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Plasma melatonin profiles in silver carp (*Hypophthalmichthys molitrix*) during natural and manipulated photoperiods

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Marit Prestrud

Abstract

Photoperiodic manipulation of reproductive events in fish is suggested to secure all-year supply of fish fry in the Nepalese aquaculture. This could significantly improve food security and decrease poverty in Nepal. In order to achieve this, knowledge about the teleost melatonin system and reproduction is needed.

The present study examined blood plasma profiles of the hormone melatonin in silver carp (*Hypophthalmichthys molitrix*) in Nepal during natural and manipulated photoperiods. Plasma melatonin levels throughout the natural 24-hour light-dark cycle were low during the light period, and increased several folds during the dark period. Peak level was reached early in the dark period, 2.5 hours after onset of darkness. Thereafter levels decreased prior to sunrise. When the silver carps were exposed to continuous darkness, the diurnal rhythmical melatonin levels observed during natural light-dark cycle were sustained. The melatonin levels during the time of the natural light period were low, and increased significantly during the time of the natural dark period, even if the fish were deprived of time signals in the light-dark cycle. In another experiment, silver carps were subjected to a period of two hours darkness at different times during the natural light period. Darkness at morning and midday did not increase plasma melatonin levels, whereas darkness during late light-phase gave a significant increase in plasma melatonin levels. The findings in this study suggest that melatonin synthesis from the pineal gland in silver carp is controlled by an endogenous biological clock. Plasma cortisol levels were measured, and were relatively high. However, it is suggested that cortisol may not have influenced the plasma melatonin levels and fluctuations in the silver carps in the present study significantly.

Sammendrag

Manipulering av fotoperiode for å kontrollere reproduksjon hos teleoster kan være med på å sikre helårstilgang av fiskeyngel i nepalsk fiskeoppdrett. Dette kan bidra til økt matsikkerhet og å redusere fattigdom i Nepal. For å kunne oppnå dette er det nødvendig med kunnskap om fiskenes melatoninsystem og reproduksjon.

Dette studiet har undersøkt plasmaprofiler av hormonet melatonin hos sølvkarpe (*Hypophthalmichthys molitrix*) i Nepal under naturlig og manipulerte fotoperioder. Melatoninnivået gjennom et døgn hos sølvkarpe ved naturlig fotoperiode var lavt gjennom dagen, og viste en flerdoblet økning om natten. Det høyeste melatoninnivået ble funnet tidlig i den naturlige mørkeperioden, 2,5 timer etter at det ble mørkt. Deretter sank melatoninnivået gradvis mot soloppgang. Da sølvkarper ble eksponert for konstant mørke, vekslet rytmen av melatoninnivå i plasma gjennom et døgn på samme måte som for sølvkarper ved naturlig fotoperiode. Melatoninnivåene var lave ved perioden for den naturlige lysfasen, og i perioden for den naturlige mørkefasen var melatoninnivåene signifikant høyere, til tross for at fiskene manglet signaler angående tid på døgnet fra omgivelsene. I et annet eksperiment ble tre grupper med sølvkarper eksponert for en periode på to timer mørke til forskjellig tid under den naturlige lysfasen. Eksponering for mørke ved morgen og formiddag førte ikke til noen endring i melatoninnivåene, mens mørke ved ettermiddagen førte til en signifikant økning i melatoninnivået hos sølvkarpe. Hovedfunnene i dette studiet indikerer at melatoninsyntesen i pinealkjertelen til sølvkarpe kontrolleres av en endogen biologisk klokke. Nivå av kortisol i plasma ble også målt. Kortisolkonsentrasjonen i sølvkarpene som ble brukt i eksperimentene var høye. Det er foreslått at de høye kortisolkonsentrasjonene sannsynligvis ikke har hatt en signifikant påvirkning på melatoninnivået og de rytmiske variasjonene av melatonin gjennom døgnet i plasma til sølvkarpe.

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1 Introduction

Teleost fishes constitute a highly diverse group of vertebrates, with more than 25 000 different species. This single group thereby accounts for almost half of all known vertebrate species today. According to Bone and Moore (2008), they inhabit almost all aquatic habitats on earth, and are adapted to a variety of abiotic and biotic factors. Fish are found in environments with remarkable differences in temperature. They are also adapted to extreme variations in salinity, and several species are capable of inhabiting both freshwater and saltwater environments. This diversity contributes to the great variety in life history strategies and reproduction among teleosts, which is seen in events like gametogenesis, spawning, and parental care. It is therefore interesting to elucidate reproductive mechanisms in teleost fishes further.

Aquaculture is the fastest growing animal food production sector in the world. In 2006, total fish production from global aquaculture was reported to be 47.3 million tons, and four years later it increased to 59.9 million tons (FAO 2012a). Understanding teleost biology is important for management and development of the aquaculture sector. Further productivity increase is especially dependent on improved delivery of fish fry. Aquaculture is also considered an important contributor to increase food security and reduce poverty in developing countries. Accordingly, manipulation and control of teleost reproduction is currently a topic for scientific studies.

1.1 Teleost reproduction

Teleost reproductive events show periodicity throughout an annual cycle, and are controlled by the neuroendocrine system. In most fish species, reproduction is synchronized with environmental changes, to ensure that spawning takes place at the time of optimum conditions for survival of the offspring (Bromage *et al.* 2001, Carnevali *et al.* 2011). Important structures involved in teleost reproduction are the brain, pituitary gland, gonad, and the pineal gland. In females, the liver is additionally involved in reproductive events (fig. 1) (Kryvi and Totland 1997).

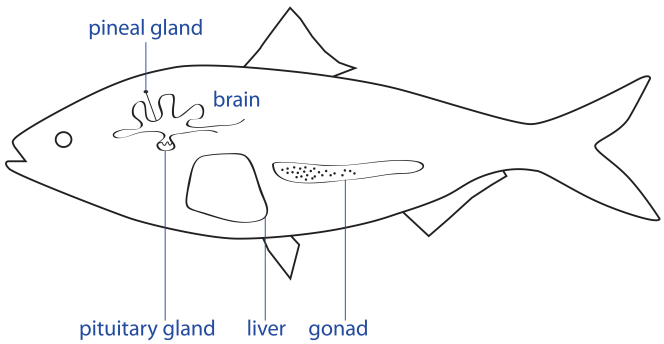

Figure 1. Anatomical structures involved in teleost reproduction (modified from Kryvi and Totland 1997).

The hypothalamic-pituitary-gonadal- (HPG) axis releases hormones involved in control of puberty, gametogenesis and spawning in fish (fig. 2). Stimuli from the external environment, such as the light-dark cycle and food availability (Falcón *et al.* 2010a), and the internal environment, such as nutritional status (Copeland *et al.* 2011), are important cues regulating the activity of the HPG-axis.

Gonadotropin-releasing hormone (GnRH), synthesized in the preoptic area (POA) of the hypothalamus, is secreted by GnRH-neurons innervating the pituitary gland. This hormone stimulates the adeno-pituitary to release the two gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the circulation (Yaron and Sivan 2006, Kah and Dufour 2011). FSH and LH binds to receptors on the gonads, and are responsible for regulating synthesis of sex steroids, the estrogen estradiol-17β (E2), the progestin 17,20β-dihydroxy-4-pregnen-3-one (DHP) and the androgen 11-ketotestosterone (11-KT) (Kazeto *et al.* 2011). These steroids are involved in controlling gamete growth, gamete maturation and spawning (Thomas and Rahman 2009). The liver is stimulated by E2 to synthesize the precursor of yolk proteins, vitellogenin (Vtg), which gives large increase in oocyte size (Thomas and Rahman 2009). Both E2 and 11-KT exerts feedback regulation on the HPG-axis (Van Der Kraak 2009).

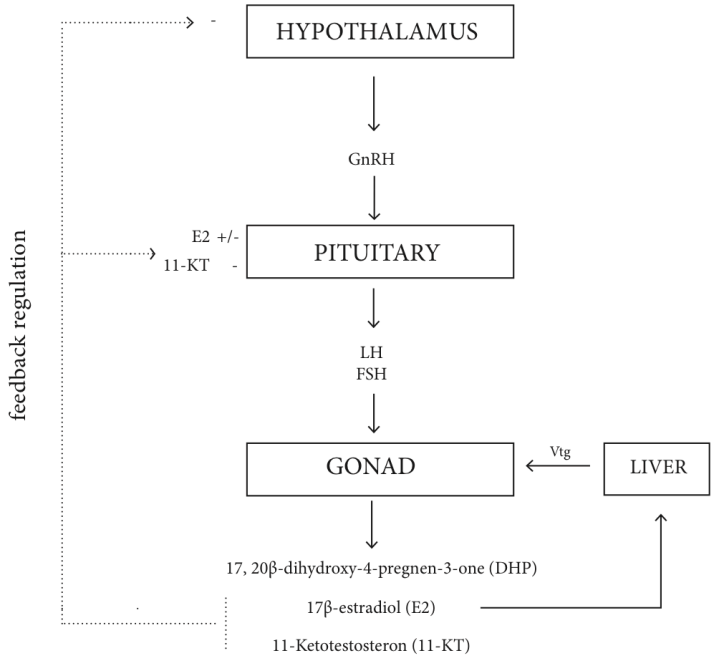

Figure 2. The hypothalamic-pituitary-gonadal axis (HPG-axis) in teleost fish (modified from Thomas and Rahman 2009).

1.2 Biological rhythms

In addition to reproduction, teleosts display rhythmicity in several other physiological and biochemical events throughout the day and year, such as food intake and growth (Falcón 1999, Cymborowski 2010). Most of these oscillations are endogenous rhythms controlled by the activity of internal biological clocks. They are synchronized to daily and seasonal changes in the surroundings by different environmental factors, called zeitgebers (Aschoff 1960). The most important zeitgeber entraining the biological clocks are changes in the light-dark cycle throughout the day and year (Roenneberg and Foster 1997, Falcón *et al.* 2011). Other factors in the external environment, such as temperature, salinity and food availability may also influence these internal oscillations (Falcón *et al.* 2007).

The biological clock functions as a timekeeper and makes the fishes capable of anticipating and preparing for changes in the environment, and thus optimize survival (Roenneberg and Foster 1997, Falcón *et al.* 2011). The clock activity continues even in the absence of time cues in the external environment. Oscillations driven by the clock run in periods close to 24 hours or a year, thus, they are called circadian or circannual rhythms, respectively (Cymborowski 2010, Falcón *et al.* 2010a). Sometimes the physiological rhythms follow the environmental cues passively, and are not driven by an endogenous biological clock (Cahill 2002, Falcón *et al.* 2010a).

The circadian system includes a clock (or pacemaker), located in the nervous system, an entrainment pathway to the clock with receptors responding to environmental factors, and one or more output signals (Falcón 1999). The clock controls processes through rhythmic neuroendocrine and nervous outputs, which impacts on various cells and tissues. The activity of the clock is based on auto-regulatory alternations between high or low levels of expression of specific clock genes, in a period of approximately 24 hours. These clock proteins regulate the activity of many other genes, which leads to a cyclical expression of their products (Cahill 2002, Falcón *et al.* 2010a). Neurons in the suprachiasmatic nucleus in the hypothalamus are the major site for the master circadian clocks in mammals, with input and output pathways located in separate areas (Falcón *et al.* 2007). In teleosts however, the master circadian clocks resides in both the pineal gland and the eyes, where photoperiodic information synchronizes the activity of the clocks (fig. 3). The two types of output signals from the clocks in the eyes and pineal gland are neural information to the brain, and hormonal information through synthesis of the hormone melatonin (N-acetyl-5-methoxytryptamine). It is also suggested that master circadian clocks might be found in the hypothalamic area in the fish brain (fig. 3) (Falcón *et al.* 2007).

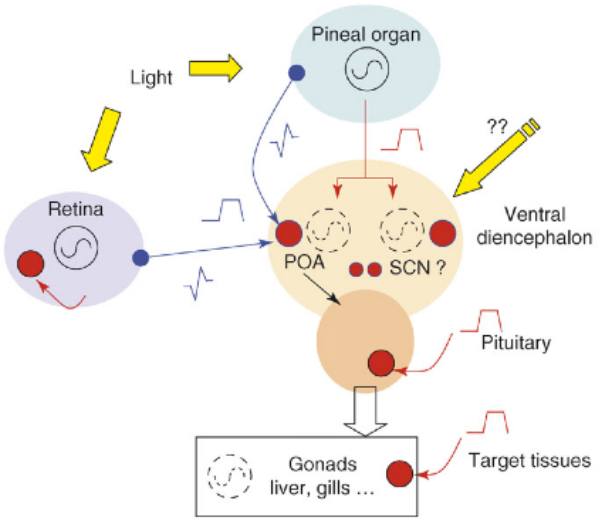

Figure 3. Photoneuroendocrine regulation in teleosts. Light synchronizes the activity of the biological clocks (black circles) inside the teleost retina and pineal gland. Light may also act on possible biological clocks in the hypothalamus. Output signals from the clocks are neural information (blue arrows), which is sent to the brain, and hormonal information by melatonin (red arrows). Pineal melatonin is released to the circulation and impacts on different target sites through melatonin receptors (red circles) (modified from Falcón *et al.* 2007).

1.3 The pineal gland and melatonin

The pineal gland in teleost fishes is a neuroendocrine gland located just underneath the skull, formed as an end-vesicle connected to diencephalon through a pineal stalk (Ekström and Meissl 1997). This gland is directly photosensitive (fig. 4) (Falcón 1999, Laurá *et al.* 2012). The part of the skull covering the gland is often thinner, and the skin is less pigmented to facilitate light penetration (Ekström 1987). There are three types of cells in the pineal gland; pinealocytes, nerve cells and interstitial cells (Ekström and Meissl 1997). The pinealocytes are the site of production of the neural and hormonal information from the pineal gland. Both of these output pathways shows rhythmicity (Erlich and Apuzzo 1985, Ekström and Meissl 1997, Boeuf and Falcón 2001).

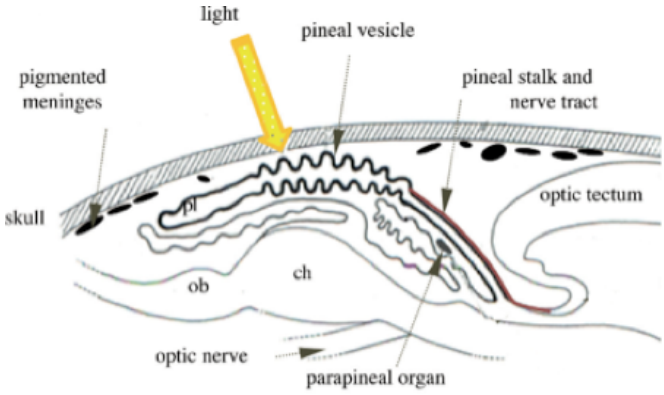

Figure 4. Fish brain cut in a sagittal plane showing the pineal vesicle located just beneath the skull. ob: olfactory bulb, ch: cerebral hemispheres, pl: pineal lumen **(**modified from Falcón *et al*. 2011).

Pinealocytes are photoreceptor cells, which convert light signals into electrical signals. In response to darkness, these cells are depolarized and release neurotransmitters, as glutamate and aspartate (McNulty *et al.* 1988, Vigh *et al.* 1995). This activates the nerve cells, and hence photoperiodic information is sent to the brain through a nervous message. In response to light, photoreceptors are hyperpolarized, inhibiting the release of neurotransmittors. Consequently, nerve cells from the pineal gland are activated during darkness (Falcón 1999, Boeuf and Falcón 2001).

Melatonin synthesized in the pinealocytes is released into the blood circulation. In teleosts, and all other vertebrates studied, the plasma melatonin levels show rhythmicity throughout the 24–hour light-dark cycle (Migaud *et al.* 2010). Plasma melatonin concentration in teleosts is low during the light phase, and higher during the dark phase (Falcón *et al.* 1987, Bolliet *et al.* 1996). The shape of the diurnal melatonin rhythms varies throughout the year, as the duration of nocturnal melatonin release and amplitude varies with season (Falcón *et al.* 2007). Thereby, the melatonin levels provide the fish information about day-length, and season (Reiter *et al.* 2010, Falcón *et al.* 2010a, Falcón *et al.* 2011). Melatonin is considered to be involved in regulating processes showing daily and annually rhythmicity, to make sure they are synchronized to the environment (Falcón *et al.* 2011).

Many effects of melatonin are mediated through specific melatonin receptors that are widely distributed in the organism. Three types of receptors have been identified in fish; the MT1, MT2 and Mel 1c. They are found in the brain, liver, kidneys, intestine, gonads and gills. The abundance of these receptors shows daily and annual variations. Thus, effects of melatonin are dependent on the availability of binding sites throughout the day and year, as well as its daily and annual rhythmic release from the pineal gland (Reiter *et al.* 2010, Falcón *et al.* 2011).

1.3.1 Melatonin synthesis and regulation

The basic mechanism of melatonin biosynthesis seems to be identical for all vertebrates (Seth and Maitra 2011). Production of pineal melatonin takes place in the photoreceptor cells, through a biosynthetic pathway consisting of four enzymatic steps (Falcón *et al.* 2011). The amino acid tryptophan is taken up from the blood, and by the action of four different enzymes; tryptophan hydroxylase (TPOH) (Lovenberg *et al.* 1967), aromatic amino acid decarboxylase (AAAD) (Lovenberg *et al.* 1962), arylalkylamin N-acetyltransferase (AANAT) (Weissbach *et al.* 1960) and hydroxyindole-O-methyltransferase (HIOMT) (Axelrod and Weissbach 1961) melatonin is synthesized.

Teleost fishes are unique because they express two different types of AANAT genes, AANAT-1 mainly in retina, and AANAT-2 mainly in the pineal gland. In some teleosts, a second type of AANAT1 gene has been detected (Coon and Klein 2006). It is well established that the activity of AANAT-2 enzymes are responsible for driving the rhythmic production of melatonin in the pineal gland (Cazaméa-Catalan *et al.* 2012). AANAT-2 levels, and thereby

also melatonin levels, are low during the light-phase and high during the dark-phase (Klein and Weller 1970, Klein *et al.* 1997).

In most teleost species studied, an intra-pineal biological clock system controls the rhythmic melatonin secretion (Bolliet *et al.* 1996, Falcón 1999). During the dark-phase the clock stimulates higher transcription of AANAT-2 mRNA, and the levels increase more than during the light-phase in most teleost species investigated. Under constant darkness, the rhythm in AANAT-2 mRNA levels is sustained, and melatonin levels continue to show internal rhythmicity for days (Bégay *et al.* 1998, Coon *et al.* 1999, Falcón *et al.* 2011). The activity of the enzyme AANAT-2 is directly inhibited by light, and thereby continuous light exposure gives constant low melatonin levels throughout 24-hours, while the rhythmicity in mRNA levels persists (Falcón *et al.* 2010a). The light-dark cycle modifies the activity of the biological clock, which adjusts the AANAT-2 rhythm and determines the phase of the melatonin rhythm (Falcón *et al.* 2001). Any changes in this zeitgeber could give a phase-shift in the oscillations of plasma melatonin (Falcón 1999, Falcón *et al.* 2010b).

Studies have demonstrated lack of circadian regulation of melatonin secretion from the pineal gland in some salmonids (Bolliet *et al.* 1996, Iigo *et al.* 1997, Masuda *et al.* 2003, Iigo *et al.* 2007). Without an intra-pineal biological clock controlling AANAT-2 mRNA levels, trout (*Oncorhynchus mykiss*) showed a constant expression of AANAT-2 mRNA throughout the 24-hour cycle (Bégay *et al.* 1998). In fishes lacking a biological clock, melatonin levels follow the light-dark cycle passively, and are high during the dark, and low during light possibly as a consequence of the lower stability of AANAT-2 proteins in light (Klein *et al.* 1997, Coon *et al.* 1998).

Non-photic factors such as temperature (Max and Menaker 1992, Zachmann *et al.* 1992) and various internal factors such as steroids, melatonin, adenosine, GABA, and catecholamines (Falcón et al. 2010b) may also be involved in regulating pineal melatonin synthesis. However, these non-photic factors are suggested to modulate the plasma amplitude melatonin, while the photoperiod regulates the melatonin rhythmicity (Migaud *et al.* 2010, Falcón *et al.* 2011).

Three different species-specific melatonin profiles throughout 24-hour for vertebrates has been described (fig. 5). These profiles are different in the time of nocturnal melatonin peak, and the duration of the peak (Reiter 1987, Reiter 1991).

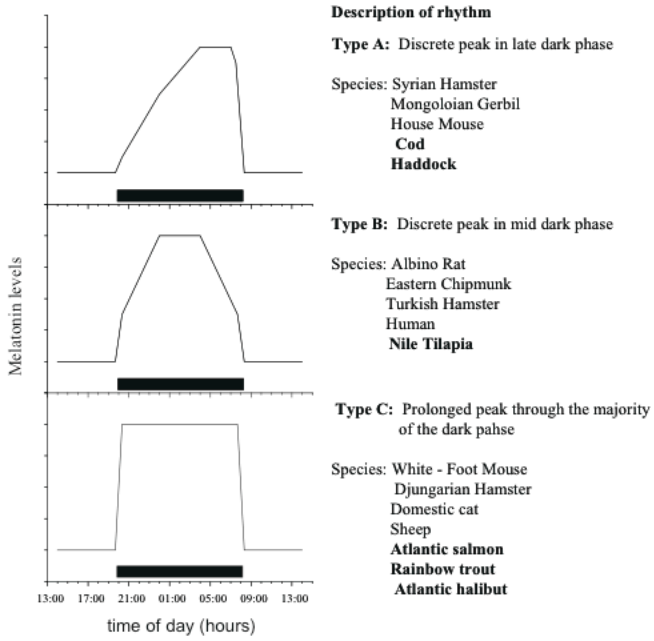

Figure 5. Vertebrate melatonin profiles (adopted from Falcón *et al.* 2010a).

1.3.2 Regulation of reproduction and effects of melatonin

The activity of the HPG-axis in teleosts is regulated by different internal and external factors (Bromage *et al.* 2001, Kah *et al.* 2010). They are unique among vertebrates because regulation of the HPG-axis takes place at both the hypothalamus and the pituitary gland (Kah and Dufour 2011).

Changes in photoperiod throughout the year are suggested to be the main environmental cue synchronizing the reproductive events with seasonal changes in temperate teleost species (Bromage *et al.* 2001). In this way, reproduction can take place at optimum environmental conditions to increase survival of the offspring (Dey *et al.* 2005). The timing of reproductive events, such as gonadal maturation, has been changed in several species by experimental manipulation of photoperiod (Bromage *et al.* 2001). Impacts from changes in photoperiod on reproductive events in fish are suggested to be mediated mainly through the action of melatonin. The changing patterns of plasma melatonin throughout the day and year, as a result of changing photoperiod, are considered to influence the HPG-axis in the fish (Bromage *et al.* 2001). However, the mechanisms on how photoperiod and melatonin influence on reproduction remains to be clarified and resolved (Bromage *et al.* 2001, Migaud *et al.* 2010, Falcón *et al.* 2011). In a review article on temperate teleost species, Migaud *et al.* (2010) indicated that a possible biological clock might be involved in mediating the photoperiodic effects on reproduction. The mechanisms of this clock, and its role in reproduction are unclear (Migaud *et al.* 2010).

Several experimental studies have demonstrated the effect of melatonin on different levels of the HPG-axis. It is considered to impact both directly and indirectly at different levels of the axis (fig. 6, p. 9). Melatonin receptors are found in different areas in the brain, such as the preoptic area, and the pituitary of some teleosts (Falcón *et al.* 2007, Sébert *et al.* 2008). It is

also suggested to act directly on the gonads (Amano *et al.* 2004, Maitra *et al.* 2005, Carnevali *et al.* 2011). Melatonin was indicated to be involved in oocyte maturation in Indian Carp (*Catla catla*), and the effects of melatonin were pro-gonadal or anti-gonadal, dependent on the stage of sexual development of the fish (Maitra *et al.* 2005). The impacts of melatonin were suggested to operate through influence on the maturation-inducing hormone (MIH) (Chattoraj *et al.* 2005, Maitra *et al.* 2005).

Treatment with melatonin increased the fecundity of zebrafish (*Danio rerio*) (Carnevali *et al.* 2011), and stimulated the release of LH in the Atlantic croaker (*Micropogonias undulatus*), acting both at the preoptic hypothalamic area, and the pituitary gland. The effects of melatonin varied with the reproductive status (Khan and Thomas 1996). In the case of European sea bass (*Dicentrarchus labrax*), artificial light changed the melatonin levels and the release of LH, and melatonin was considered to be involved in regulating reproduction (Bayarri *et al.* 2004a). Melatonin treatment gave a reduction in the activity of the pituitary-gonad axis in the eel (*Anguilla anguilla*), suggesting a negative effect of melatonin (Sébert *et al.* 2008).

Several experiments show that effects of melatonin on reproduction depend on the annual developmental stage of the experimental fish. Whether it is a first time spawner, immature veteran spawner, or maturing fish will give different endocrine settings and expectations for the physiological effects of the melatonin molecule. The effects of melatonin may also depend on other factors, such as the species, temperature and sex (Zachmann *et al.* 1992, Dufour *et al.* 2010). This could explain some of the different results from experiments investigating the effect of melatonin on reproduction.

Recently, it is been indicated that some of the effects of melatonin on the HPG-axis are mediated through interactions with the dopaminergic-system (Popek *et al.* 2005, Sébert *et al.* 2008, Dufour *et al.* 2010). The dopaminergic system has inhibitory impact on the HPG-axis in teleosts (Van Der Kraak 2009). Effects of dopamine (DA) are mediated through the DA-D1 and DA-D2 receptors found in the brain and the pituitary. The inhibitory effects from DA on reproductive events are carried out through interactions with the GnRH-neurons, and direct actions on gonadotropin release of FSH and LH (Peter *et al.* 1986, Vacher *et al.* 2000, Dufour *et al.* 2010).

Kisspeptins are neuropeptides synthesized in the brain, and are involved in the regulation of reproduction in vertebrates, including teleosts. Their effects are mediated through the kisspeptin receptor GPR54 (Kah *et al.* 2010). The kisspeptidergic-system is assumed to be important in mediating effects from environmental or internal cues on reproduction, such as photoperiod and energetic status (Kah *et al.* 2010). The kisspeptins may act on the HPG-axis at both the hypothalamus and the pituitary (Elizur and Nocillado 2008, Martinez-Chavez *et al.* 2008a, Chang *et al.* 2012). A study on Nile tilapia (*Oreochromis niloticus*) indicated that photoperiod could have an effect on the expression of GPR54 genes (Martinez-Chavez *et al.* 2008a). In another study, melatonin increased the gene transcription of kisspeptins, consistent with an increase in fecundity of zebrafish (Carnevali *et al.* 2011). This supports the

indications that melatonin mediates the effects from photoperiodic information on reproduction through the kisspeptin system. However, potential interactions of melatonin with the kisspeptidergic-system are far from clarified, and future studies are needed to confirm the involvement of melatonin on kisspeptin neurons in teleost fishes (Migaud et al. 2010).

Gonadotropin-inhibitory hormone (GnIH) is a recent discovery in the field of reproduction, and is considered to play a key role in regulation of the HPG-axis in vertebrates (Tsutsui *et al.* 2012). GnIH is also demonstrated in teleosts, and acts on the HPG-axis (Zhang *et al.* 2010, Moussavi *et al.* 2012). The mechanisms of GnIH are not clear, but GnIH is considered to be involved in regulating release of hormones from the pituitary (Kah and Dufour 2011). Ongoing investigations are looking at a possible effect from melatonin on GnIH function.

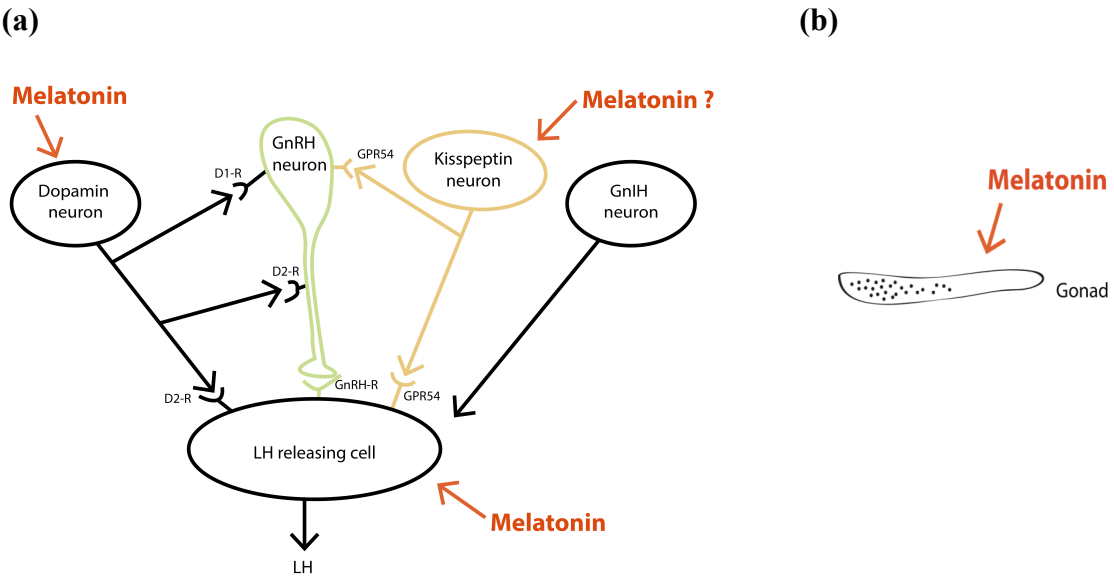

Figure 6. Possible melatonin influence on the HPG-axis in teleost fish. **(a)** Hypothalamus and pituitary **(b)** gonad (modified from Kah *et al.* 2010, and Kah and Dufour 2011).

The hormone leptin is involved in the regulation of food intake, body weight, and energy balance. It has been suggested to synchronize the energetic status of the organism with reproductive events through regulation of FSH and LH secretion (Weil *et al.* 2003). Stress is also a factor demonstrated to influence on reproduction in teleosts (Bonga 1997). In response to a stressor, catecholamines are released from the chromaffin tissue in head kidney, through activation of the sympatic nervous system, and then the hypothalmus-pituitary-interrenal (HPI)- axis is activated. This gives release of the hormone cortisol from the inter-renal tissue (Bonga 1997). In teleosts, the HPG and HPI-axes interact at several levels (Fuzzen *et al.* 2011).

1.4 Application of knowledge about the melatonin system

1.4.1 Photoperiodic manipulation

It has been known for a long time that changes in photoperiod is the primary event in synchronizing the seasonality of reproduction in free living fish species in the temperate zones (Maitra *et al.* 2006). Control of spawning through photoperiodic manipulation of reproduction has made it possible to increase the supply of eggs and fry out of season. In this way, a steady supply of fingerlings and fish production throughout the year to meet the demands of continuous supply of fish from aquaculture can be ensured (Bromage *et al.* 2001). Manipulation of photoperiod to change spawning time has been carried out in a range of temperate fish species (Taranger *et al.* 1998, Hansen *et al.* 2001, Rodríguez *et al.* 2001, Davies and Bromage 2002).

The main environmental factors influencing the seasonal variations in reproduction in sub-tropical and tropical fish species are less distinct (Guerrero *et al.* 2009). Their environment does not show the same considerable seasonal variations in the 24-hour light-dark cycle throughout the year, as species living in mid- to high latitudes. Hence, photoperiod has not been thought of as an effective cue in synchronizing reproductive events to the environment in tropical fish species (Takemura *et al.* 2004, Bapary *et al.* 2009). Some factors suggested to be important are the lunar cycle, rainfall, food availability and physical factors, like pH and salinity (Takemura *et al.* 2004, Guerrero *et al.* 2009).

Recently, however, several studies have indicated that photoperiod and temperature may be important cues in the regulation of reproduction, also in some sub-tropical and tropical fish species. Studies on the free-living Indian sub-tropical major carp, indicated that seasonal variations in photoperiod, probably also in combination with changes in temperature throughout the year, controlled the seasonal maturation of ovaries (Dey *et al.* 2004). In the same species, photoperiod was involved in regulation of seasonal events in the gonads (Dey *et al.* 2005). A study by Sarkar *et al.* (2010) altered spawning time in the major carps rohu (*Labeo rohita*), Indian carp and mrigal (*Cirrhinus mrigala*) through photothermal manipulation. Photoperiodic manipulation was also likely to have a direct influence on the maturation of the gonads in the tropical Nile tilapia (Campos-Mendoza *et al.* 2004) and the sapphire devil (*Chrysiptera cyanea*) (Bapary *et al.* 2009, Bapary and Takemura 2010).

1.4.2 Aquaculture

In order to increase food security and to reduce poverty in developing countries, aquaculture is considered a potentially significant contributor (Ahmed and Lorica 2002, Subasinghe 2005, FAO 2011). Fish is particularly rich in animal proteins, micronutrients, minerals and essential fatty acids, and is therefore an important food source to improve human nutrition (Kawarazuka and Béné 2010). Small-scale aquaculture contributes to the livelihood of people in developing countries, through generating increased supply of food with high nutritional value, income and employment (Edwards 2000, Tacon 2001). A future increase in demand for

fish in developing countries is expected (FAO/NACA 2012). Moreover, the fish farming industry is more efficient and environmentally friendly, compared to other animal protein industries such as the production of livestock (Hall *et al.* 2011).

1.4.3 The Sustainable Poverty Reduction in Nepal Program (SPRN) and Nepal

SPRN started in 2006, with the Norwegian University of Science and Technology (NTNU) as main initiator. The goal of the SPRN research program is to contribute to the reduction of poverty, and increase food security in the rural hilly areas in Nepal (SPRN 2008).

Nepal is among some of the least developed countries of the world, and is ranked number 157 out of 187 countries in the Human Development Index from 2011 (UNDP 2011). In this part of Asia, 43 % of children under the age of five are underweight, showing the highest numbers of underweight children in the world (UNICEF 2009, UN 2011). The hunger situation in Nepal is categorized as "serious", and close to "alarming", based on the number of 19.9 in the Global Hunger Index score[1] (IFPRI 2011). Furthermore, the proportion of animal protein intake is low, and the main food items in the Nepalese diet are cereals and root crops (Hirai *et al.* 1993, Buhjel *et al.* 2008). Consequently, it is important to increase food security and the living conditions in Nepal, and aquaculture could be an important contributor to achieve this.

In 2009, total output from Nepalese aquaculture was 26 730 tons, valued 45 million USD. In comparison, another developing country, Bangladesh, had a production of 1 million tons valued 2.35 billion USD in 2009 (FAO 2012b). This illustrates that the productivity in Nepalese fish farming is low. With rich freshwater and fish resources, and a high demand for fish and animal protein, there is a high potential for increasing the production of fish from aquaculture in Nepal (Sharma and Leung 1998, Sharma and Leung 2000). In order to improve the efficiency and productivity of this industry, the input of fish seed, fertilizer, labor and feed have to be increased (Sharma and Leung 1998).

One of the major problems related to aquaculture in Nepal today is the lack of stable supply of carp fingerlings (SPRN 2008). Manipulation of reproductive events in carps may be a possible way to significantly increase access of fingerlings throughout the year, and thus be an important contribution to increase productivity in the Nepalese aquaculture. In order to achieve stable supply of fingerlings by manipulating reproduction, it is necessary to gain more knowledge about the periodicity and regulation of reproduction in the species of interest, and to look at possible ways to manipulate it (SPRN 2008).

[1] Global Hunger Index Score combines three equally weighted indicators in one index number. The indicators are undernourishment, child underweight and child mortality. It ranks countries on a 100-point scale in five categories: ≤ 4.9 low, 5.0-9.9 moderate, 10.0-19.9 serious, 20.0-29.9 alarming, ≥30.0 extremely alarming (IFPRI 2011)

1.5 Silver carp (*Hypophthalmichthys molitrix*)

Silver carp (*Hypophthalmichthys molitrix*) belongs to the family Cyprinidae, the largest family of freshwater fish in the world. In 2010, global aquaculture production of silver carp was reported to be 4.1 million tons, which makes silver carp among one of the most cultured cyprinid species (FAO 2012c). They are planktivores, and feed mainly on phytoplankton and organic particles (Spataru and Gophen 1985, Kolar *et al.* 2005). Being a planktivore, no supplementary food is needed in the fish farming of silver carp. Fish farming of silver carp is consequently considered an environmental friendly way of increasing production of animal protein, at low manufacturing costs. People can afford to buy it frequently, due to low market price (FAO 2012c). Silver carp is characterized by being a surface feeder, and is exposed to natural changes in the surrounding light-dark cycle (Sukumaran *et al.* 1968). Hence, silver carp is considered a well-suited candidate in both fish farming as well as studying the effects of photoperiod in carps.

1.6 Study aims

Securing all-year supply of fish fry is important to improve the productivity in Nepalese aquaculture. The hormone melatonin gives the fish information about changes in photoperiod, and may be involved in controlling reproductive events. Maturational control in teleost fish from temperate zones is documented to involve both the HPG-axis, and the pineal complex. Carp fish at low latitudes does also exhibit a melatonin producing pineal complex. Photo-manipulation of these species may therefore contribute to secure stable supply of fish fry throughout the year.

In order to improve our understanding of how photoperiodic manipulation can be used to change the timing of cyclic reproductive events in silver carp, a fish commonly used in Nepalese aquaculture, it is necessary to achieve knowledge about the melatonin system.

The main aims of the present thesis were to answer the following questions:

1. How do silver carp plasma melatonin levels change throughout natural or manipulated photoperiod in silver carp?

2. Does the silver carp pineal melatonin synthesis involve a biological clock control system?

3. Is there a correlation between silver carp plasma cortisol and melatonin levels?

2 Materials and methods

2.1 Study site

Experiments were performed at Mandal Fish Farm, near Butwal City (27°42'N 83°26'E) in Rupandehi District, Lumbini Zone, Nepal (map 1). Nepal is a country in South-Asia, located between India and China. The country is climatically and topographically diverse with three main climatic zones, the Himalayan range, Hilly midlands and Terai plain. Butwal is situated in the Terai plain in Southern-Nepal, recognized by flat sub-tropical lowland (CIA 2012). Fieldwork was carried out from the 27th to the 31st of March 2011, in collaboration with Master student Ingun Næve (Næve, 2012). The weather was mostly sunny, with air temperatures around 30° C during midday.

Map 1. Map of Nepal. Study site is indicated by a red asterisk (modified from Discover Asia 2008).

Mandal fish farm is a privately operated carp hatchery producing fry and fingerlings for commercial local sale. It has 16 fishponds and various indoor and outdoor tanks. All the facilities were newly built and of good standard.

Six rectangle shaped concrete tanks (2.3 m x 1.0 m x 0.9 m) containing about 750 L water were provided for the experiments in the present study. They were numbered one to six. The tanks were situated outdoor under a ceiling (fig. 7). Continuously flow of water through (7 L/min) in the tanks secured stable oxygen levels throughout the experiments. Oxygen level was 7.1 mg/L (YSI ProODO). The water temperature in the open tanks was 24° C at 12:00. In tanks covered with black plastic the water temperatures were 25° C at 12:00, and 22° C at 05:00. Fishes were transferred to the tanks 24 hours prior to experimental start.

Figure 7. Fish tanks one to six. Tanks were situated outdoor under a ceiling, exposed to natural photoperiod. (**a)** Uncovered tanks used during experiments under natural photoperiod. (**b)** Covered tanks used during experiments under manipulated photoperiod.

The intensity of light in ambient air and inside the fish tanks was measured using a lux meter (INS-DX 200) to demonstrate daily changes in the light-dark cycle. Sunrise was at 06:00, and sunset at 18:20 (fig. 8).

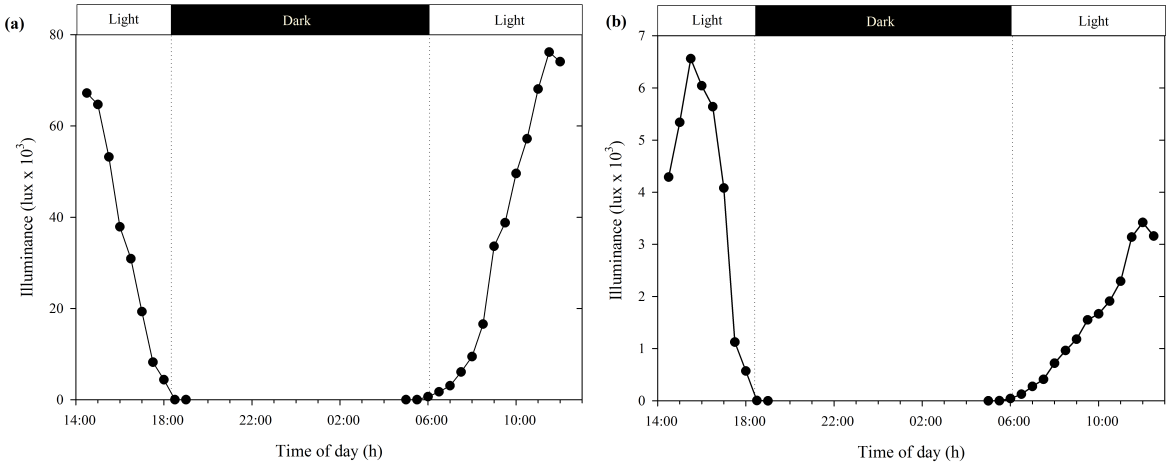

Figure 8. (**a)** Illuminance (lux) in ambient light-dark cycle at the study site and **(b)** inside the outdoor fish tanks.

2.2 Experimental animal

About 250 silver carps were bought from a local fish farmer. Randomly chosen fish were dissected to determine reproductive status by examining the gonads, and all were shown to be barren.

2.3 Blood sampling

Red light (Petzl Tikka XP^2) was used during sampling in dark-phase to prevent degradation of melatonin (Bayarri *et al.* 2002). Fish were anaesthetized in fresh water using 100 mg MS-222 / L water (Tricaine Methane Sulphonate 100 % w/w, PHARMAQ Ltd). Heparinized syringes (16.5 IE, LEO Pharma AS) were used to collect blood from the carp caudal vein complex. Fish were killed after blood sampling. Blood was centrifuged (3-4000 rpm, 5 min, Hettich EBA 3S), and plasma was obtained and stored in a freezer (-20 °C).

2.4 Experimental design

2.4.1 Experiment 1: Plasma melatonin profile during natural photoperiod.

The purpose of experiment 1 was to establish the 24-hour plasma melatonin profile in silver carp kept under natural (outdoor) photoperiod. Fish were distributed in groups of 14-18 individuals in each of the six outdoor tanks, one day prior to experimental start. At ten time points during 24-hours (12:00, 17:00, 19:00, 21:00, 23:00, 01:00, 03:00, 05:00, 07:00 and 12:00) groups of six fish were taken from a tank and blood samples collected. Fish were caught using hand net from the tanks in order one to six (tank one to four were used twice). Average body weight and length of fish are given in Table 1(a) (p. 28). No food was provided during the experiment.

2.4.2 Experiment 2: Plasma melatonin profile during continuous darkness

The purpose of experiment 2 was to establish a plasma melatonin profile during a 24-hour period in silver carp exposed to continuous experimental darkness, and compare this melatonin profile to the profile demonstrated under natural photoperiod. Fish were distributed in groups of 14-18 individuals in each of the six outdoor tanks, 24 hours prior to start of experiment. At sunset, 18 hours prior to the first time of blood sampling, all tanks were covered with black plastic. Fish tanks were kept covered throughout the entire 24-hour period when blood samples were collected. Light intensity in the covered tanks was measured to be 0.01 lux. At eight different time points during a time period of 24-hours (12:00, 15:30, 19:00, 22:00, 02:00, 05:00, 8:30 and 12:00) groups of six fish were collected from one tank using hand nets, and blood samples were taken. To secure that no fish had been exposed to light during the experiment, tanks used twice were those where fish was collected during natural dark-phase the first time. During blood sampling carried out in the light-phase, head of fish were covered with paper to prevent exposure of light. Average body weight and length of fish are given in Table 1(b) (p. 28). No food was provided during the experiment.

2.4.3 Experiment 3: Short time (2 hours) darkness during natural light period

Experiment 3 was designed to investigate any changes in the daytime concentrations of plasma melatonin in silver carp after exposure to two hours experimental darkness during the natural light phase. Three groups of seven to eight fish were placed in three outdoor tanks. Each tank was covered with black plastic two hours prior to blood sampling, at 10:00 (group 1), 13:00 (group 2) and 16:00 (group 3). After exposure to two hours experimental darkness, six fish were caught using hand nets, and blood samples were collected. Blood sampling time points were 12:00 (group 1), 15:00 (group 2) and 18:00 (group 3). Average body weight and length of fish are given in table 1(c) (p. 29). No food was provided during the experiment.

2.5 Plasma analysis

Plasma samples were analyzed to determine the concentration of melatonin. Concentration of plasma cortisol was also measured in some samples from each experiment. Determination of hormone levels in plasma samples was done using the radioimmunoassay (RIA) technique. The analyses were performed at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway.

2.5.1 Radioimmunoassay theory

Basic components in a radioimmunoassay are radioactive labeled antigen (Ag*), unlabeled antigen (Ag) and antibody (Ab). Ag is the component to be quantitatively determined (melatonin or cortisol) in the samples. The principle of this technique is based on a reversible competitive reaction between known concentrations of Ag*, and unknown concentrations of Ag. Ag* and Ag compete for binding to Ab, which is in limited amount in the mixture (1). Ratio between bound and free Ag and Ag* is related to the total concentration of Ag in the sample, and is used to determine its concentration. Higher concentration of Ag in a solution gives fewer Ag*-Ab complexes and more free Ag* (Berson and Yalow 1968, Chard 1995, Wild 2005).

$$\begin{matrix}[\mathrm{Ag}] \\ [\mathrm{Ag}^*]\end{matrix} \quad + \quad [\mathrm{Ab}] \quad \leftrightarrows \quad \begin{matrix}[\mathrm{Ag}-\mathrm{Ab}\,] + [\mathrm{Ag}^*] \\ [\mathrm{Ag}^*-\mathrm{Ab}] + [\mathrm{Ag}]\end{matrix} \qquad (1)$$

Bound and free fraction of Ag* in samples were separated using a precipitating agent. Radioactivity (counts per minute) in bound fraction was measured in a gamma counter. Radioimmunoassay does not determine concentrations of Ag directly. A standard curve (fig. 9) was made from solutions of known concentrations of Ag. This curve express percentage bound Ag^* at different concentrations of Ag. Results from measurement of percentage bound Ag^* in a mixture of unknown Ag concentration, is compared to the standard curve. Thus, it is possible to determine concentration of Ag in the sample. The steepest part of the standard curve represents the most effective range (Chard 1995).

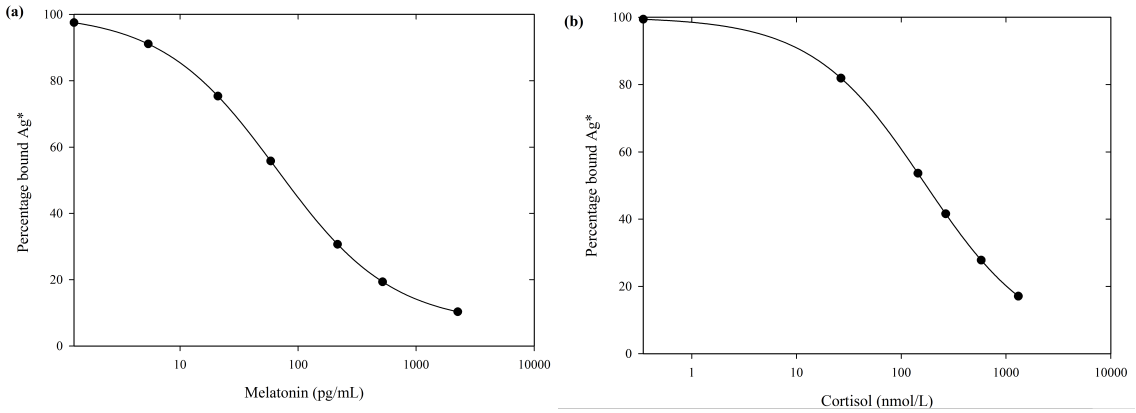

Figure 9. (a) Standard curve from melatonin radioimmunoassay based on measure of bound fraction of Ag* (radioactive labeled melatonin). Validation parameters were: Sensitivity: 17.5 pmol/L, Recovery: 89.9 %, Parallelism: Samples were parallel to the standard curve **(b)** Standard curve from cortisol radioimmunoassay based on measure of bound fraction of Ag* (radioactive labeled cortisol).

Determination of plasma melatonin concentration in the samples was carried out using I^{125}-radioimmunoassay kit (Melatonin Research RIA, Labor Diagnostika Nord GmbH & Co. KG, Germany) for direct quantitative determination of melatonin in biological fluids. Procedure was done according to the standard protocol of the kit. A test run of the kit was performed prior to analysis of the plasma samples.

Plasma cortisol concentration was determined using a commercial radioimmunoassay kit (Coat-A-Count Cortisol, Siemens Healthcare Diagnostics, USA). Procedure was done according to the standard protocol of the kit. Running random plasma samples in a test kit demonstrated high cortisol levels (values were above the highest standard). Thus, samples were diluted (1:2), using one portion sample and two portions standard zero solution. Cortisol values presented are multiplied with the dilution factor three.

2.5.2 Radioimmunoassay validation

Using commercial radioimmunoassay kits requires a species-specific validation. This is to test for any interference in the assay affecting the results. This could be components in plasma interfering with the assay, and thus incorrectly give too high, or low, concentrations of the substance to be measured (Irwin *et al.* 1999, Vera *et al.* 2011). Validation was performed on melatonin kit by testing sensitivity, recovery and parallelism of the system.

Sensitivity

Sensitivity is the lowest measurable amount of melatonin different from zero, the lower limit detection of the assay. It was determined by calculating mean percentage bound melatonin* in six samples stripped for melatonin, minus 2 standard deviation (SD). Lower limit detection was then detected by using the standard curve. Lower limit detection was set to be 17.5 pmol/L. Detection limit calculated by gamma counter was 5.4 pmol/L.

Recovery

Recovery examines if the system is able to detect the accurate concentration of a sample, thus it gives information about the accuracy of the assay. Optimum recovery is 100 %, but recovery > 50 % is acceptable. Six spiked plasma samples (200 pg/mL melatonin) were measured, and percentage recovery calculated. Recovery of the assay was 89.8 %.

Parallelism

Parallelism tests if components in the kit respond to other substances than intended, thus it tests the specificity of the kit. Serial diluted spiked plasma samples (1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 0 pg/mL) were measured, and parallelism with the standard curve was tested. Non-parallelism indicates cross-reactivity in the assay (Chard 1995, Welp *et al.* 2010). The diluted spiked plasma samples were parallel to the standard curve.

2.6 Conversion factors

Hormone concentrations are given in SI-units. Conversions of values to conventional units in the literature are demonstrated in (2) for melatonin and (3) for cortisol. In discussion, values from literature are converted to the SI-units used in the present study.

- Melatonin:

  pmol/L = pg/mL x 4.3 (2)

- Cortisol:

  nmol/ = ng/mL x 2.75 (3)

2.7 Statistical analysis

The statistical analyses and making of graphs were performed using the graphing and statistical software SigmaPlot Version 12.0 (Systat Software, inc., San Jose California, USA). Statistical significance was set at $p < 0.05$.

Data were analyzed using the parametric One-Way Analysis of Variance (ANOVA), to determine any significant differences in hormone concentrations at the different times in an experiment. Data that did not meet the underlying assumptions for One-Way ANOVA (normality and homogeneity of variances), were analyzed using a nonparametric analysis of variance (ANOVA on ranks), the Kruskal-Wallis One Way Analysis of Variance on Ranks test (Zar 1984). If significant differences were found, the ANOVA tests were followed by a parametric or non-parametric Tukey Test. This is a post-hoc multiple comparison procedure used to detect between which groups the significant difference occurred.

Results from the ANOVA are presented as: H or F-value, (Degrees of Freedom), p-value.

The non-parametric Spearman Rank Order Correlation test was used to test for any correlation between plasma melatonin and cortisol concentrations.

Plasma melatonin and cortisol concentrations are presented as mean values ± standard deviations (SD).

3 Results

3.1 Experiment 1: Plasma melatonin profile during natural photoperiod

Blood plasma melatonin concentrations in silver carp were low during light-phase, and increased rapidly following onset of darkness (fig. 10). Maximum plasma melatonin concentration was reached in early dark-phase, at 21:00 (1717 ± 2193 pmol/L). Thereafter, melatonin levels gradually decreased to a low level of 82 ± 67 pmol/L at sunrise. A significant difference among samples in dark-phase and light-phase was detected (Kruskal-Wallis: H (9) =36.370, $p < 0.001$). The melatonin level at 12:00 in the second photoperiod was significantly different from the levels at 19:00, 21:00 and 23:00. Level of melatonin at 07:00 was statistically significant different from levels sampled at 21:00 and 23:00 (Tukey test).

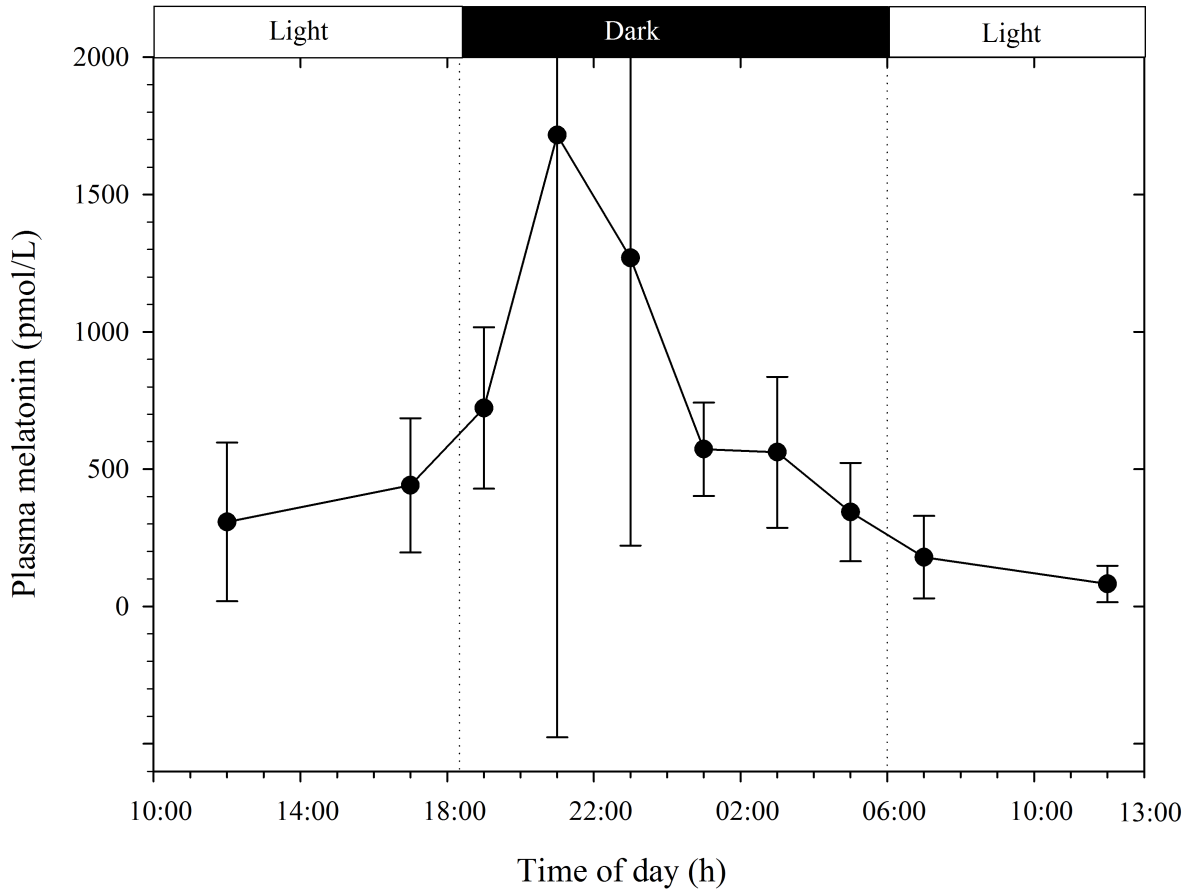

Figure 10. Plasma melatonin concentrations in silver carp (*Hypophthalmichthys molitrix*) kept under natural photoperiod. Values are mean ±SD (N=6). Bar on top of graph illustrates light-dark phases. Stippled lines denote sunset and sunrise.

3.1.1 Plasma cortisol levels during natural photoperiod

The plasma cortisol levels in silver carp kept under natural photoperiod were high during the light-phase, and low during the dark-phase (fig. 11). In fish from the first light period at 12:00, the cortisol level was 1352 ± 607 nmol/L. One hour after sunset, cortisol levels decreased to 662 ± 426 nmol/L. A comparable cortisol level was seen at end of the dark period, followed by another high level of 1418 ± 380 nmol/L in the next light period. A statistically significant difference was detected (ANOVA: F (4) = 2.768, p = 0.049). Tukey test could not detect at which times cortisol levels were significantly different.

There was a significant negative correlation between melatonin and cortisol (r = -0.407, p = 0.0258, n = 30).

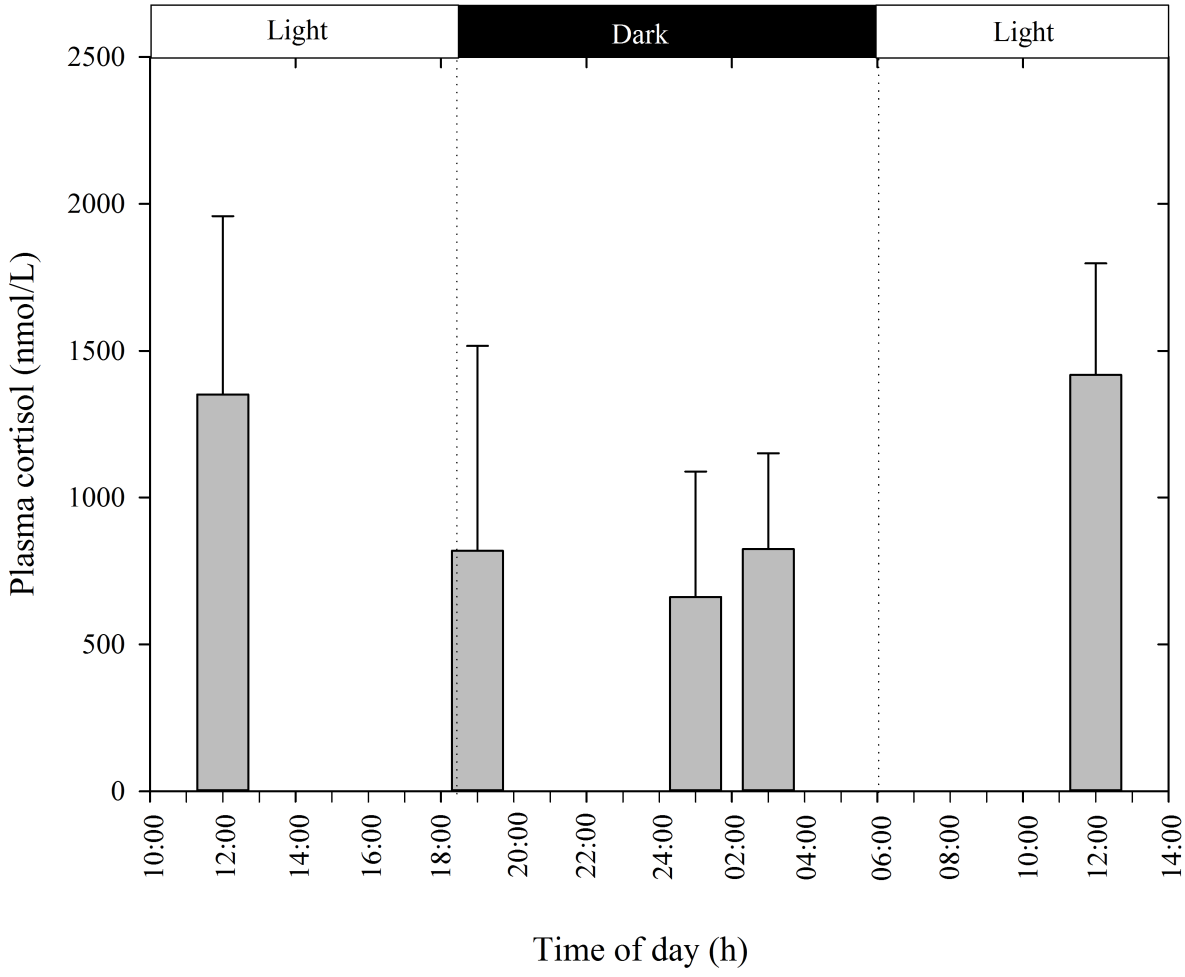

Figure 11. Plasma cortisol concentrations in silver carp (*Hypophthalmichthys molitrix*) under natural photoperiod. Values are mean +SD (N=6). Bar on top of graph illustrates natural light-dark phases. Stippled lines denote sunset and sunrise

3.2 Experiment 2: Plasma melatonin profile during continuous darkness

Melatonin levels in silver carp exposed to continuous darkness were low throughout the natural light period, and increased significantly during the natural dark period (fig. 12). The lowest level was at 12:00 (102 ± 40 pmol/L) in the initial light-phase. After onset of darkness, melatonin peaked at 05:00 (1651 ± 657 pmol/L). Subsequently, melatonin levels quickly decreased to 124 ± 30 pmol/L at 08:30. Differences between melatonin concentrations from samples in dark-phase and light-phase were significant (Kruskal-Wallis: $H(7) = 38.726$, $p < 0.001$). The peak level of melatonin at 05:00 was significantly different from all melatonin levels during the natural light-phase. Melatonin level at 22:00 was significantly different from 12:00, 8:30 and 12:00. At 02:00, the melatonin level was significant different from the levels at 12:00 in both of the natural light periods.

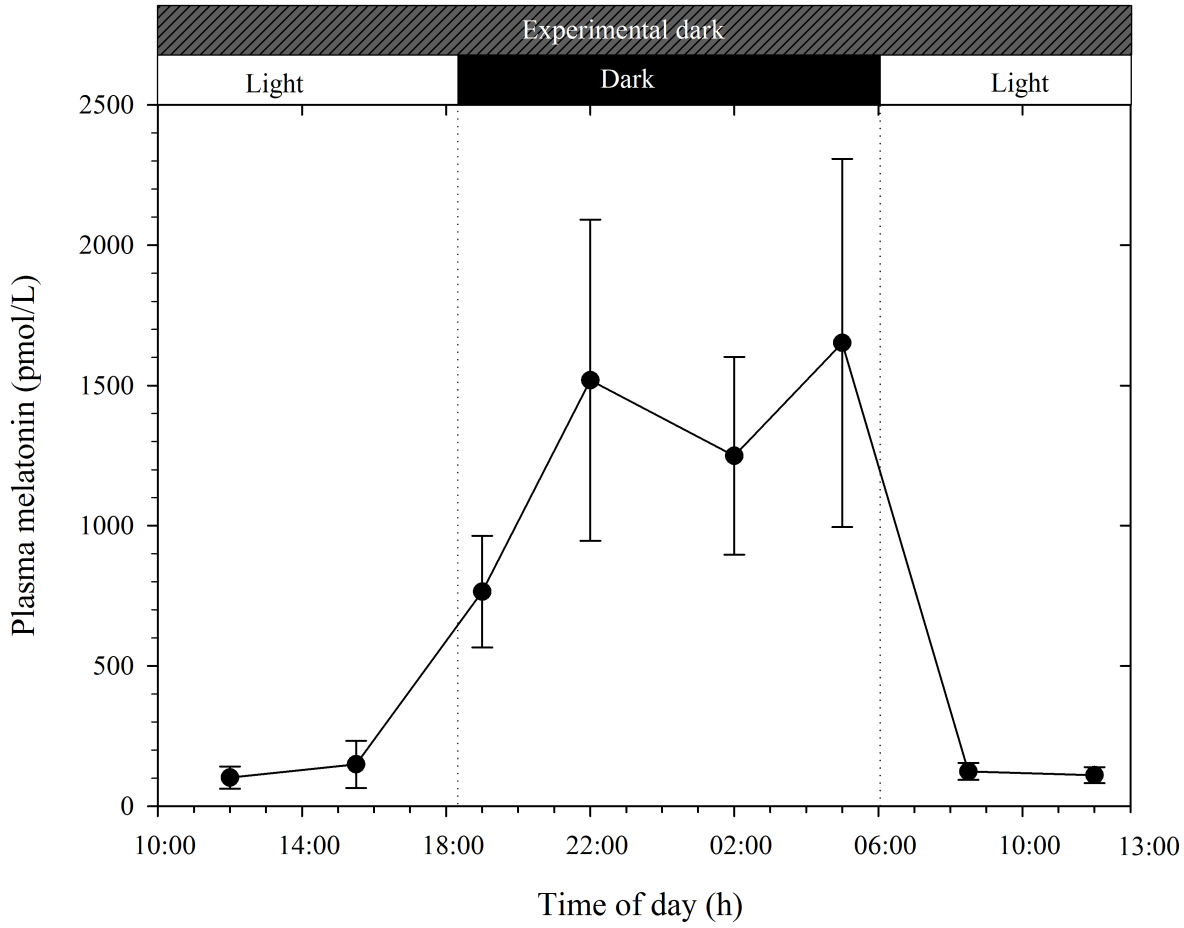

Figure 12. Plasma melatonin concentrations silver carp (*Hypophthalmichthys molitrix*) under continuous darkness. The two bars on top of the graph illustrate the manipulated photoperiod and the natural photoperiod. Stippled lines denote sunset and sunrise. Values are mean ±SD (N=6).

3.2.1 Plasma cortisol levels during continuous darkness

The highest cortisol level in silver carps exposed to continuous darkness was at 12:00 in the initial light period (1238 ± 567 nmol/L). Levels decreased to 668 ± 186 nmol/L and 627 ± 272 nmol/L during the dark period. In the following light-phase, lowest concentration of cortisol in the experiment was at 12:00 (478 ± 211 nmol/L) (fig. 13). A significant difference was detected between cortisol level at 12:00 in first photoperiod and 12:00 in second photoperiod (Kruskal-Wallis: H (3) = 9.213, p=0.027).

No correlation was found between melatonin and cortisol concentrations in experiment 2 (r = 0.251, p = 0.233, n = 24).

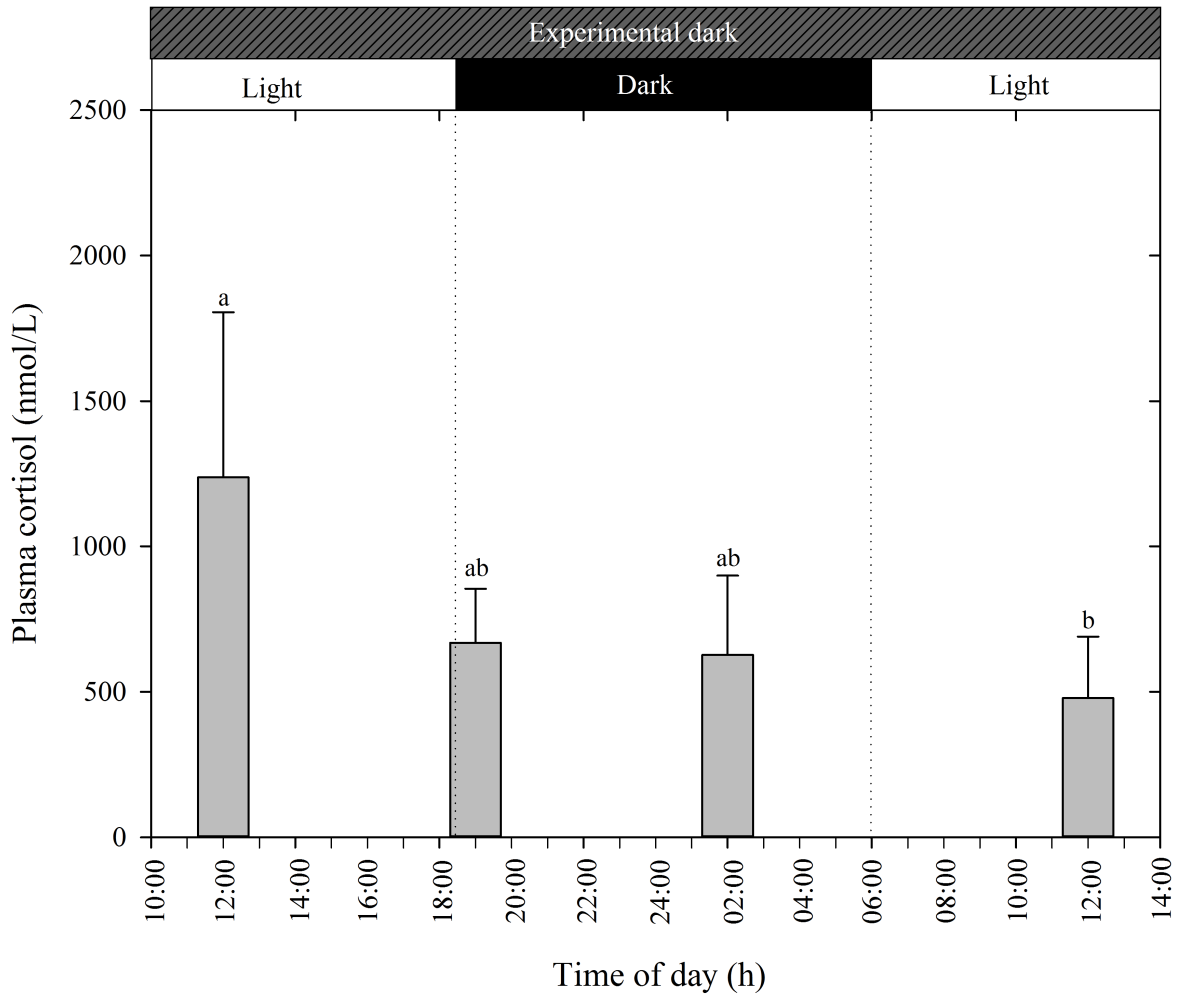

Figure 13. Plasma cortisol concentrations in silver carp (*Hypophthalmichthys molitrix*) under continuous darkness. The two bars on the top of the graph represent the manipulated photoperiod and the natural photoperiod. Stippled lines denote sunset and sunrise. Values are mean +SD (N=6). Different letters indicate statistically significant difference.

3.3 Experiment 3: Melatonin levels after 2 hours darkness during natural light period

The melatonin levels in silver carp were low after two hours exposure of darkness at both 12:00 (132 ± 52 pmol/L) and 15:00 (109 ± 80 pmol/L). At 18:00, the melatonin levels increased significantly to 376 ± 77 pmol/L (fig. 14) (ANOVA: $F(2) = 26.332$, $p < 0.001$, followed by Tukey test).

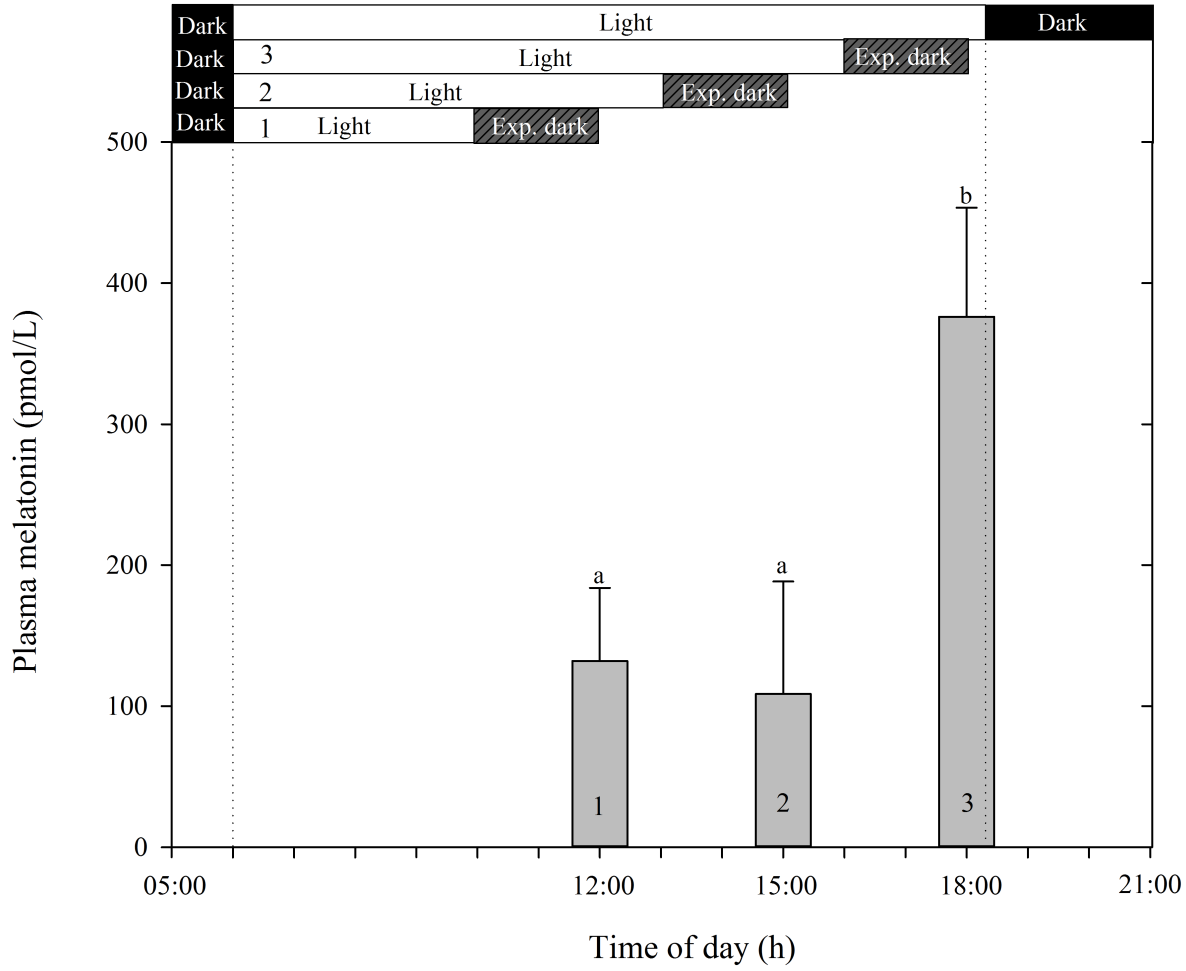

Figure 14. Plasma melatonin concentrations in silver carp (*Hypophthalmichthys molitrix*) after exposure to two hours darkness during natural light phase. Each of the three groups of carps was exposed to darkness at different times. Samples were collected at 12:00 (group 1), 15:00 (group 2) and 18:00 (group 3). Values are mean + SD (N=6). Bar on top of graph illustrates changes in natural photoperiod, and the three following bars below demonstrate light exposure of the different groups. Stippled lines denote sunrise and sunset. Different letters indicate statistically significant differences between the groups.

3.3.1 Cortisol levels after 2 hours darkness during the natural light period

Plasma cortisol levels in silver carp showed no significant differences throughout experiment 3 (fig. 15). Levels at 12:00 (1239 ± 567 nmol/L), 15:00 (1066 ± 664 nmol/L) and 18:00 (1176 ± 475 nmol/L) remained high, and showed little variation. No statistical difference was found (ANOVA: F (2)=0.139, p=0.872).

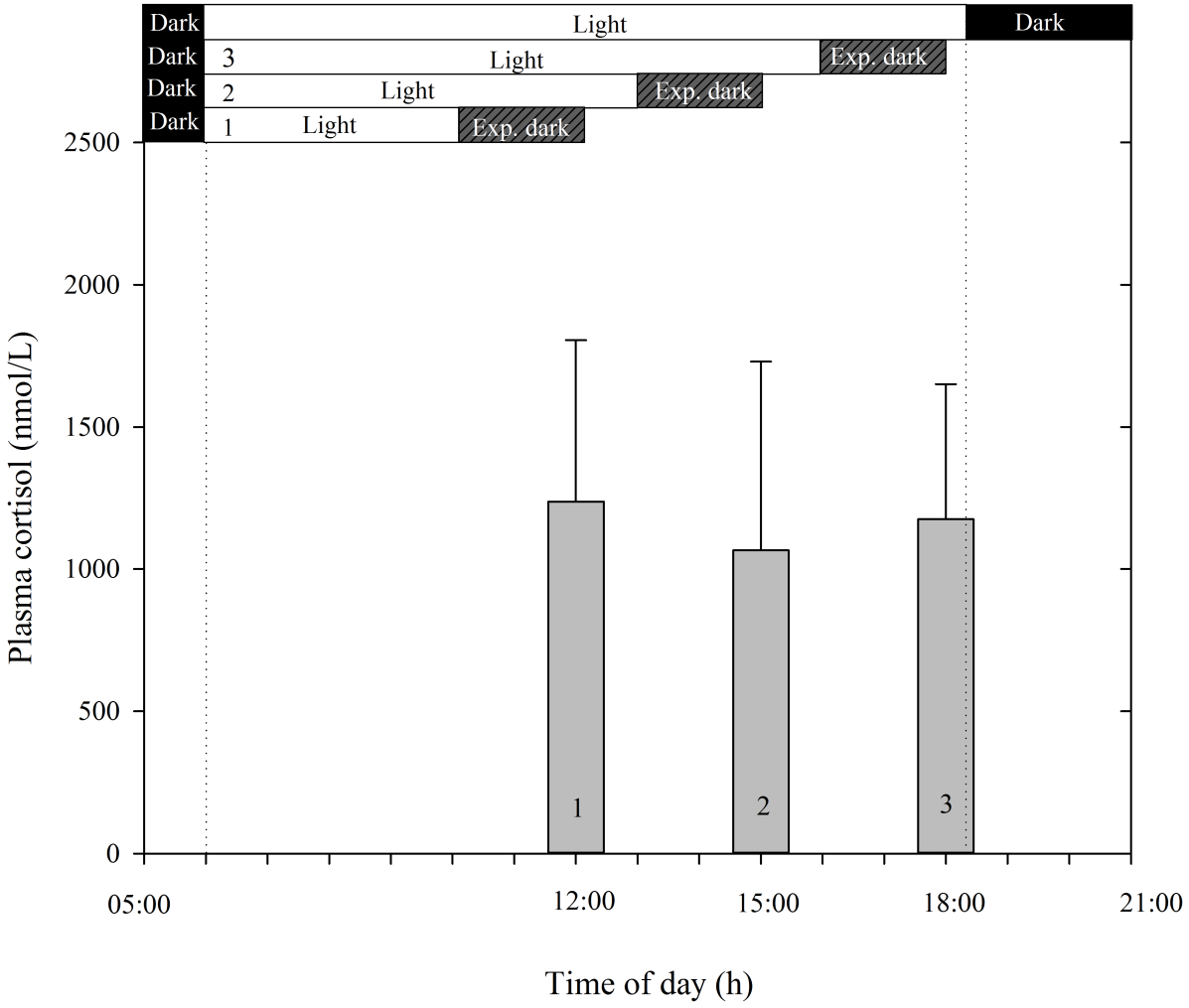

Figure 15. Plasma cortisol concentrations in silver carp (*Hypophthalmichthys molitrix*) after exposure to two hours darkness during natural light-phase. Each of the three groups of carps was exposed to darkness at different times. Samples were collected at 12:00 (group 1), 15:00 (group 2) and 18:00 (group 3). Values are mean + SD (N=6). Bar on top of graph illustrates changes in natural photoperiod, and the three following bars demonstrate light exposure of the different groups. Stippled lines denote sunrise and sunset.

3.4 Correlation between melatonin and cortisol

The regression line between individual plasma melatonin (pmol/L) and cortisol (nmol/L) concentrations in silver carp from all three experiments showed a low and non-significant decrease (R^2=0.119) (fig.16).

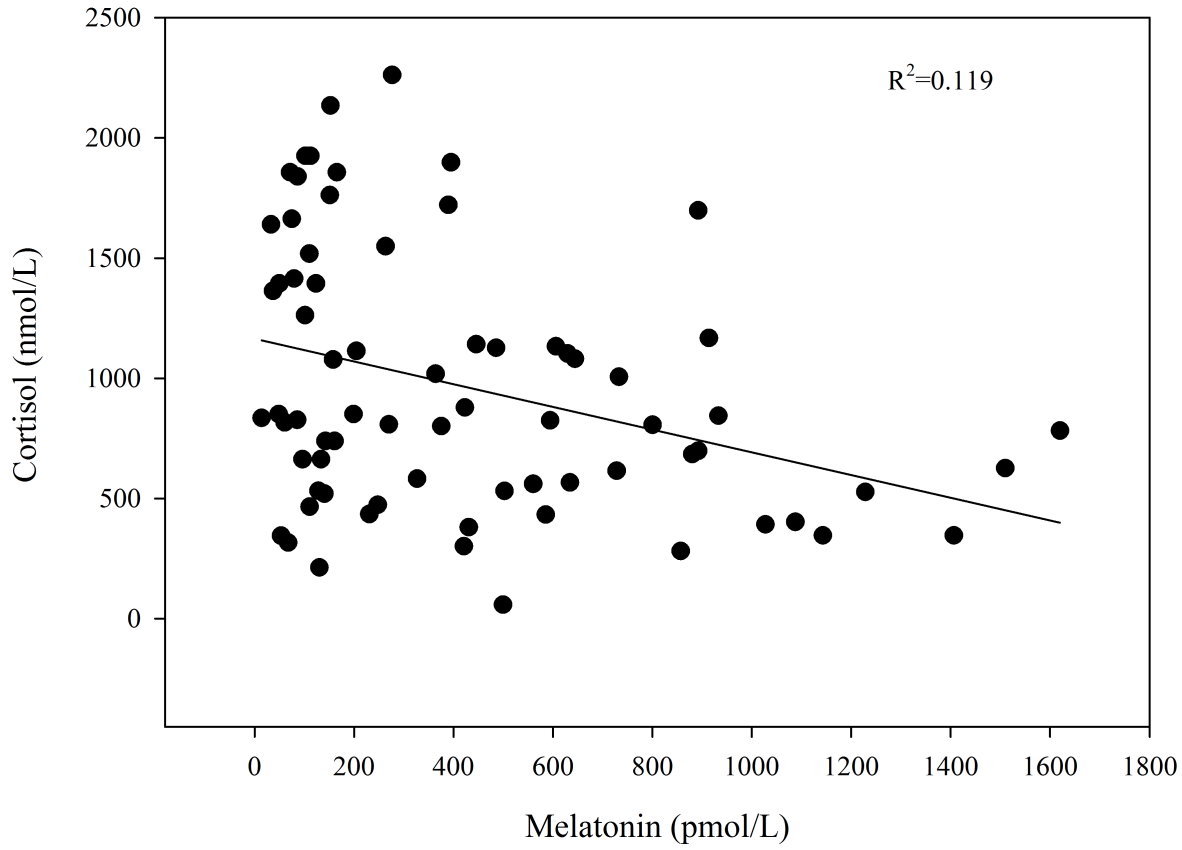

Figure 16. Correlation between melatonin and cortisol. Individual melatonin (pmol/L) and cortisol (nmol/L) levels in silver carp (*Hypophthalmichthys molitrix*) in samples from all three experiments.

3.5 Body length and weight of silver carp

Average length ranged between 24 ± 4 cm and 32 ± 2 cm, and average weight ranged between 185 ± 35 g and 304 ± 80 g in silver carp during natural photoperiod (experiment 1) (Table 1a). No statistical significant difference was found between groups in length (Kruskal-Wallis: H (9)=10.759, p=0.293). A significant difference was found between weight of fish from sampling time 12:00 and 21:00 (ANOVA: F (9)=2.658, p=0.013, followed by Tukey test).

Table 1 (a) Body length (cm) and weight (g) of silver carp (*Hypophthalmichthys molitrix*) under natural photoperiod (experiment 1). Values are mean ± SD (N=6).

Sampling time (h)	Length (cm)	Weight (g)
12:00	32 ± 2	304 ± 80
17:00	28 ± 4	245 ± 35
19:00	30 ± 2	258 ± 50
21:00	30 ± 1	185 ± 35
23:00	29 ± 1	258 ± 27
01:00	29 ± 1	258 ± 45
03:00	29 ± 1	241 ± 22
05:00	29 ± 2	271 ± 69
07:00	28 ± 1	238 ± 21
12:00	29 ± 1	235 ± 26

Average length ranged between 30 ± 1 cm and 31 ± 1 cm, and average weight ranged between 243 ± 44 g and 272 ± 28 g in silver carp subjected to continuous darkness (experiment 2) (Table 1b). No significant differences were found between groups in length (ANOVA: F (7) = 1.146, p=0.355), or weight (ANOVA: F (7)=1.067, p=0.402)

Table 1 (b). Body length (cm) and weight (g) of silver carp (*Hypophthalmichthys molitrix*) subjected to continuous darkness (experiment 2). Values are mean ± SD (N=6).

Sampling time (h)	Length (cm)	Weight (g)
12:00	31 ± 1	264 ± 19
15:30	31 ± 1	269 ± 28
19:00	31	248 ± 11
22:00	31 ± 1	253 ± 33
02:00	30 ± 1	243 ± 44
05:00	31 ± 1	251 ± 35
08:30	31 ± 1	272 ± 28
12:00	30 ± 1	258 ± 24

Average length ranged between 28 ± 1 cm and 29 ± 2 cm, and average weight ranged between 241 ± 32 g and 262 ± 74 g in silver carp exposed to short time darkness (2 hours) during the natural light-phase (experiment 3) (table 1c). No significant differences were found between groups in length (Kruskal-Wallis: H (9)= 0.667, p=0.716) or weight (Kruskal-Wallis: H (9)= 0.636, p=0.728).

Table 1(c). Body length (cm) and weight (g) of Silver carp (*Hypophthalmichthys molitrix*) exposed to short time (2 hours) darkness during daytime (experiment 3). Values are mean ± SD (N=6).

Sampling time (h)	Length (cm)	Weight (g)
12:00	29 ± 2	241 ± 32
15:00	29 ± 3	262 ± 74
18:00	28 ± 1	255 ± 21

4 Discussion

4.1 The silver carp melatonin profile under different photoperiods

4.1.1 Natural photoperiod

Blood plasma melatonin concentration in silver carp under natural photoperiod was low during daytime (photophase), and increased considerably during nighttime (scotophase). Similar melatonin levels and rhythmicity during the natural 24-hour light-dark cycle have been documented in other teleost species (Pavlidis *et al.* 1999, Migaud *et al.* 2010). This indicates that changes in pineal melatonin release provide the silver carp with temporal information used in the timing and control of physiological and behavioral mechanisms, as previously suggested in other fish species (Reiter 1987, Maitra *et al.* 2006, Vera *et al.* 2007).

After peak level of melatonin in early scotophase, the levels decreased noticeably prior to onset of light in the following photophase. These results are in accordance with findings in recent studies on other carp species from Nepal (Guttu 2011, Holtan 2011, Næve 2012), in Nile tilapia (Martinez-Chavez *et al.* 2008b) and in European sea bass (Migaud *et al.* 2006).

The reduction in melatonin concentration during late scotophase indicates either a decreased pineal melatonin synthesis, or increased melatonin removal from the circulation. A diurnal change in melatonin half-life ($t_{1/2}$) seems unlikely, but should be tested in future experiments by determination of melatonin $t_{1/2}$ at different times throughout the 24-hour light-dark cycle. Melatonin drop during the scotophase is more likely the result of reduced hormonal release from the pineal complex. This could be caused by exhaustion of components involved in biosynthesis of melatonin during the first half of scotophase. Alternatively, reduced melatonin synthesis might be the result of an internal timed control mechanism blocking the pineal melatonin synthesis in the late part of the scotophase.

4.1.2 Continuous darkness

During the time of the natural photophase, melatonin levels in silver carp subjected to an 18-hour period of continuous darkness were low, and during the time of natural scotophase the melatonin levels increased significantly. The diurnal rhythmical melatonin levels observed during the natural light-dark cycle were consequently sustained in silver carps subjected to continuous darkness, even if the fish were deprived of time signals in the light-dark cycle.

In accordance with the results in this study, several other studies have shown that fish pineal melatonin synthesis during constant darkness may continue in the same rhythmical manner as observed during natural photoperiod (Bolliet *et al.* 1996, Falcón *et al.* 2011). Maintained rhythmicity in plasma melatonin during a natural light-dark cycle have also been shown in other carps from Nepal subjected to an extended dark-phase (Guttu 2011, Holtan 2011, Næve 2012). *In vitro* studies on pineal glands from several teleost species have also demonstrated that rhythmical melatonin secretion persisted when exposed to constant darkness (Iigo *et al.*

1991, Bolliet *et al.* 1996, Cahill 1996, Oliveira *et al.* 2009). In Nile tilapia, melatonin rhythms were maintained for 18 days under constant darkness (Martinez-Chavez *et al.* 2008b).

If the synthesis of melatonin followed the changes in the light-dark cycle without any control mechanism, it would have been expected that melatonin levels remained continuously high throughout the 24-h cycle when exposed to continuous darkness. However, the diurnal rhythmicity in melatonin release during natural photoperiod was maintained even at the end of the period of constant darkness. This sustained diurnal rhythmicity during continuous darkness indicates that exhaustion of the pineal system, or changes in melatonin metabolism, may not be factors involved in affecting the plasma melatonin levels in the silver carps. Based on this, these factors are suggested not to have contributed to the observed melatonin drop during late scotophase in fish under natural photoperiod. Rather, the findings indicates the presence of an endogenous control mechanism involved in regulating the rhythmical melatonin synthesis in the pineal gland of silver carp through the 24-hour light-dark cycle. This mechanism must be partly independent of direct information from the ambient light cycle in the control of the rhythmic melatonin secretion.

4.1.3 Short time (2 hours) darkness during natural light period

Melatonin levels in silver carps exposed to two hours experimental darkness at morning (10:00-12:00) or midday (13:00-15:00) did not increase. These results suggest that silver carp pineal gland does not respond to darkness by increased melatonin synthesis during morning and midday. Exposure to darkness during the evening (16:00-18:00), however, significantly increased the plasma melatonin levels. This indicates that the pineal gland is refractory during parts of the natural light-phase, which is in accordance of findings in other teleost species (Falcón *et al.* 1989, Næve 2012). This observed refractory period of the silver carp pineal gland also suggests a pineal timing mechanism regulating the synthesis of pineal melatonin.

4.1.4 Control of silver carp melatonin release and profile

Altogether, the results from the three experiments indicate the presence of a control mechanism regulating pineal melatonin synthesis in silver carp. This mechanism is able to sustain the diurnal rhythmical pineal melatonin synthesis observed during natural photoperiod, even when subjected to constant darkness. Thus, the presence of an intra-pineal biological clock controlling the pineal melatonin synthesis in silver carp is suggested. This proposition is in accordance with most teleost species investigated, including goldfish (*Carassius auratus*) (Iigo *et al.* 1991), zebrafish (*Danio rerio*) (Cahill 1996), European sea bass (Bayarri *et al.* 2004b), Nile tilapia (*Oreochromis niloticus*) (Martinez-Chavez *et al.* 2008b) and tench (*Tinca tinca*) (Oliveira *et al.* 2009).

Based on knowledge from studies of other teleost species described in the introduction, and the findings in silver carp in the present study, it can be assumed that a biological clock is controlling the AANAT-2 mRNA levels in the pineal gland of the silver carp. The clock is partly independent of the light-dark cycle in controlling melatonin synthesis. Activity of the

biological clock is suggested to give low AANAT-2 mRNA levels during the natural light-phase, and increased levels during the natural dark-phase. This rhythm sustains during constant conditions, and could explain why the rhythmical diurnal melatonin patterns persist in fish subjected to continuous darkness. The activity of a biological clock could have caused reduction in AANAT-2 mRNA levels, causing the decrease in melatonin levels in late scotophase during the natural photoperiod. The observed refractory period during natural light-phase could also be explained by the presence of an intra-pineal biological clock. At morning and midday, the clock may give low levels of mRNA AANAT-2, and exposure to darkness will not increase melatonin levels. In late day/beginning of evening, however, the system may be preparing for the following scotophase by increasing the level of AANAT-2 mRNA present. When silver carp is exposed to experimental darkness at this time of the day, AANAT-2 levels increases, and consequently there is an increase in plasma melatonin levels.

The observed melatonin levels throughout 24-hours in silver carp kept under natural photoperiod display a melatonin profile resembling the type B melatonin profile (Reiter 1987). This profile is characterized by a peak in mid dark-phase, followed by a gradual decrease of melatonin levels prior to onset of light (fig. 5, p. 7). However, the nocturnal melatonin levels in silver carp exposed to the manipulated photoperiod showed more resemblance to a type A melatonin profile, described by a peak of melatonin in late dark-phase. This may indicate that there has been a shift in the rhythm of melatonin secretion when there is no synchronization to the light-dark cycle. This indicates that it may be possible to change the rhythm of melatonin synthesis in silver carp by photoperiodic manipulation.

Some teleost species do not have an intra-pineal biological clock (Falcón *et al.* 2011). Isolated pineal glands of rainbow trout (*Salmo gairdneri*) showed no endogenous rhythmicity in melatonin synthesis when subjected continuous darkness (Gern and Greenhouse 1988). In the sockeye salmon (*Oncorhynchus nerka*) the pineal gland released constant high levels of melatonin during continuous darkness (Iigo *et al.* 2007). According to the descriptions of different melatonin patterns by Reiter (1987), a type C melatonin synthesis pattern is characteristic of the salmonids. Thus, photomanipulation regimes used in farming of salmonids might not be useful in silver carp, and development of photomanipualtion regimes should be further investigated in silver carp.

4.2 Non-photic influence on plasma melatonin levels

In addition to photoperiod, various other factors may have an impact on pineal melatonin synthesis in teleost fishes.

4.2.1 Season and temperature

Changes in ambient temperature may affect the synthesis of melatonin from the teleost pineal gland, especially the amplitude of melatonin levels (García-Allegue *et al.* 2001, Popek and Ćwioro 2010, Falcón *et al.* 2011). A specific combination of photoperiod and temperature is

believed to give the fish precise information about season. This is seen in Atlantic salmon (*Salmo salar*), where diurnal variations in both amplitude and time of peak were (in addition to photoperiod) influenced by temperature (Randall *et al.* 1995). Different seasonal melatonin profiles were also described in the sea bass (García-Allegue *et al.* 2001) and Senegal sole (*Solea senegalensis*) (Vera *et al.* 2007). In the case of Indian carp, temperature was involved in the changes in melatonin secretion observed throughout an annual cycle (Chattoraj *et al.* 2009). These results illustrate the importance of considering season and temperature when studying melatonin profiles in teleost fishes. The silver carp melatonin profile could consequently be different at the different times of the year.

4.2.2 Maturation and sex steroids

Several studies indicate that sex steroids may influence the pineal gland melatonin synthesis in fish (Bégay *et al.* 1994, Yanthan and Gupta 2007). Some cyprinids changed melatonin profile according to maturation status. The annual variations in melatonin profiles in maturing Indian carps were in addition to photoperiod and temperature, influenced by the endocrine activity of the ovary, as an inverse relationship between gonad and pineal gland was reported (Maitra *et al.* 2005, Chattoraj *et al.* 2009). Consequently, sex steroids might also be involved in the seasonal changes observed in melatonin profiles in maturing carps.

4.2.3 Stress

Handling and transport may initiate stress responses in fish, which gives elevated levels of circulating catecholamines (adrenaline and noradrenaline), and cortisol (Barton and Iwama 1991). To prevent initiating a stress response during handling of silver carps, the fish were sedated using the anesthetic agent MS-222 upon netting from the tanks. It is reported that MS-222 inhibits the stress response during handling by suppressing the nervous system. This results in paralysis and reduces the perception of stress (Carter *et al.* 2011, Zahl *et al.* 2012).

The basal plasma cortisol levels in non-stressed fish are generally reported to be less than 80 nmol/L (Barton and Iwama 1991). In response to exposure of an acute stressor, an increase in plasma cortisol levels between 80-825 nmol/L is representative for various teleost species (Barton and Iwama 1991). The levels in silver carps in this study were ranging between 500-1400 nmol/L, and 600-800 nmol/L during light and dark periods, respectively. These high values indicate that the experimental fishes were somewhat stressed. During a transport experiment, the cortisol levels were 300-550 in unstressed silver carps, and 700-800 nmol/L in stressed silver carps (Hasan and Bart 2007). The stress response, and magnitude of cortisol release during exposure of a stressor is found to vary between different species, and also within the same species (Barton 2002). Holtan (2011), Guttu (2011) and Næve (2012) reported similar high cortisol levels as reported in this study in other carp species from Nepal.

The high cortisol levels reported in the silver carps could have been caused by a change of environment from fishponds to the experimental tanks. However, MS-222 may cause an

increase in cortisol levels in some teleosts (Small 2003, Davis and Griffin 2004, King *et al.* 2005), and the use of this anesthetic could have contributed to elevate the cortisol levels.

Cortisol and melatonin concentrations in the silver carps showed a weak inverse correlation during natural photoperiod. This could indicate a relationship between melatonin and cortisol. However, no such correlation was found in fish exposed to continuous darkness. The low and non-significant correlation based on individual cortisol and melatonin levels from all three experiments, indicates spurious relationship between melatonin and cortisol. In any case, a correlation analysis does not tell anything about causality between the two factors. The correlation found in fish under natural photoperiod, could be a result of the natural diurnal variations in the two hormones, and not because of interactions between them.

Cortisol and catecholamines can influence pineal melatonin synthesis in teleosts, as some species has receptors for catecholamines and glucocorticoids in the pineal gland (Benyassi *et al.* 2001, Nikaido *et al.* 2010). It has been shown that corticosteroids could directly inhibit AANAT activity, and thus melatonin synthesis (Yanthan and Gupta 2007). Nikaido et al. (2010) showed that cortisol reduced pineal melatonin synthesis. In contrast, social stressed rainbow trout had significantly higher levels of nocturnal melatonin compared to non-stressed individuals (Larson *et al.* 2004).

The high cortisol levels reported in the silver carps during the experiments might thus have influenced the results concerning melatonin. However, the lack of correlation between the plasma levels of the two hormones indicated that has not influenced on the melatonin levels. Even though cortisol might have had an effect, it is the fluctuations occurring in melatonin levels throughout the 24-hour cycle that is the important findings of this study. The rhythmicity in melatonin levels through 24-hours in silver carp was similar to what has been reported in other teleost fish. Highest levels of cortisol were found in the light phase, while in this study the nocturnal levels of melatonin were of importance. All of the fishes were treated the same way during the different experiments, and a possible effect from cortisol is thus considered not to impair the significance of the melatonin profiles. Consequently, it is suggested that cortisol and stress may not have influenced plasma melatonin levels and fluctuations in the silver carps significantly.

4.2.4 Extra-pineal melatonin

The retina is an important extra-pineal site of melatonin synthesis in fish. The main effects of retinal melatonin are believed to be local in the retina, through autocrine and paracrine effects (Falcón *et al.* 2011). A high deacetylase activity exists in retina, which breaks down melatonin. This prevents melatonin release into the general blood circulation (Grace *et al.* 1991).

The gastrointestinal tract (GIT) is also found to be a site for extra-pineal-melatonin synthesis in teleosts (Bubenik and Pang 1997, Velarde *et al.* 2010). Melatonin synthesis in the GIT is probably linked to food intake (Bubenik 2002). Little is known about the effects of

gastrointestinal melatonin in fish. However, it is suggested to mainly show autocrine or paracrine actions (Velarde *et al.* 2010). Melatonin from the GIT may circulate from the intestine to the liver for degradation (Herrero *et al.* 2007). Hence, intestinal melatonin may most likely not be released into the circulation.

Since melatonin from other tissues than the pineal gland are suggested to have local effects, and may not be released into the circulation, melatonin released from the pineal gland is considered to be the main source of this hormone in the blood circulation of fish (Falcón *et al.* 1989, Reiter 1993, Hardeland *et al.* 2006, Falcón *et al.* 2010a). If pineal-melatonin is mixed with melatonin from extra-pineal sites in the circulation, the effects of melatonin as a signal to the organism about time of the day and season could be disturbed.

4.3 The validity of melatonin results

4.3.1 Assay performance

Results from the plasma melatonin analyses were considered to be accurate, as the different validation parameters, sensitivity, recovery and parallelism, fulfilled the criteria to verify the validity of the assay.

4.3.2 Individual variations

Plasma melatonin and cortisol levels varied considerably between individual fishes sampled at the same time during the experiments. Natural biological variations are commonly observed. This is likely to have contributed to the high SD reported in this study. Future studies should investigate the melatonin release in individual fishes throughout the 24-hour light-dark cycle.

4.3.3 Silver carp body size

A significant difference was found between the weights of fish sampled at 12:00 and 21:00 under natural photoperiod. However, this is not considered to have an impact on the results as this significance only occurred between two groups.

4.3.4 Light intensity in fish tanks

The measured light intensity inside the fish tanks was considerably lower than the surroundings. However, melatonin synthesis is highly sensitive to light, with a threshold of about 10 lux (Bayarri *et al.* 2002). The levels measured in silver carps during natural light phase were low. Consequently, light intensity inside the tanks was sufficient to reduce melatonin synthesis under natural light.

4.4 Experimental summary

The main results in the present study can be summarized as:

- Plasma melatonin levels were low during the natural light phase, and increased during the natural dark-phase. Melatonin levels started to decrease during late scotophase, prior to onset of light.

- The rhythmicity in plasma melatonin throughout 24-hours during the natural photoperiod was maintained during extended period of darkness.

- A pineal refractory period was observed during early and mid photophase, but not in late photophase just prior to onset of natural darkness.

4.5 Conclusions

1. Plasma melatonin levels in silver carps under natural photoperiod were low during the photophase, and increased significantly during the scotophase. During exposure to continuous darkness this diurnal rhythmicity in melatonin persisted. Silver carp pineal gland is refractory during parts of the photophase.

2. A biological clock is suggested to control pineal melatonin synthesis in silver carp.

3. There was no correlation between cortisol and melatonin in silver carp, and cortisol was not assumed to influence on the melatonin levels significantly.

4.6 Perspectives and recommendations

The suggested intra-pineal biological clock in silver carps provide relevant information to future studies on how to develop photomanipulation regimes which could control the timing of reproductive events in this species. These studies should examine how the suggested endogenous rhythm controlling melatonin secretion could be manipulated by changing the photoperiod. Impacts from non-photic factors, such as season, temperature, and sex steroids on the melatonin system must also be investigated. Future experiments should then investigate if photoperiodic manipulation could change the timing of maturation.

Further studies are needed to understand the impacts of photoperiod and melatonin on the teleost HPG-axis. Improvement of knowledge about regulation of the HPG-axis by melatonin through different factors like the kisspetidergic and dopaminergic-systems is suggested. It should also be important to explore if melatonin is involved in influencing on teleost reproduction through the recently discovered GnIH. Furthermore, examining the effects of other factors influencing on reproductive events is important in future studies on photoperiodic manipulation. One such factor is indicated to be the energetic status of the fish. In order to achieve a successful initiation of maturation by photoperiodic manipulation, it is important to improve our understanding on how the energetic condition of the fish impacts on maturation.

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