian border, to tundra and glaciers near Mount Everest (8848 metres above sea level) in the Himalayas (Haugan 2012).

Figure 6: Map of Nepal. Experiments were conducted at 27° northern latitude close to Butwal city (red dot) in the Terai area of Nepal. Modified from Lonely Planet (2011).

Outdoor photoperiod at study site was measured (INS DX-200, Digital illumination meter), results are given in Figure 7A. Corresponding changes in luminance were also documented in the outdoor experimental tanks (Fig. 7B).

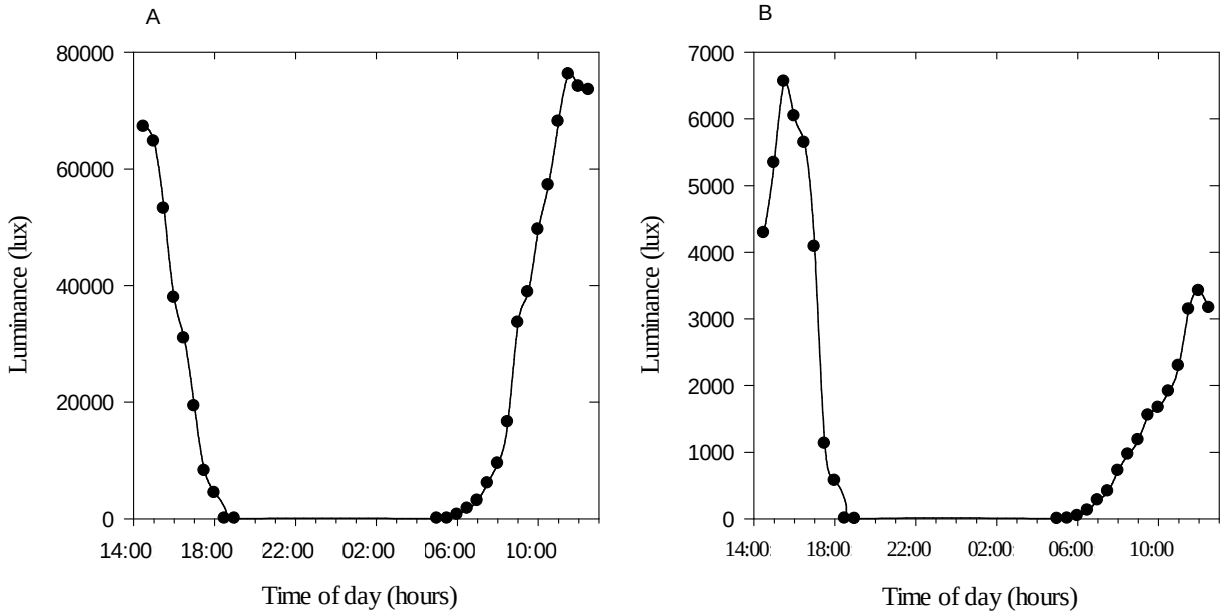

Figure 7: Recorded luminance of ambient outdoor light (A) and ambient light within experimental tanks (B).

Experimental animals and design

Mrigal carp (*Cirrhinus mrigala*) was used in experiments designed to document plasma melatonin levels during natural and manipulated photoperiods. Research animals were bought at the Mandal fish farm, and kept in holding tanks (Fig. 8) at the farm (2,5m x 1,5m, 1m deep). These fish, aversaging 67,0 ± 15,9 g and 20,2 ± 1,3 cm, were in a pre-pubertal life stage, as no external or internal signs of maturation could be documented.

Figure 8: Working site with experimental tanks exposed to natural outdoor light conditions (A,B), and covered up with black plastic to simulate extended darkness (C).

Experimental animals were fed a mix of rice bran (40%), wheat flour (40%) and mustard oil cake (20%). In preparation of experiments, fish were transferred from the holding tank to experimental tanks (1m x 2m, 1m deep) (Fig. 8B). Animals were allowed to settle down for minimum 24 hours before experiments started, and were not fed after transfer. Experimental tanks were supplied with running water to ensure sufficient oxygen levels. Temperature in the tanks varied during a 24 hour cycle between 22°C and 25°C.

Three experiments were performed in order to examine some of the melatonin dynamics in the mrigal carp.

Experiment 1: Plasma melatonin profile during natural outdoor photoperiod

This experiment was designed to measure the plasma melatonin levels of mrigal carp during one 24 hour outdoor light-dark cycle. Thirty fish were netted and transferred from the holding tank to each of the six experimental tanks. At regular intervals, 6 – 9 fish were captured and rapidly sedated (MS-222 (tricaine methane sulphonate), 75 mg L^{-1}, PHARMAQ Ltd). Blood was collected from the caudal vein system by use of 1mL heparinised syringes (approx. 20 IE ml^{-1} blood, LEO Pharma, 5000 IE ml^{-1}). Blood plasma obtained after five minutes of centrifugation at 3-4000 rpm (Hettich Universal EBA 3s) was kept on ice, and later frozen at -18°C until analyses were performed. Sampling during the natural dark phase was done under dim red light (Petzl E99 PG, headlight) (Bayarri *et al.* 2002).

Experiment 2: Plasma melatonin levels during daytime dark exposures

This experiment examined the mrigal carp melatonin production during the daytime in a period of darkness. Approximately twenty fish were transferred to each of the three experimental tanks. The first tank (group 1) was covered by thick, black plastic from 10:00 until 12:00 in the morning. At 12:00 6 – 9 fish were rapidly caught with a land net and sedated in a bucket (MS-222, 75 mg L^{-1}, PHARMAQ Ltd) covered with a lid to avoid any unwanted light exposure. To avoid light exposure during sampling, the head and eyes of the fish were covered with paper. Blood plasma was collected as described for experiment 1. The same procedure was repeated for groups 2 and 3, kept in darkness from 13:00 until 15:00, and from 16:00 until 18:00, respectively.

Experiment 3: Plasma melatonin profile during continuous darkness

The third experiment was designed to examine the effect of long lasting darkness on the mrigal plasma melatonin profile. Approximately thirty fish were transferred from the holding tank to each of the six experimental tanks. At the approximate time of sunset (18:30) these six

experimental tanks were covered with thick, black plastic blocking out all light (0,0 lux measured). The plastic was kept on the tanks from Monday at 18:30 until sampling started Friday at 12:00 (89,5 hours of darkness for the animals sampled first). Two tanks were only sampled once each (12:00 and 15:30), while the four remaining tanks were sampled twice in two rounds. 6 – 9 fish were rapidly netted and sedated in a bucket (MS-222, 75 mg L^{-1}, PHARMAQ Ltd) with a lid to avoid any exposure to light during the daytime samplings. The head and eyes of the animal were covered with paper during sampling. Night time samplings were done with dim, red light. Blood plasma was collected as described for experiment 1.

Hormone analysis

Blood plasma melatonin and cortisol levels were analysed by use of radioimmuno assay (RIA-system). This method uses radioactivity to indirectly measure amount of a substance based on the specificity of the antibody. The technique is based on unknown amounts of plasma hormone (Ag) competing with known amounts of radioactively labelled hormone (Ag*) for binding to a hormone specific antibody (Ab) present in limited amount.

$$\begin{array}{ccccccc} Ag & & & & Ag\text{-}Ab & & Ag \\ & + & Ab & \leftrightarrow & & + & \\ Ag^* & & & & Ag^*\text{-}Ab & & Ag^* \end{array} \quad (1)$$

After separating free from bound antigen the radioactive amount within one of the fractions is measured (beta or gamma counter) and the results are compared to a standard curve (Davies 2005).

Analysis of plasma melatonin content was done with Melatonin Research RIA kit (Labor Diagnostika Nord GmbH & Co), as validated by Guttu (2011) and Holtan (2011), following the procedure given in the protocol. Standard curve for Melatonin Research RIA is found in Figure 9A. There was one unintended deviation from protocol analysing samples from experiment 1, as the samples were incubated in a refrigerator (4°C) instead of at room temperature. Plasma melatonin values that were too low to be detected in the system were defined to the detection limit of the system (7,0 pmol L^{-1} for experiment 1 and 5,0 pmol L^{-1} for experiments 2 and 3).

Analysis of plasma cortisol was done with Coat-A-Count cortisol kit (Siemens), following the procedure given in the protocol. In accordance with previous results (Guttu 2011, Holtan 2011), samples for cortisol testing were diluted 1:1 using standard A (cortisol: 0 nmol L^{-1}) to

ensure that the hormone concentrations were within the range of the standard curve (Fig. 9B). A few samples from experiment 1 overshot the standard curve, and based on their count per minute (CPM) number, they were defined to be equal to the highest standard in the system (1380 nmol L^{-1}).

Melatonin conversion factor is:

$$pg\ mL^{-1} \times 4{,}305 = pmol\ L^{-1} \quad (2)$$

while cortisol conversion factor is:

$$ng\ mL^{-1} \times 2{,}75 = nmol\ L^{-1} \quad (3)$$

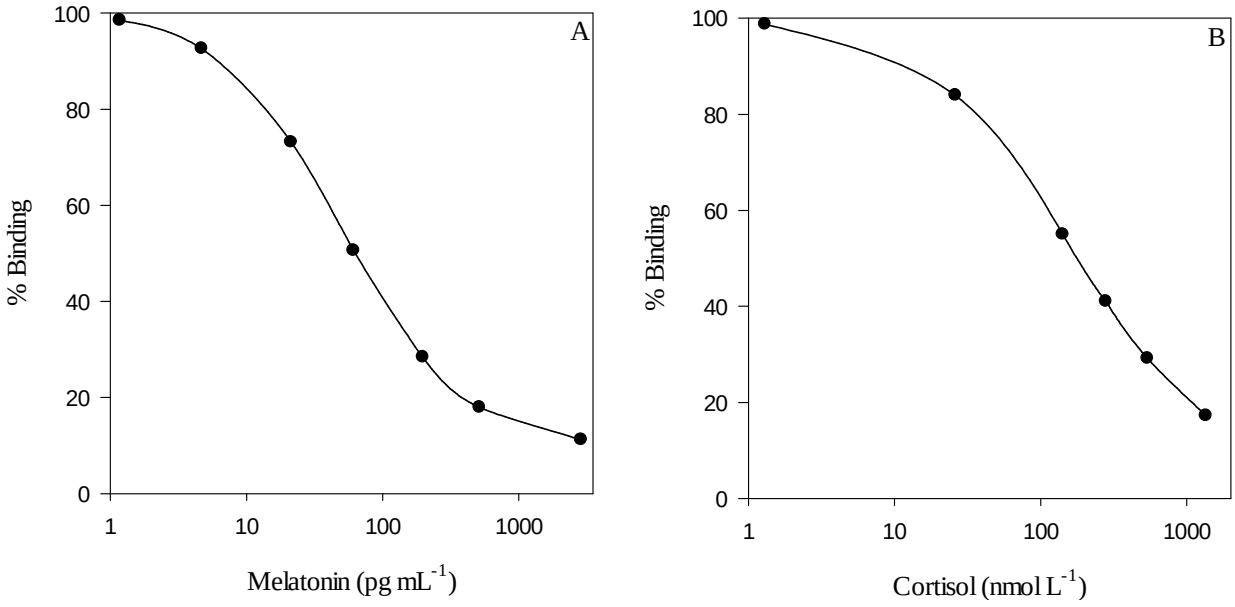

Figure 9: Standard curves for melatonin (A), and cortisol (B).

Statistical analysis and graphs

Graphics were made using SigmaPlot 12.0 (Systat Software, Inc.) for Microsoft Windows. Modification of figures was done using Paint for Microsoft Windows.

Statistical analysis of the data was done using SigmaPlot 12.0 (Systat Software, Inc.) for Microsoft Windows. Data were checked for normality using Shapiro – Wilk-test ($P < 0{,}05$).

One way analysis of variance (ANOVA) and ANOVA on ranks (Kruskal-Wallis) ($P < 0{,}05$) was used to test for significant differences between the experimental groups for parametric and nonparametric data, respectively.

If significant differences were found, Tukey post hoc test ($P < 0,05$) was used to identify groups that were different from each other for both parametric and nonparametric (Tukey on rank sum mean) data.

To test for correlation between plasma melatonin and cortisol levels Pearson product moment correlation and Spearman rank order correlation ($P < 0,05$) was used for parametric and nonparametric data, respectively.

Results

Experiment 1: Plasma melatonin profile during natural outdoor photoperiod

The aim of this experiment was to investigate the blood plasma melatonin concentration of mrigal carp kept under a natural outdoor photoperiod. Time of sampling and body measures for the experimental animals, are given in Table 1.

Table 1: Average body length and weight of mrigal carp sampled during a 24 hour natural outdoor photoperiod (experiment 1) in Nepal.

Time of sampling	**N**	**Weight (g)**	**Length (cm)**
12:00	6	69,3 ± 9,1	20,3 ± 1,0
17:00	6	64,5 ± 4,9	19,5 ± 1,1
19:00	6	65,7 ± 4,6	20,0 ± 0,5
21:00	6	66,8 ± 7,9	20,1 ± 0,8
23:00	6	70,8 ± 15,9	20,2 ± 1,1
01:00	6	73,7 ± 11,5	20,6 ± 1,1
03:00	6	66,7 ± 6,4	20,1 ± 0,5
05:00	6	62,7 ± 14,3	19,6 ± 1,4
07:00	6	69,3 ± 7,0	20,6 ± 0,6
12:00	6	62,0 ± 13,3	19,4 ± 2,1

Average mrigal carp body weight and body length at the sampling points of experiment 1 varied from 62,0 ± 13,3 g to 73,7 ± 11,5 g and from 19,4 ± 2,1 cm to 20,6 ± 1,1 cm, respectively. There were no significant differences between the experimental groups (ANOVA on ranks; P = 0,52; H = 8,09; 9 degrees of freedom (weight) and P = 0,34; H = 10,12; 9 degrees of freedom (length)).

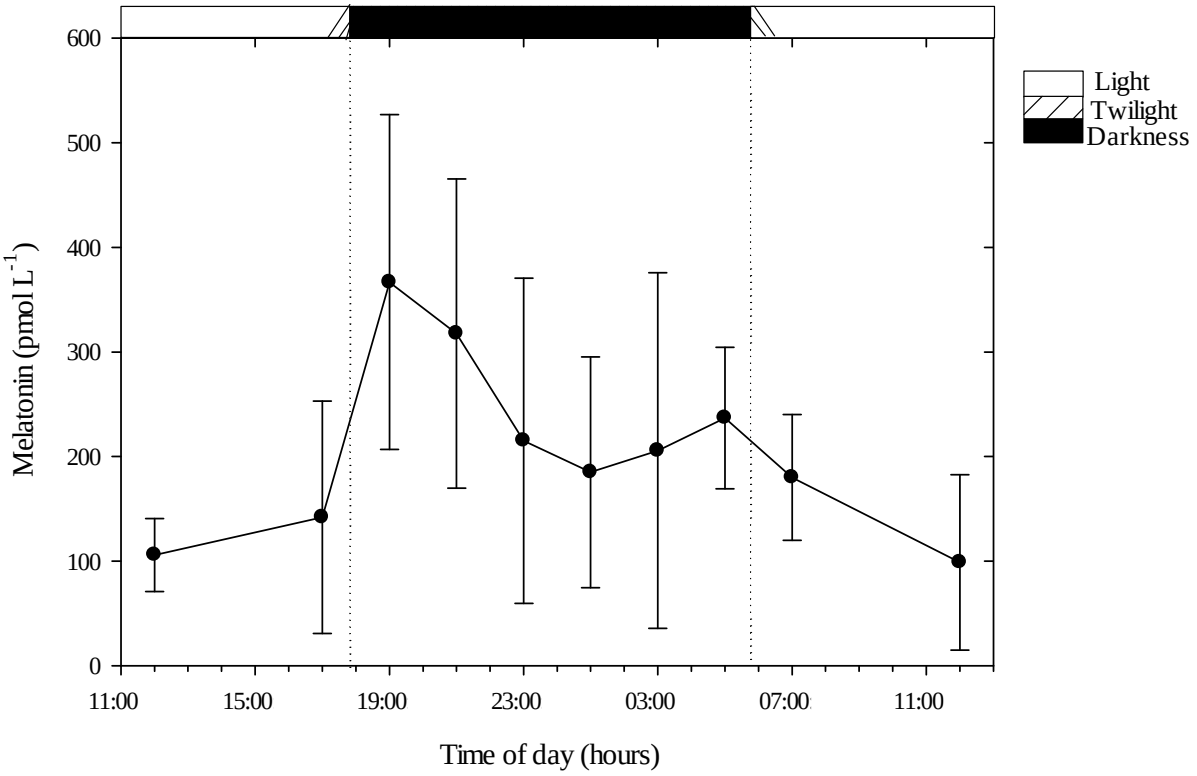

Figure 10: Plasma melatonin concentrations in mrigal carp (*Cirrhinus mrigala*) sampled during natural outdoor photoperiod at 27° northern latitude in Nepal. Values are mean ± SD (N = 6).

During a normal outdoor light-dark cycle, measured plasma melatonin level of mrigal carp (Fig. 10) increased from a low level of 106 pmol L^{-1}in daylight (12:00), to an average maximum of 367 pmol L^{-1}at the end of twilight and beginning of darkness (19:00). From this time there is a tendency of hormonal decline throughout the night, and average melatonin levels are down to 237 pmol L^{-1} at dawn (05:00). The following noon average blood plasma melatonin level dropped further to 145 pmol L^{-1}. ANOVA on ranks (Kruskal-Wallis) identified significant differences between the experimental groups (P = 0,014; H = 20,62; 9 degrees of freedom), but Tukey test could not identify which groups were different from each other (P > 0,05) (Appendix 1).

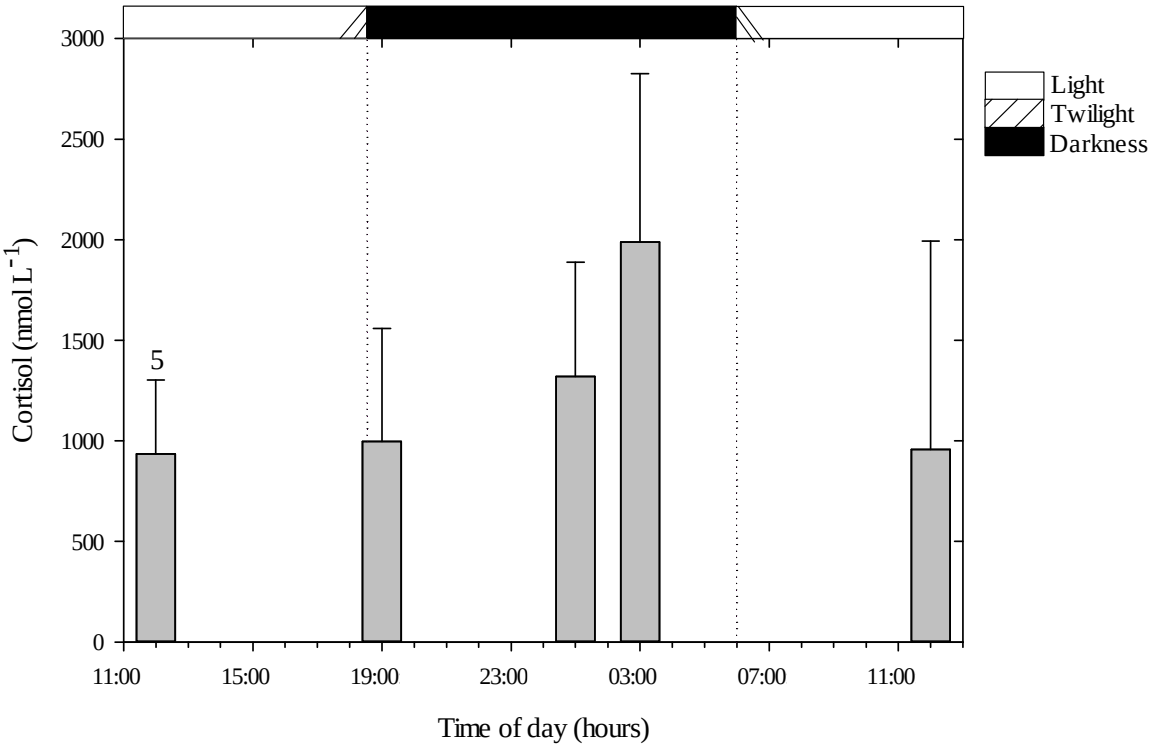

Figure 11: Plasma cortisol concentration in mrigal carp (*Cirrhinus mrigala*) sampled during natural outdoor photoperiod at 27° northern latitude in Nepal. Values are mean ± SD (N = 6 unless otherwise shown).

Plasma cortisol levels in mrigal carp under natural outdoor photoperiod (Fig. 11) increase from the first sampling point at 12:00 (935 nmol L^{-1}) until a peak is reached at 03:00 (1989 nmol L^{-1}), before declining to daytime levels (956 nmol L^{-1}) at 12:00 the following day. No significant differences were detected between the experimental groups (ANOVA; P = 0,095). Testing for a correlation between plasma melatonin and cortisol levels (Spearman rank order correlation) gave no correlation between melatonin and cortisol (P > 0,05), except at one sampling point (01:00), where there was a positive correlation (P = 0,033, correlation coefficient = 0,88) between the two variables.

Experiment 2: Plasma melatonin levels during daytime dark exposures

This experiment was designed to investigate melatonin production at different times of day following a short period of introduced darkness. Time of sampling and body measures for the experimental animals, are given in Table 2.

Table 2: Average body length and weight of mrigal carp after two hours of daytime dark exposure (experiment 2).

Time of sampling	N	Weight (g)	Length (cm)
12:00	6	63,1 ±10,4	20,2 ± 1,3
15:00	6	63,1 ± 9,6	20,1 ± 1,2
18:00	6	63,8 ± 11,8	20,3 ± 1,5

Average mrigal carp body weight and body length at the sampling points of experiment 2 varied from 63,1 ± 9,6 g to 63,8 ± 11,8 g and 20,1 ±1,2 cm and 20,3 ± 1,5 cm, respectively. There were no significant differences between the experimental groups (ANOVA; P = 0,98 (weight) and P = 0,96 (length).

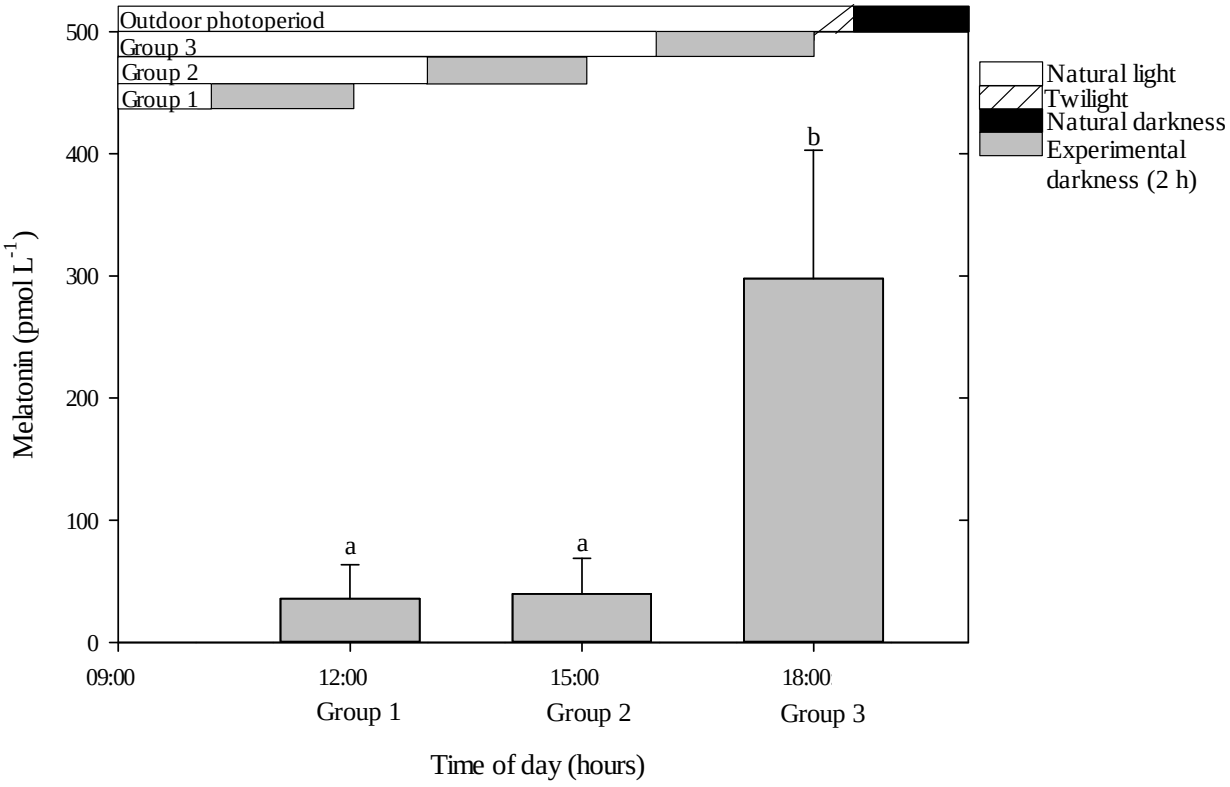

Figure 12: Plasma melatonin concentration in groups of mrigal carp (Cirrhinus mrigala) sampled after daytime dark exposures at 27° northern latitude in Nepal. Values are ± SD (N = 6). Letters denote significant differences.

Experimental group 1, held in darkness for two hours from 10:00 until 12:00 did not show an increase of melatonin production (Fig. 12) above expected daytime levels (42 pmol L^{-1}). The same was seen for group 2, held in darkness from 13:00 until 15:00 (57 pmol L^{-1}). Experimental group 3, held in darkness from 16:00 until 18:00 showed an increased plasma

melatonin concentration (298 pmol L^{-1}) compared to experimental groups 1 and 2 (ANOVA; $P < 0,001$; Tukey test $P < 0,05$).

Plasma cortisol levels of the mrigal carp experimental groups 1 and 2 (Fig. 13) are approximately equal (618 nmol L^{-1} and 598 nmol L^{-1}, respectively), while there is a small decrease in plasma cortisol levels for experimental group 3 (379 nmol L^{-1}). No significant differences were detected between the experimental groups (ANOVA; $P = 0,413$). No correlation could be detected between melatonin and cortisol (Pearson product moment correlation $P > 0,05$).

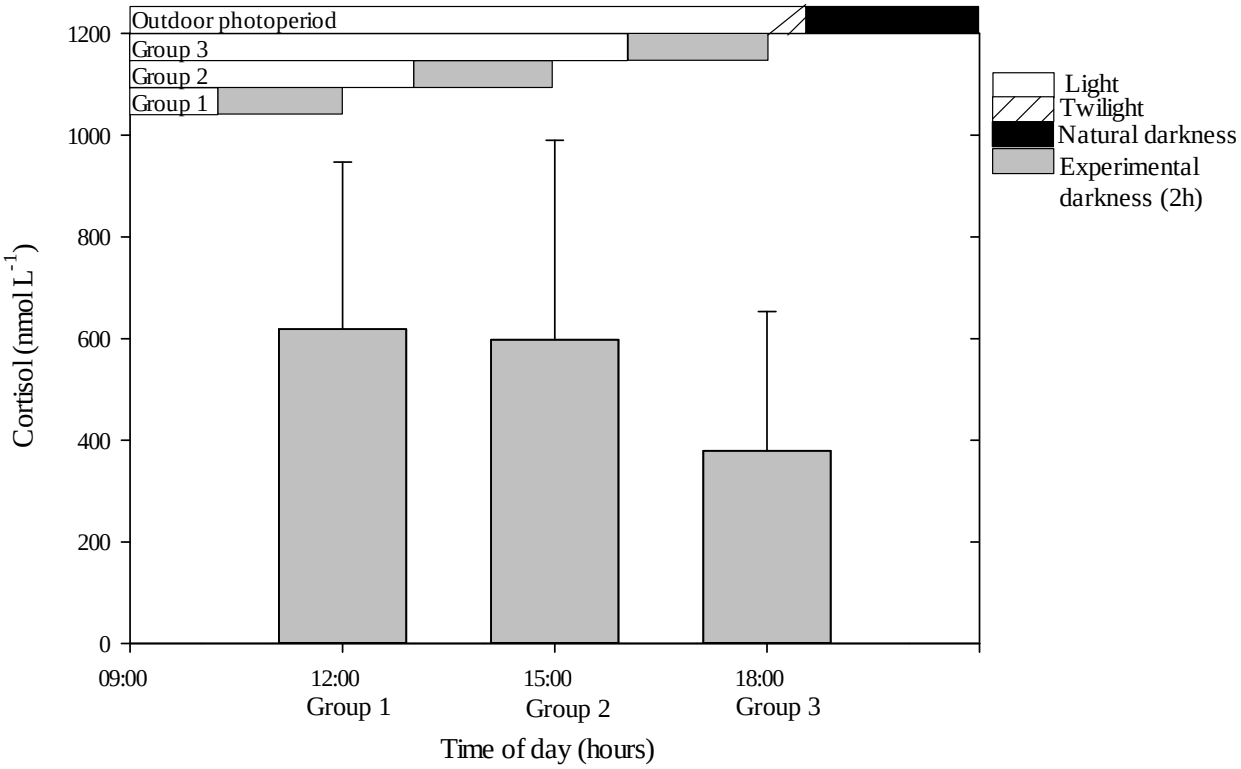

Figure 13: Plasma cortisol concentration in mrigal carp (*Cirrhinus mrigala*) sampled after daytime dark exposures at 27° northern latitude in Nepal. Values are mean ± SD (N = 6).

Experiment 3: Plasma melatonin profile during continuous darkness

To investigate the effect of prolonged darkness on pineal melatonin production in mrigal carp, experimental animals were kept in darkness for more than 3 light-dark cycles. Time of sampling and body measures for the experimental animals, are given in Table 3.

Table 3: Average body length and weight of mrigal carp sampled during a 24 hour cycle while kept in continuous darkness (experiment 3).

Time of sampling	**N**	**Weight (g)**	**Length (cm)**
12:00	6	62,5 ± 5,1	20,2 ± 0,6
15:30	6	67,3 ±11,7	20,6 ± 1,0
19:00	6	52,9 ± 16,5	18,4 ± 2,1
22:00	6	71,1 ± 13,1	20,7 ± 1,1
02:00	6	68,8 ± 8,8	20,6 ± 0,7
05:00	6	62,8 ± 10,9	20,2 ± 1,3
08:30	6	67,1 ± 13,9	20,4 ± 1,3
12:00	6	64,9 ± 10,6	20,8 ± 0,7

Average mrigal carp body weight and body length at the sampling points of experiment 3 varied from 52,9 ± 16,5 g to 71,1 ± 13,1 g and 18,4 ± 2,1 cm and 20,8 ± 0,7 cm, respectively. There were no significant differences between the experimental groups (ANOVA; P = 0,98 (weight) and ANOVA on ranks; P = 0,2; H = 9,72; 7 degrees of freedom (length).

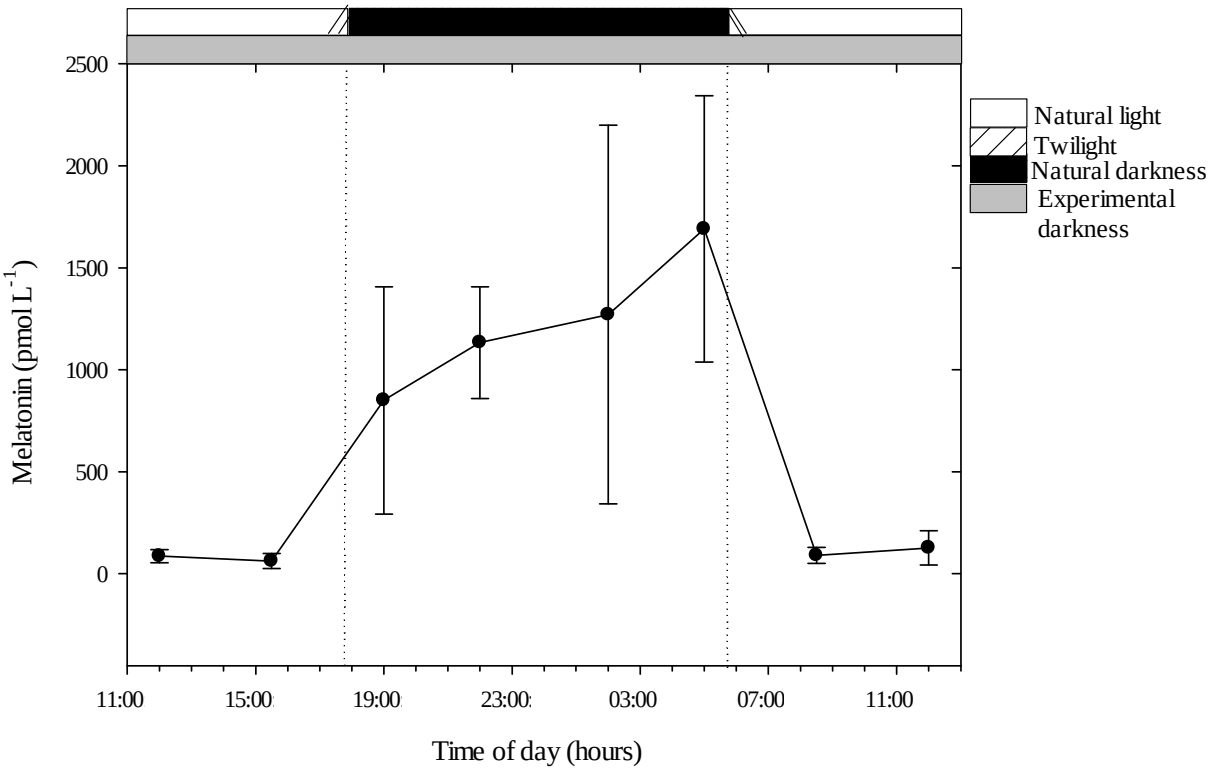

Figure 14: Plasma melatonin concentration in the mrigal carp (*Cirrhinus mrigala*), sampled while being kept at constant darkness (>72 hours) at 27° northern latitude in Nepal. Values are mean ± SD (N = 6). Peak value is significant different from all light values.

Plasma melatonin concentration in mrigal carp kept under continuous darkness (>72 hours) (Fig. 14) rise from a subjective daytime level of 62 pmol L^{-1} (15:30) to a peak level of 1690 pmol L^{-1} late in the subjective night (05:00). Levels then fall abruptly at the approximate time of sunrise and reaches daytime values at 08:30 (90 pmol L^{-1}). Peak value is significantly different from all light values (ANOVA on ranks (Kruskal-Wallis; $P < 0,001$; $H = 36,571$; 7 degrees of freedom), Tukey test; $P < 0,05$). Details regarding the statistics are found in Appendix 2.

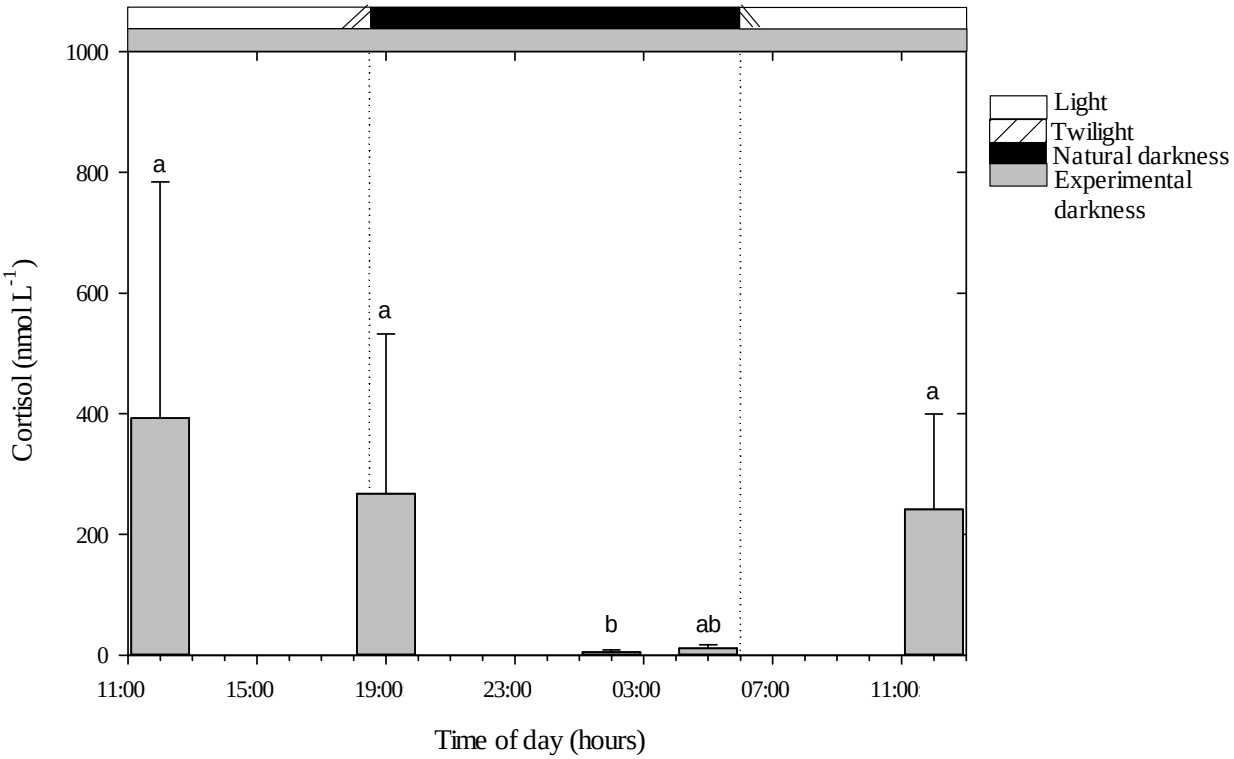

Figure 15: Plasma cortisol concentration in mrigal carp (*Cirrhinus mrigala*) sampled while being kept in constant darkness (>72 hours) at 27° northern latitude in Nepal. Values are mean ± SD (N=6). Letters denote significant differences.

Plasma cortisol concentration of mrigal carp kept under continuous darkness (Fig. 15) show a tendency of declining from the daytime (12:00) level at 393 nmol L^{-1} until 02:00, when the lowest level (5 nmol L^{-1}) is measured. This is followed by an increased plasma cortisol level at 12:00 the following day (241 nmol L^{-1}). Two experimental tanks (05:00 and 12:00-2) were sampled twice at an interval of 10 hours and 9 hours, respectively. Letters denote significant differences (ANOVA on ranks (Kruskal-Wallis; $P < 0,001$; $H = 20,834$; 4 degrees of freedom), Tukey test; $P < 0,05$). There was no correlation (Pearson product moment correlation) between cortisol and melatonin, except at 12:00 at the end of the experiment, where there was a positive correlation ($P = 0,003$, correlation coefficient = 0,95).

Discussion

A well developed aquaculture with carps in developing countries could increase income for the rural people and improve the under- and malnourishment prevalent in these areas (WFP 2012). One major challenge in developing the Asian aquaculture industry is the limited availability of carp fries (Sarkar *et al*. 2010). In the intensive salmon farming in Norway, this has been omitted by use of photoperiodic manipulations to delay or accelerate maturation and reproduction according to need. This is based on knowledge about the function of the salmon melatonin system, and the effect of environmental cues on puberty and maturation (Bromage *et al*. 2001, Taranger *et al*. 2010). A pineal melatonin system has also been shown to exist and function in tropical and subtropical carp species (Bolliet *et al*. 1996, Oliveira *et al*. 2009a, Seth and Maitra 2010, Guttu 2011, Holtan 2011). Knowledge about how this system conveys photoperiodic changes to brain centres, can be the basis for manipulation of the reproductive system in tropical and subtropical carp species.

The mrigal carp melatonin response to ambient light-dark changes

The mrigal carp is one of the seven major carp species commonly kept in Asian pond polyculture systems. At early life stages, mrigal carp inhabits surface layers of the water, while it as an adult becomes a bottom dweller, feeding on decayed plant material, phytoplankton and zooplankton. A high growth potential under the prevalent conditions can make this an important species for Nepalese aquaculture in the Terai region (FAO 2012), especially if out of season production of fries can be achieved.

Natural outdoor photoperiod

Blood plasma melatonin of mrigal carp kept under natural outdoor photoperiod show a rapid increase at onset of darkness, and reaches a peak early in the dark phase. From this time and onwards there is a tendency of decreasing plasma melatonin level until dawn. This early melatonin peak indicates that the pineal gland has a rapid endocrine response at onset of darkness. The following decrease in plasma melatonin levels indicates that the pineal gland does not mount a full response throughout the night, as seen in salmonids (Falcón *et al*. 2010). Instead, there seems to be a down-regulation of melatonin release from the pineal gland after peak level is reached. Accordingly, the plasma melatonin profile of the mrigal resembles that of a type B response (Falcón *et al*. 2010). This response is typical for Nile tilapia (Fig. 4), and has recently been reported for common carp (*Cyprinus carpio*) kept under indoor simulated natural photoperiod (Holtan 2011). It is also in accordance with plasma melatonin profile in

copper mahseer (*Neolissochilus hexagonolepis*) during a natural outdoor light-dark cycle (Guttu 2011).

Daytime dark exposures

A second experiment was carried out to investigate the mrigal carp pineal melatonin response when subjected to darkness during daytime. The results demonstrate that mrigal carp subjected to darkness in morning or midday does not induce an increase in blood plasma melatonin. However, if exposed to darkness in the afternoon, a large increase in the mrigal plasma melatonin level is seen. These endocrine results give reason to assume that the pineal gland has a refractory period during daytime, where it is insensitive to dark stimuli. This has also been suggested for other teleosts (Falcón *et al.* 1989, 1992), as wells as rats (Binkley *et al.* 1973) and chicken (Binkley *et al.* 1975). These results indicate that pineal melatonin production in the mrigal carp might be controlled by a circadian clock.

Continuous darkness

After being subjected to continuous darkness for more than three 24 hour cycles, mrigal carp show a rapid increase in plasma melatonin level at the start of subjective night. This further adds to the impression that a circadian clock initiates production of melatonin, even without a change from light to darkness. The increase in plasma melatonin levels continues until a peak is reached late at subjective night. From this peak level there is a decline in plasma melatonin coinciding with onset of subjective day. Thus, production of melatonin in the mrigal pineal gland terminates at the end of subjective night, keeping the melatonin cycle even after four 24 hour periods in darkness. This supports the theory of a circadian clock controlling pineal melatonin secretion.

Plasma melatonin levels in darkness during the subjective daytime are as low as expected in light, and this supports previous results of a refractory period of the pineal gland. This is also a clear indication that there is an endogenous circadian clock controlling mrigal pineal melatonin production. There is an increase in plasma melatonin level through the subjective dark phase under continuous darkness, which contrasts somewhat to the decline seen under natural photoperiod. Thus, after a period of continuous darkness, the plasma melatonin profile resembles a type A, more than a type B response (Fig. 4). This shift of the plasma melatonin profile from a peak at the onset of darkness, to a peak in the transition between subjective darkness and light, may suggest that the circadian clock free runs with a period of slightly

more than 24 hours. Such results have also been demonstrated for species such as European sea bass (Bayarri *et al.* 2004) and pike (*Esox lucius* L.) (Bolliet *et al.* 1997).

The alternative to pineal melatonin production being controlled by a circadian clock, is a passive hormonal release during darkness, as seen in salmonids (Max and Menaker 1992, Iigo *et al*. 2007a). If this was the case for mrigal carp, one would expect plasma melatonin levels to remain equally high through the whole sampling period, and not show a profile pattern with high levels during subjective darkness, and low levels during subjective light. As one clearly can identify a profile that is shifted, but still present, there is no reason to expect that pineal melatonin production is under direct control of light-dark conditions. The effect of light on pineal melatonin production in mrigal carp seem to be indirect, and to control the pineal production of melatonin by resetting the circadian clock, as previously experienced from ayu (*Plecoglossus altivelis*) studies (Iigo *et al.* 2003).

A possible relation between photoperiod and maturation

Results from previous investigations indicate that a pineal-melatonin-gonadal axis exists in the mrigal carp (Sarkar *et al*. 2010). This is in accordance with results from studies on major carp (*Catla catla*), in which both manipulation of photoperiod and exogenous melatonin injections are shown to affect testicular development and function. Constant light gave precocious maturation of testes during the pre-spawning phase, while constant darkness or injections with exogenous melatonin either stimulated (preparatory phase), inhibited (pre-spawning and spawning phases), or had no effect (post-spawning phase) on testicular activity. (Bhattacharya *et al.* 2007).

These effects of changing photoperiods and exogenous melatonin administrations, shows that melatonin may influence and control reproductive functions in tropical and subtropical teleosts. Several mechanisms for the influence of melatonin on reproductive functions are suggested. Studies of rohu (*Labeo rohita*) and major carp have shown that incubation of mature oocytes with melatonin previous to maturation inducing hormone, gave earlier final oocyte maturation (Chattoraj *et al.* 2005, Maitra *et al.* 2005). Melatonin has also been documented to inhibit dopamine release in the hypothalamus, and thus melatonin can have a stimulatory effect on reproduction by removing the inhibition that dopamine exerts (Popek *et al.* 2005, 2006). Experimental evidence also indicates a functional relationship between gonadal sex steroids and changes in photoperiod, represented by melatonin (Chattoraj *et al.* 2009).

The mrigal carp is a bottom dweller in free living and captive adult life (Sarkar *et al*. 2010, FAO 2012), and accordingly, little work has been done to test whether photoperiod can affect the reproductive physiology of this animal. In the present work, the mrigal carp pineal is shown to respond to changes in natural light-dark conditions with increased plasma melatonin in darkness, and to have a circadian clock controlling pineal secretion of melatonin. This indicates that despite its bottom dwelling adult life style, the mrigal carp has a melatonin system that responds to the ambient light-dark cycle, and probably can be used for manipulation of the mrigal maturation. Others (Sarkar *et al*. 2010) have shown that the mrigal carp is responsive to photothermal manipulations of reproduction. In fish such as mrigal, with a circadian clock controlling the pineal production of melatonin, endogenous endocrine rhythms and environmental temperature shifts, must be taken into consideration when designing manipulatory regimes for control of maturation.

The validity of the experimental results

Stress, cortisol release, and melatonin levels

Many different stimuli in wild life and culture settings can initiate a stress response that can affect several aspects of the animal physiology (Iwama 1998, Barton 2002). Studies on the impact of artificial changes in photoperiod on plasma cortisol levels have given differing results; either showing an effect (Leonardi and Klempau 2003), or no effect (Biswas *et al.* 2004, 2006). A few studies have indicated that plasma melatonin levels can be affected by stress and plasma cortisol release in fish (Benyassi *et al.* 2001, Larson *et al.* 2004).

Animals kept under continuous darkness for more than three 24 hour cycles can be assumed to have settled down, and reached a non-stressed state. As expected, plasma cortisol levels of these animals were down to an average low of 5 nmol L^{-1}. This plasma cortisol level should be representative for unstressed mrigal carp. In mammals, there is a clearly defined circadian pattern in plasma cortisol levels, with a decline towards the evening and night, and a subsequent rise at the time of awakening in the morning (Weitzman *et al.* 1971, 1976, Wisser and Breuer 1981). Studies investigating the circadian rhythm of cortisol secretion in teleosts have reported either a similar pattern as seen in humans (Boujard and Leatherland 1992), or an opposite pattern, with a maximum plasma cortisol level at night (Laidley and Leatherland 1988). Such circadian plasma cortisol rhythm might also exist for mrigal carp, even so, the low plasma cortisol values reported herein are in agreement with cortisol results reported for unstressed common carp (Ruane *et al.* 2001) and mrigal (Das *et al.* 2009).

Plasma cortisol levels in mrigal carp kept under natural outdoor photoperiod, and subjected to daytime dark exposures, were quite high compared to resting values reported in this work and others. It is therefore assumed that these experimental animals were stressed. Other, similar work on common carp and silver carp (*Hypophthalmichthys molitrix*) also gave plasma cortisol levels in the range reported here (Holtan 2011, Prestrud 2012 (in prep.)). Experimental animals used in the present work were sedated before blood samples were collected. The MS-222 sedation is known to inhibit the secretion of cortisol by blocking release of adrenocorticotopic hormone (ACTH) from the pituitary of salmon (Olsen *et al.* 1995). MS-222 sedation of mrigal carp in this experiment should also block the release of cortisol. It is more likely that the transfer to a new environment (e.g. to experimental tanks and different social ranking), might have been perceived as stressful (Kurogi and Lida 1999).

Analysis of the relation between plasma melatonin and cortisol levels in all experiments resulted in only two positive correlations (under natural outdoor photoperiod at 01:00 and under continuous darkness at 12:00). These variables are therefore assumed not to be correlated. In addition, the present work has shown plasma melatonin profiles even in individuals assumed to be stressed. Hence, a possible effect of cortisol on pineal melatonin secretion is not expected.

Individual melatonin level variation

It was not possible to identify any significant difference in plasma melatonin levels between experimental groups kept under natural outdoor photoperiod. Visual inspection of the results does, however, make it clear that there is an increase in average plasma melatonin from daytime to average melatonin peak is reached. Experimental animals may qualitatively mount the same endocrine responses to ambient changes, but the quantitative hormone changes might differ both in amplitude and timing. This is probably the case in this experiment, as standard deviations for the sampling groups are quite large. In order to verify present findings, future experiments should look at individual melatonin responses over time.

Assaying conditions

There was an unintended deviation from protocol analysing samples from the experiment under natural outdoor photoperiod, as they were incubated at 4°C instead of at room temperature. This may have caused the enzymatic reaction where plasma melatonin and radioactively labelled melatonin compete for binding to the antibody, to proceed more slowly, and not come to completion. The consequence could be a decreased measuring sensitivity,

and accordingly, lowered melatonin values. However, as standards and samples all received the same treatment, such effect should not seriously affect the shape of the melatonin profile.

Influence from other melatonin sources

Melatonin may be produced by several organs including the pineal gland, brain, liver, heart, gastrointestinal system and retina (Falcón *et al*. 2010). There is a very large production of melatonin in the gastrointestinal system, with melatonin values 10 – 100 times higher than those seen in the general circulation. Gut melatonin is however assumed to undergo enterohepatic cycling, and hence, do not reach the general circulation (Messner *et al*. 2001) (If gastrointestinal melatonin reached the general circulation it would disturb the pineal clock and calendar function (Reiter 1993) – which we know from experience, do not happen). Melatonin production in other tissues and organs is only known to exhibit local paracrine effects (Falcón *et al*. 2011). Thus, in the present investigation there are no reasons to believe that extrapineal melatonin sources would have added to the hormonal concentrations measured in collected blood samples.

Experimental summary

- Mrigal pineal melatonin increase at onset of darkness. Plasma melatonin levels decline through the dark phase, indicating a down-regulation of pineal melatonin production after peak is reached. Resemblance with profile type B was shown.
- Daytime dark exposures show that the mrigal pineal gland has a refractory period where it does not respond to darkness with increased melatonin secretion.
- Continuous dark exposure gave a plasma melatonin profile in accordance with subjective day and night, and show that the mrigal pineal secretion of melatonin is under control of a circadian clock. Resemblance with profile type A was shown.
- Cortisol has not been shown to affect plasma melatonin levels in mrigal carp.

Conclusions

- Mrigal pineal melatonin production increase in darkness, but seems to be controlled by an endogenous circadian clock.
- The mrigal carp seems to be a good candidate for photo-controlled out of season maturation and production of fries.

Perspectives

In order to achieve out of season maturation in mrigal carp it is necessary to further expand knowledge of the pineal melatonin dynamics in this fish species.

Experiments are also needed to examine the impact from nutrition and ambient temperature on puberty and maturation, directly, or indirectly through regulating factors like kisspeptins and dopamine. In addition, the correlation between the melatonin system and the HPG-axis must be explored. This can be done through experiments where groups of carp are subjected to different photo regimes, while melatonin levels and gonadal maturation are investigated simultaneously.

References

AKSNES, A., GJERDE, B. & ROALD, S. O. 1986. Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar*. *Aquaculture,* 53**,** 7-20.

ASCHOFF, J. 1965. The phase-angle difference in circadian periodicity. *In:* ASCHOFF, J. (ed.) *Circadian clocks.* Amsterdam: North-Holland Publishing Company.

ASHLEY, P. J. 2007. Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science,* 104**,** 199-235.

BARTON, B. A. 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology,* 42**,** 517-525.

BAYARRI, M. J., MADRID, J. A. & SÁNCHEZ-VÁZQUEZ, F. J. 2002. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. *Journal of Pineal Research,* 32**,** 34-40.

BAYARRI, M. J., DE LAMA, M. A. R., MADRID, J. A. & SANCHEZ-VAZQUEZ, F. J. 2003. Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in sea bass. *Brain Research,* 969**,** 175-182.

BAYARRI, M. J., GARCÍA-ALLEGUE, R., LÓPEZ-OLMEDA, J. F., MADRID, J. A. & SÁNCHEZ-VÁZQUEZ, F. J. 2004. Circadian melatonin release *in vitro* by European sea bass pineal. *Fish Physiology and Biochemistry,* 30**,** 87-89.

BENFEY, T. J. 2011. Aquaculture: a sustainable future for aquatic food production. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* 89**,** V-VII.

BENYASSI, A., SCHWARTZ, C., DUCOURET, B. & FALCÓN, J. 2001. Glucocorticoid receptors and serotonin N-acetyltransferase activity in the fish pineal organ. *Neuroreport,* 12**,** 889-892.

BESSEAU, L., BENYASSI, A., MØLLER, M., COON, S. L., WELLER, J. L., BOEUF, G., KLEIN, D. C. & FALCÓN, J. 2006. Melatonin pathway: breaking the 'high-at-night' rule in trout retina. *Experimental Eye Research,* 82**,** 620-627.

BHATTACHARYA, S., CHATTORAJ, A. & MAITRA, S. K. 2007. Melatonin in the regulation of annual testicular events in carp *Catla catla*: evidence from the studies on the effects of exogenous melatonin, continuous light, and continuous darkness. *Chronobiology International,* 24**,** 629-650.

BILLARD, R., BRETON, B., FOSTIER, A., JALABERT, B. & WEIL, C. 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors: salmonid and cyprinid models. *In:* GAILLARD, P. J. & BOER, H. H. (eds.) *Comparative endocrinology.* Amsterdam: Elsevier/ North-Holland Biomedical press.

BINKLEY, S. A., KLEIN, D. C. & WELLER, J. L. 1973. Dark induced increase in pineal serotonin N-acetyltransferase activity: a refractory period. *Cellular and Molecular Life Sciences,* 29**,** 1339-1340.

BINKLEY, S. A., MACBRIDE, S. E., KLEIN, D. C. & RALPH, C. L. 1975. Regulation of pineal rhythms in chicken: refractory period and nonvisual light perception. *Endocrinology,* 96**,** 848-853.

BISWAS, A. K., MAITA, M., YOSHIZAKI, G. & TAKEUCHI, T. 2004. Physiological responses in Nile tilapia exposed to different photoperiod regimes. *Journal of Fish Biology,* 65**,** 811-821.

BISWAS, A. K., SEOKA, M., TAKII, K., MAITA, M. & KUMAI, H. 2006. Stress response of red sea bream *Pagrus major* to acute handling and chronic photoperiod manipulation. *Aquaculture,* 252**,** 566-572.

BOLLIET, V., ALI, M. A., LAPOINTE, F. J. & FALCÓN, J. 1996. Rhythmic melatonin secretion in different teleost species: an in vitro study. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 165**,** 677-683.

BOLLIET, V., BÉGAY, V., TARAGNAT, C., RAVAULT, J. P., COLLIN, J. P. & FALCÓN, J. 1997. Photoreceptor cells of the pike pineal organ as cellular circadian oscillators. *European Journal of Neuroscience,* 9**,** 643-653.

BOUJARD, T. & LEATHERLAND, J. F. 1992. Circadian pattern of hepatosomatic index, liver glycogen and lipid content, plasma non-esterified fatty acid, glucose, T_3, T_4, growth hormone and cortisol concentrations in *Oncorhynchus mykiss* held under different photoperiod regimes and fed using demand-feeders. *Fish Physiology and Biochemistry,* 10**,** 111-122.

BROMAGE, N., JONES, J., RANDALL, C., THRUSH, M., DAVIES, B., SPRINGATE, J., DUSTON, J. & BARKER, G. 1992. Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* 100**,** 141-166.

BROMAGE, N., PORTER, M. & RANDALL, C. 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture,* 197**,** 63-98.

BUBENIK, G. A. & PANG, S. F. 1997. Melatonin levels in the gastrointestinal tissues of fish, amphibians, and a reptile. *General and Comparative Endocrinology,* 106**,** 415-419.

BUBENIK, G. A. 2002. Gastrointestinal melatonin: Localization, function, and clinical relevance. *Digestive Diseases and Sciences,* 47**,** 2336-2348.

BYE, V. J. 1984. The role of environmental factors in the timing of reproductive cycles. *In:* POTTS, G. W. & WOOTTON, R. J. (eds.) *Fish reproduction: strategies and tactics.* London: Academic Press.

CAHILL, G. M., GRACE, M. S. & BESHARSE, J. C. 1991. Rhythmic regulation of retinal melatonin: Metabolic pathways, neurochemical mechanisms, and the ocular circadian clock. *Cellular and Molecular Neurobiology,* 11**,** 529-560.

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

CAMPOS-MENDOZA, A., MCANDREW, B. J., COWARD, K. & 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.

CHALLET, E. 2007. Minireview: entrainment of the Suprachiasmatic clockwork in diurnal and nocturnal mammals. *Endocrinology,* 148**,** 5648-5655.

CHATTORAJ, A., BHATTACHARYYA, S., BASU, D., BHATTACHARYA, S., BHATTACHARYA, S. & MAITRA, S. K. 2005. Melatonin accelerates maturation inducing hormone (MIH): induced oocyte maturation in carps. *General and Comparative Endocrinology,* 140**,** 145-155.

CHATTORAJ, A., SETH, M., BASU, A., SHRIVASTAV, T. G., PORTA, S. & MAITRA, S. K. 2009. Temporal relationship between the circulating profiles of melatonin and ovarian steroids under natural photo-thermal conditions in an annual reproductive cycle in carp *Catla catla*. *Biological Rhythm Research,* 40**,** 347-359.

COON, S. L., BÉGAY, V., DEURLOO, D., FALCÓN, J. & KLEIN, D. C. 1999. Two arylalkylamine N-acetyltransferase genes mediate melatonin synthesis in fish. *Journal of Biological Chemistry,* 274**,** 9076-9082.

DAS, T., PAL, A. K., CHAKRABORTY, S. K., MANUSH, S. M., DALVI, R. S., APTE, S. K., SAHU, N. P. & BARUAH, K. 2009. Biochemical and stress responses of rohu

Labeo rohita and mrigal *Cirrhinus mrigala* in relation to acclimation temperatures. *Journal of Fish Biology,* 74**,** 1487-1498.
DAVIES, C. 2005. Introduction to Immunoassay Principles. *In:* WILD, D. (ed.) *The Immunoassay Handbook.* 3rd ed. Amsterdam: Elsevier.
DAVIES, P. R., HANYU, I., FURUKAWA, K. & NOMURA, M. 1986. Effect of temperature and photoperiod on sexual maturation and spawning of the common carp: III. Induction of spawning by manipulating photoperiod and temperature. *Aquaculture,* 52**,** 137-144.
DEY, R., BHATTACHARYA, S. & MAITRA, S. K. 2005. Importance of photoperiods in the regulation of ovarian activities in Indian major carp *Catla catla* in an annual cycle. *Journal of Biological Rhythms,* 20**,** 145-158.
DRORI, S., OFIR, M., LEVAVI-SIVAN, B. & YARON, Z. 1994. Spawning induction in Common carp (*Cyprinus carpio*) using pituitary extract or GnRH superactive analogue combined with metoclopramide: analysis of hormone profile, progress of oocyte maturation and dependence of temperature. *Aquaculture,* 119**,** 393-407.
DUFOUR, S., SEBERT, M. E., WELTZIEN, F. A., ROUSSEAU, K. & PASQUALINI, C. 2010. Neuroendocrine control by dopamine of teleost reproduction. *Journal of Fish Biology,* 76**,** 129-160.
EKSTRÖM, P. & MEISSL, H. 1997. The pineal organ of teleost fishes. *Reviews in Fish Biology and Fisheries,* 7**,** 199-284.
FALCÓN, J., MARMILLON, J. B., CLAUSTRAT, B. & COLLIN, J. P. 1989. Regulation of melatonin secretion in a photoreceptive pineal organ: an *in vitro* study in the pike. *Journal of Neuroscience,* 9**,** 1943-1950.
FALCÓN, J., THIBAULT, C., BEGAY, V., ZACHMANN, A. & COLLIN, J. P. 1992. *Regulation of the rhythmic melatonin secretion by fish pineal photoreceptor cells,* New York, Plenum Press Div Plenum Publishing Corp.
FALCÓN, J., GALARNEAU, K. M., WELLER, J. L., RON, B., CHEN, G., COON, S. L. & KLEIN, D. C. 2001. Regulation of arylalkylamine N-acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the fish pineal organ: evidence for a role of proteasomal proteolysis. *Endocrinology,* 142**,** 1804-1813.
FALCÓN, J., BESSEAU, L., SAUZET, S. & BOEUF, G. 2007. Melatonin effects on the hypothalamo–pituitary axis in fish. *Trends in Endocrinology and Metabolism,* 18**,** 81-88.
FALCÓN, J., BESSEAU, L., FUENTÈS, M., SAUZET, S., MAGNANOU, E. & BOEUF, G. 2009. Structural and functional evolution of the pineal melatonin system in vertebrates. *In:* VAUDRY, H., ROUBOS, E. W., COAST, G. M. & VALLARINO, M. (eds.) *Trends in Comparative Endocrinology and Neurobiology.* Oxford: Blackwell Publishing.
FALCÓN, J., MIGAUD, H., MUÑOZ-CUETO, J. A. & CARRILLO, M. 2010. Current knowledge on the melatonin system in teleost fish. *General and Comparative Endocrinology,* 165**,** 469-482.
FALCÓN, J., BESSEAU, L., MAGNANOU, E., HERRERO, M. J., NAGAI, M. & BOEUF, G. 2011. Melatonin, the time keeper: biosynthesis and effects in fish. *Cybium,* 35**,** 3-18.
FAO 2010. State of the world fisheries and aquaculture. Rome: Food and Agriculture Organization of the United Nations.
FAO 2012. *Cultured Aquatic Species Programme Cirrhinus mrigala* [Online]. FAO. Available: http://www.fao.org/fishery/culturedspecies/Cirrhinus_mrigala/en [Accessed 06.02 2012].

GARCÍA-ALLEGUE, R., MADRID, J. A. & SÁNCHEZ-VÁZQUEZ, F. J. 2001. Melatonin rhythms in European sea bass plasma and eye: influence of seasonal photoperiod and water temperature. *Journal of Pineal Research,* 31**,** 68-75.

GARCÍA-LÓPEZ, A., PASCUAL, E., SARASQUETE, C. & MARTÍNEZ-RODRÍGUEZ, G. 2006. Disruption of gonadal maturation in cultured Senegalese sole *Solea senegalensis* Kaup by continuous light and/or constant temperature regimes. *Aquaculture,* 261**,** 789-798.

GERN, W. A. & GREENHOUSE, S. S. 1988. Examination of *in vitro* melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination or continuous darkness. *General and Comparative Endocrinology,* 71**,** 163-174.

GLASSER, F., MIKOLAJCZYK, T., JALABERT, B., BAROILLER, J. F. & BRETON, B. 2004. Temperature effects along the reproductive axis during spawning induction of grass carp (*Ctenopharyngodon idella*). *General and Comparative Endocrinology,* 136**,** 171-179.

GUTTU, A. M. 2011. *Plasma melatonin profiles in copper mahseer (Neolissochilus hexagonolepis) kept under natural and manipulated photoperiods.* Master of Science, Norwegian University of Science and Technology (NTNU).

HAUGAN, S. 2012. *Nepal* [Online]. Store Norske Leksikon. Available: http://snl.no/Nepal [Accessed 19.04 2012].

HERRERO, M. J., MARTÍNEZ, F. J., MÍGUEZ, J. M. & MADRID, J. A. 2007. Response of plasma and gastrointestinal melatonin, plasma cortisol and activity rhythms of European sea bass (*Dicentrarchus labrax*) to dietary supplementation with tryptophan and melatonin. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 177**,** 319-326.

HOLTAN, M. B. 2011. *Plasma melatonin profiles in Common carp (Cyprinus carpio) exposed to indoor photoperiods.* Master of Science, Norwegian University of Science and Technology (NTNU).

HUETHER, G. 1993. The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. *Experientia,* 49**,** 665-670.

IIGO, M., FURUKAWA, K., HATTORI, A., OHTANI-KANEKO, R., HARA, M., SUZUKI, T., TABATA, M. & AIDA, K. 1997a. Ocular melatonin rhythms in the goldfish, *Carassius auratus*. *Journal of Biological Rhythms,* 12**,** 182-192.

IIGO, M., SÁNCHEZ-VÁZQUEZ, F. J., MADRID, J. A., ZAMORA, S. & TABATA, M. 1997b. Unusual responses to light and darkness of ocular melatonin in European sea bass. *Neuroreport,* 8**,** 1631-1635.

IIGO, M., MIZUSAWA, K., YOKOSUKA, M., HARA, M., OHTANI-KANEKO, R., TABATA, M., AIDA, K. & HIRATA, K. 2003. In vitro photic entrainment of the circadian rhythm in melatonin release from the pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-through culture. *Brain Research,* 982**,** 131-135.

IIGO, M., ABE, T., KAMBAYASHI, S., OIKAWA, K., MASUDA, T., MIZUSAWA, K., KITAMURA, S., AZUMA, T., TAKAGI, Y., AIDA, K. & YANAGISAWA, T. 2007a. Lack of circadian regulation of *in vitro* melatonin release from the pineal organ of salmonid teleosts. *General and Comparative Endocrinology,* 154**,** 91-97.

IIGO, M., FURUKAWA, K., NISHI, G., TABATA, M. & AIDA, K. 2007b. Ocular melatonin rhythms in teleost fish. *Brain Behavior and Evolution,* 69**,** 114-121.

IUVONE, P. M., TOSINI, G., POZDEYEV, N., HAQUE, R., KLEIN, D. C. & CHAURASIA, S. S. 2005. Circadian clocks, clock networks, arylalkylarnine N-acetyltransferase, and melatonin in the retina. *Progress in Retinal and Eye Research,* 24**,** 433-456.

IWAMA, G. K. 1998. Stress in Fish. *Annals of the New York Academy of Sciences,* 851**,** 304-310.
KAH, O. & DUFOUR, S. 2011. Conserved and divergent features of reproductive neuroendocrinology in teleost fishes. *In:* NORRIS, D. O. & LOPEZ, K. H. (eds.) *Hormones and Reproduction of Vertebrates.* London: Academic Press.
KNAPP, R. & CARLISLE, S. L. 2011. Testicular function and hormonal regulation in fishes. *In:* NORRIS, D. O. & LOPEZ, K. H. (eds.) *Hormones and Reproduction of Vertebrates.* London: Academic Press.
KUROGI, J. & LIDA, T. 1999. Social stress suppresses defense activities of neutrophils in tilapia. *Fish Pathology,* 34**,** 15-18.
LAIDLEY, C. W. & LEATHERLAND, J. F. 1988. Circadian studies of plasma cortisol, thyroid hormone, protein, glucose and ion concentration, liver glycogen concentration and liver and spleen weight in rainbow trout, *Salmo gairdneri* richardson. *Comparative Biochemistry and Physiology Part A: Physiology,* 89**,** 495-502.
LARSON, E. T., WINBERG, S., MAYER, I., LEPAGE, O., SUMMERS, C. H. & ØVERLI, Ø. 2004. Social stress affects circulating melatonin levels in rainbow trout. *General and Comparative Endocrinology,* 136**,** 322-327.
LEONARDI, M. O. & KLEMPAU, A. E. 2003. Artificial photoperiod influence on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) in the Southern Hemisphere. *Aquaculture,* 221**,** 581-591.
LONELYPLANET. 2011. *Map of Nepal* [Online]. Available: http://www.lonelyplanet.com/maps/asia/nepal/ [Accessed 19.04. 2012].
MAITRA, S. K., CHATTORAJ, A. & BHATTACHARYYA, S. 2005. Implication of melatonin in oocyte maturation in Indian major carp *Catla catla*. *Fish Physiology and Biochemistry,* 31**,** 201-207.
MAITRA, S. K. & CHATTORAJ, A. 2007. Role of photoperiod and melatonin in the regulation of ovarian functions in Indian carp *Catla catla*: basic information for future application. *Fish Physiology and Biochemistry,* 33**,** 367-382.
MAX, M. & MENAKER, M. 1992. Regulation of melatonin production by light, darkness, and temperature in the trout pineal. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* 170**,** 479-489.
MESSNER, M., HUETHER, G., LORF, T., RAMADORI, G. & SCHWÖRER, H. 2001. Presence of melatonin in the human hepatobiliary-gastrointestinal tract. *Life Sciences,* 69**,** 543-551.
MIGAUD, H., DAVIE, A., MARTINEZ CHAVEZ, C. C. & AL-KHAMEES, S. 2007. Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *Journal of Pineal Research,* 43**,** 327-335.
MIGAUD, H., DAVIE, A. & TAYLOR, J. F. 2010. Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. *Journal of Fish Biology,* 76**,** 27-68.
MYLONAS, C. C., FOSTIER, A. & ZANUY, S. 2010. Broodstock management and hormonal manipulations of fish reproduction. *General and Comparative Endocrinology,* 165**,** 516-534.
OKUZAWA, K. 2002. Puberty in teleosts. *Fish Physiology and Biochemistry,* 26**,** 31-41.
OLIVEIRA, C., GARCIA, E. M., LÓPEZ-OLMEDA, J. F. & SÁNCHEZ-VÁZQUEZ, F. J. 2009a. Daily and circadian melatonin release *in vitro* by the pineal organ of two nocturnal teleost species: Senegal sole (*Solea senegalensis*) and tench (*Tinca tinca*). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology,* 153**,** 297-302.

OLIVEIRA, C., VERA, L. M., LOPÉZ-OLMEDA, J. F., GUZMÁN, J. M., MAÑANÓS, E., RAMOS, J. & SÁNCHEZ-VÁZQUEZ, F. J. 2009b. Monthly day/night changes and seasonal daily rhythms of sexual steroids in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental conditions. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology,* 152**,** 168-175.

OLSEN, Y. A., EINARSDOTTIR, I. E. & NILSSEN, K. J. 1995. Metomidate anesthesia in Atlantic salmon, *Salmo salar*, prevents plasma-cortisol increase during stress. *Aquaculture,* 134**,** 155-168.

OMURA, Y. & OGURI, M. 1969. Histological studies on the pineal organ og 15 species of teleosts. *Bulletin of the Japanese Society for the Science of Fish,* 35**,** 991-1000.

OPPEDAL, F., TARANGER, G. L., JUELL, J. E., FOSSEIDENGEN, J. E. & HANSEN, T. 1997. Light intensity affects growth and sexual maturation of Atlantic salmon (*Salmo salar*) postsmolts in sea cages. *Aquatic Living Resources,* 10**,** 351-357.

PANKHURST, N. W. & PORTER, M. J. R. 2003. Cold and dark or warm and light: variations on the theme of environmental control of reproduction. *Fish Physiology and Biochemistry,* 28**,** 385-389.

PANKHURST, N. W. 2011. The endocrinology of stress in fish: an environmental perspective. *General and Comparative Endocrinology,* 170**,** 265-275.

PETER, R. E. & YU, K. L. 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. *Reviews in Fish Biology and Fisheries,* 7**,** 173-197.

POPEK, W., ŁUSZCZEK-TROJNAR, E., DRĄG-KOZAK, E., FORTUNA-WROŃSKA, D. & EPLER, P. 2005. Effect of the pineal gland and melatonin on dopamin release from perifused hypothalamus of mature female carp during spawning and winter regression. *Acta Ichthyologica et Piscatoria,* 35**,** 65 - 71.

POPEK, W., ŁUSZCZEK-TROJNAR, E., DRĄG-KOZAK, E., RZĄSA, J. & EPLER, P. 2006. Effect of melatonin on dopamin secretion in the hypothalamus of mature female common carp, *Cyprinus carpio* L. *Acta Ichthyologica et Piscatoria,* 36**,** 135 - 141.

PRASAI, M. J., PERNICOVA, I., GRANT, P. J. & SCOTT, E. M. 2011. An endocrinologist's guide to the clock. *Journal of Clinical Endocrinology & Metabolism,* 96**,** 913-922.

PRESTRUD, M. 2012 (in prep.). *Plasma melatonin profiles in silver carp (Hypophthalamichtys molitrix) exposed to natural and experimental photoperiod.* Master of science, Norwegian University of Science and Technology (NTNU).

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

RUANE, N. M., HUISMAN, E. A. & KOMEN, J. 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. *Journal of Fish Biology,* 59**,** 1-12.

SARKAR, S. K., SAHA, A., DASGUPTA, S., NANDI, S., VERMA, D. K., ROUTRAY, P., DEVARAJ, C., MOHANTY, J., SARANGI, N., EKNATH, A. E. & AYYAPPAN, S. 2010. Photothermal manipulation of reproduction in Indian major carp: a step forward for off-season breeding and seed production. *Current Science,* 99**,** 960-964.

SCHULZ, R. W., ANDERSSON, E. & TARANGER, G. L. 2006. Photoperiod manipulation can stimulate or inhibit pubertal testis maturation in Atlantic salmon (*Salmo salar*). *Animal Reproduction,* 3**,** 121-126.

SELYE, H. 1973. The evolution of the stress concept: the originator of the concept traces its development from the discovery in 1936 of the alarm reaction to modern therapeutic applications of syntoxic and catatoxic hormones. *American Scientist,* 61**,** 692-699.

SETH, M. & MAITRA, S. K. 2010. Photoreceptor proteins and melatonin rhythm generating AANAT in the carp pineal: temporal organization and correlation with natural photo-thermal cues. *Journal of Photochemistry and Photobiology B: Biology,* 99**,** 21-28.

SIMONNEAUX, V. & RIBELAYGA, C. 2003. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacological Reviews,* 55**,** 325-395.

SPRN 2008. Sustainable poverty reduction in Nepal by combining fish farming and education with eco-friendly hydropower (2009-2012). Kathmandu: Sustainable Poverty Reduction in Nepal (SPRN).

STACEY, N. E. 1984. Control of the the timing of ovulation by exogenous and endogenous factors. *In:* POTTS, G. W. & WOOTTON, R. J. (eds.) *Fish reproduction: strategies and tactics.* London: Academic Press.

SUSILO, E. S., HARNADI, L. & TAKEMURA, A. 2009. Tropical monsoon environments and the reproductive cycle of the orange-spotted spinefoot *Siganus guttatus*. *Marine Biology Research,* 5**,** 179-185.

TACON, A. G. J. & METIAN, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* 285**,** 146-158.

TARANGER, G. L., CARRILLO, M., SCHULZ, R. W., FONTAINE, P., ZANUY, S., FELIP, A., WELTZIEN, F.-A., DUFOUR, S., KARLSEN, Ø., NORBERG, B., ANDERSSON, E. & HANSEN, T. 2010. Control of puberty in farmed fish. *General and Comparative Endocrinology,* 165**,** 483-515.

THORPE, J. E., TALBOT, C., MILES, M. S. & KEAY, D. S. 1990. Control of maturation in cultured Atlantic salmon, *Salmo salar*, in pumped seawater tanks, by restricting food intake. *Aquaculture,* 86**,** 315-326.

THORPE, J. E. 2004. Life history responses of fishes to culture. *Journal of Fish Biology,* 65**,** 263-285.

URBATZKA, R., ROCHA, M. J. & ROCHA, E. 2011. Regulation of ovarian development and function in teleosts. *In:* NORRIS, D. O. & LOPEZ, K. H. (eds.) *Hormones and Reproduction of Vertebrates.* London: Academic Press.

VELARDE, E., CERDÁ-REVERTER, J. M., ALONSO-GÓMEZ, A. L., SANCHÉZ, E., ISORNA, E. & DELGADO, M. J. 2010. Melatonin-synthesizing enzymes in pineal,retina, liver, and gut of the goldfish (caurassius): mRNA expression pattern and regulation of daily rhythms bt lighting conditions. *Chronobiology International,* 27**,** 1178-1201.

WALTON, J. C., WEIL, Z. M. & NELSON, R. J. 2011. Influence of photoperiod on hormones, behavior, and immune function. *Frontiers in Neuroendocrinology,* 32**,** 303-319.

WEITZMAN, E. D., FUKUSHIMA, D., NOGEIRE, C., ROFFWARG, H., GALLAGHE.T.F. & HELLMAN, L. 1971. 24 hour pattern of episodic secretion of cortisol in normal subjects. *Journal of Clinical Endocrinology & Metabolism,* 33**,** 14.

WEITZMAN, E. D. 1976. Circadian rhythms and episodic hormone secretion in man. *Annual Review of Medicine,* 27**,** 225-243.

WENDELAAR BONGA, S. E. 1997. The stress response in fish. *Physiological Reviews,* 77**,** 591-625.

WFP 2012. *Nepal overview* [Online]. World Food Programme. Available: http://www.wfp.org/countries/Nepal/Overview [Accessed 26.04. 2012].

WISSER, H. & BREUER, H. 1981. Circadian changes of clinical, chemical and endocrinological parameters. *Journal of Clinical Chemistry and Clinical Biochemistry,* 19**,** 323-337.

ZHANG, L., WENG, W. & GUO, J. 2011. Posttranscriptional mechanisms in controlling eukaryotic circadian rhythms. *FEBS Letters,* 585**,** 1400-1405.

Appendix 1: Statistics experiment 1

One Way Analysis of Variance onsdag, mars 28, 2012, 13:10:39

Data source: Statistics experiment 1

Normality Test (Shapiro-Wilk) Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks onsdag, mars 28, 2012, 13:10:39

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
12:00	6	0	98,928	82,625	145,171
17:00	6	0	136,266	40,876	231,575
19:00	6	0	421,150	312,974	446,922
21:00	6	0	265,276	233,821	376,862
23:00	6	0	215,044	97,242	307,071
01:00	6	0	185,816	71,825	273,861
03:00	6	0	176,484	82,832	328,030
05:00	6	0	235,908	179,824	288,916
07:00	6	0	161,621	133,021	234,565
12:00-2	6	0	109,832	7,060	155,130

H = 20,698 with 9 degrees of freedom. (P = 0,014)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,014)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0,05
19:00 vs 12:00	188,000	4,395	No
19:00 vs 12:00-2	185,000	4,325	Do Not Test
19:00 vs 17:00	144,000	3,366	Do Not Test
19:00 vs 01:00	109,000	2,548	Do Not Test
19:00 vs 07:00	104,000	2,431	Do Not Test
19:00 vs 03:00	104,000	2,431	Do Not Test
19:00 vs 23:00	86,000	2,010	Do Not Test
19:00 vs 05:00	51,000	1,192	Do Not Test
19:00 vs 21:00	9,000	0,210	Do Not Test
21:00 vs 12:00	179,000	4,184	Do Not Test
21:00 vs 12:00-2	176,000	4,114	Do Not Test
21:00 vs 17:00	135,000	3,156	Do Not Test
21:00 vs 01:00	100,000	2,338	Do Not Test
21:00 vs 07:00	95,000	2,221	Do Not Test
21:00 vs 03:00	95,000	2,221	Do Not Test
21:00 vs 23:00	77,000	1,800	Do Not Test
21:00 vs 05:00	42,000	0,982	Do Not Test
05:00 vs 12:00	137,000	3,203	Do Not Test
05:00 vs 12:00-2	134,000	3,132	Do Not Test
05:00 vs 17:00	93,000	2,174	Do Not Test
05:00 vs 01:00	58,000	1,356	Do Not Test
05:00 vs 07:00	53,000	1,239	Do Not Test

05:00 vs 03:00	53,000	1,239	Do Not Test
05:00 vs 23:00	35,000	0,818	Do Not Test
23:00 vs 12:00	102,000	2,384	Do Not Test
23:00 vs 12:00-2	99,000	2,314	Do Not Test
23:00 vs 17:00	58,000	1,356	Do Not Test
23:00 vs 01:00	23,000	0,538	Do Not Test
23:00 vs 07:00	18,000	0,421	Do Not Test
23:00 vs 03:00	18,000	0,421	Do Not Test
03:00 vs 12:00	84,000	1,964	Do Not Test
03:00 vs 12:00-2	81,000	1,893	Do Not Test
03:00 vs 17:00	40,000	0,935	Do Not Test
03:00 vs 01:00	5,000	0,117	Do Not Test
03:00 vs 07:00	0,000	0,000	Do Not Test
07:00 vs 12:00	84,000	1,964	Do Not Test
07:00 vs 12:00-2	81,000	1,893	Do Not Test
07:00 vs 17:00	40,000	0,935	Do Not Test
07:00 vs 01:00	5,000	0,117	Do Not Test
01:00 vs 12:00	79,000	1,847	Do Not Test
01:00 vs 12:00-2	76,000	1,777	Do Not Test
01:00 vs 17:00	35,000	0,818	Do Not Test
17:00 vs 12:00	44,000	1,029	Do Not Test
17:00 vs 12:00-2	41,000	0,958	Do Not Test
12:00-2 vs 12:00	3,000	0,0701	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

Appendix 2: Statistics experiment 3

One Way Analysis of Variance lørdag, mai 05, 2012, 15:52:06

Data source: Statistics experiment 3

Normality Test (Shapiro-Wilk) Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks lørdag, mai 05, 2012, 15:52:06

Data source: Data 1 in Statistikk melatonin forsøk 3

Group	N	Missing	Median	25%	75%
12:00-1	6	0	93,513	50,551	114,454
15:30	6	0	72,345	26,649	90,398
19:00	6	0	699,824	533,431	1186,494
22:00	6	0	1145,804	848,039	1385,655
02:00	6	0	994,485	600,207	2169,582
05:00	6	0	1592,926	1071,157	2324,029
08:30	6	0	81,331	65,060	121,513
12:00-2	6	0	121,862	54,627	195,612

H = 36,571 with 7 degrees of freedom. (P = <0,001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0,05
05:00 vs 15:30	196,000	5,715	Yes
05:00 vs 12:00-1	172,000	5,016	Yes
05:00 vs 08:30	171,000	4,986	Yes
05:00 vs 12:00-2	161,000	4,695	Yes
05:00 vs 19:00	63,000	1,837	No
05:00 vs 02:00	38,000	1,108	Do Not Test
05:00 vs 22:00	31,000	0,904	Do Not Test
22:00 vs 15:30	165,000	4,811	Yes
22:00 vs 12:00-1	141,000	4,112	No
22:00 vs 08:30	140,000	4,082	Do Not Test
22:00 vs 12:00-2	130,000	3,791	Do Not Test
22:00 vs 19:00	32,000	0,933	Do Not Test
22:00 vs 02:00	7,000	0,204	Do Not Test
02:00 vs 15:30	158,000	4,607	Yes
02:00 vs 12:00-1	134,000	3,908	Do Not Test
02:00 vs 08:30	133,000	3,878	Do Not Test
02:00 vs 12:00-2	123,000	3,587	Do Not Test
02:00 vs 19:00	25,000	0,729	Do Not Test
19:00 vs 15:30	133,000	3,878	No
19:00 vs 12:00-1	109,000	3,179	Do Not Test
19:00 vs 08:30	108,000	3,149	Do Not Test
19:00 vs 12:00-2	98,000	2,858	Do Not Test
12:00-2 vs 15:30	35,000	1,021	Do Not Test
12:00-2 vs 12:00-1	11,000	0,321	Do Not Test

12:00-2 vs 08:30	10,000	0,292	Do Not Test
08:30 vs 15:30	25,000	0,729	Do Not Test
08:30 vs 12:00-1	1,000	0,0292	Do Not Test
12:00-1 vs 15:30	24,000	0,700	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.