NTNU

Norwegian University of
Science and Technology

Plasma melatonin profiles in Common carp (*Cyprinus carpio*) exposed to indoor photoperiods

Marte Berg Holtan

Biology
Submission date: August 2011
Supervisor: Kjell J. Nilssen, IBI

Norwegian University of Science and Technology
Department of Biology

Acknowledgments

Thank you, my supervisor, professor Kjell Nilssen, for introducing me to the fantastic, but weird field of endocrinology, where everything is connected in curious ways, and thank you for giving me the opportunity to travel to the wonderful, mighty Nepal, a country I hope to visit again and again; Dhanjabad to my Nepalese friends Rakesh, Agni, Mr. Bistha and Uttam for hospitality, organizing at the research centre, and guidance through the Nepalese traditions; thank you Maria Guttu, for being a perfect travel-companion and partner during the field-work; thank you Henriette Vaagland for your always sparkling mood and care for all people around you; thank you Grethe S. Eggen for always rapid answers and solutions for my laboratory FAQs; thank you, Ida Caspersen, my statistical guru; thank you all my inspiring friends from many, many coffee-breaks with enlightening talks, thank you Stine Ims for great company in DU2-100 and for reading at the last end; thank you Siv Rørvik for razor-sharp proofreading; thank you to my supportive, fantastic family; and a very special thank you to Fredrik for all your love and support, without you, I never would have managed.

hjernen er alene

Trondheim, August 2011

_____

Marte Berg Holtan

Abstract

An intervention against extreme poverty and hunger was introduced in year 2000, when all members of the United Nations agreed on the Millennium Development Goals (MDG). Today, 1.4 billion people live in poverty and hunger, with poor prospects for increased living standards. Nepal is one of the world's poorest countries with most of its population living ruraly on low-income agriculture. Due to ongoing climatic changes and financial instability, the international food security is threatened. Inexpensive and low water consuming food production will therefore be an important development for times to come. In line with the MDGs, the NTNU initiated programme Sustainable Poverty Reduction in Nepal (SPRN) have started to utilize Nepal's rich freshwater resources to develop fish farms in tandem with eco-friendly hydropower projects. The main target is to develop year-round delivery of carp fingerlings in hilly rural areas.

Fish are seasonally breeding animals that use environmental signals to coordinate and control their biological rhythms. Photoperiod represents an accurate indicator of time of day and season, and may be translated into a chemical body signal, melatonin, by the pineal gland. Pineal melatonin is released during night and the secretory pattern – which reflects the environmental light/dark cycle – may exhibit one of three known patterns. A daily and annual rhythmic production of melatonin may provide the fish with a physiological capacity to anticipate and prepare for upcoming seasonal changes. Manipulation of the photoperiodic control of pineal melatonin release has been successfully used to initiate biorhythms like spawning in cultured finfish species at mid and high latitudes.

The current study was performed to describe the day/night plasma melatonin levels in Common carp (*Cyprinus carpio*) during November at mid-hills Nepal (28 ºN). It represents the first part of possible development of a maturation control system for low latitude carps.

Plasma melatonin levels exhibited a single peak profile during late darkphase and decreased to low daytime levels before the onset of light. When subjected to an extended night period, carp plasma melatonin rhythm appeared to repeat this profile from natural photoperiod, which may indicate a circadian clock system at work. Blood plasma cortisol levels were elevated during these experiments but are not expected to have stimulated the melatonin release. These results demonstrate a possible complex melatonin control system of type B in the Common carp kept at low latitude (Nepal).

Contents

Introduction

In September 2000, all member states adopted the United Nations Millennium Declaration, a global action plan to fight extreme poverty in its many dimensions. This declaration consists of eight goals[1], known as the Millennium Development Goals (MDGs) and is set to be achieved by 2015 (UN, 2010b). The top targets are to fight the poverty and hunger that 1.4 billion people in the world experience every day (Chen & Ravallion, 2010; UN, 2010b; PRB, 2011). The importance of these goals is also illustrated by the estimation that 129 million children under age of five are underweight (UNICEF, 2009). It is furthermore noteworthy that 46 % of these children live in Southern Asia (Figure 1) (UN, 2010a).

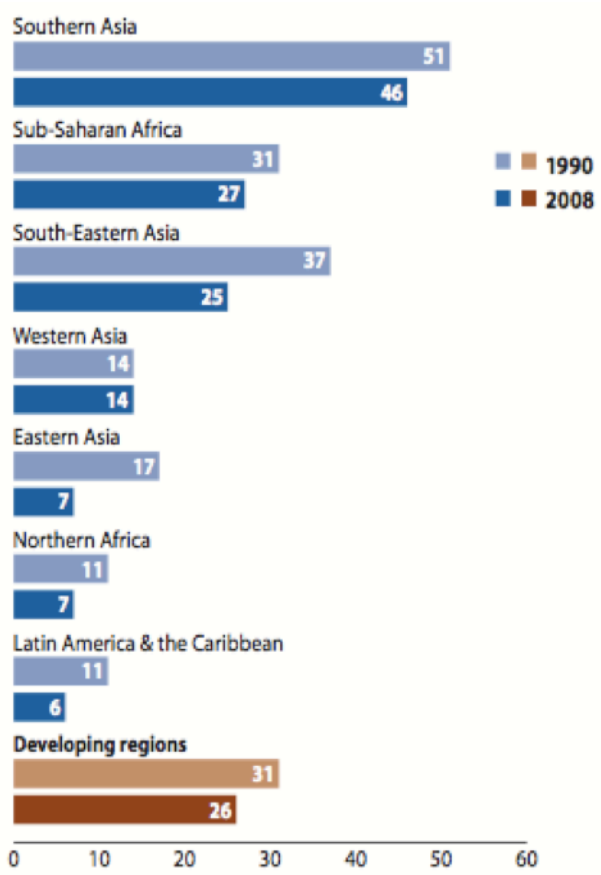

Figure 1: Proportion of children under age five who are underweight in 1990 and 2008 (percentage). Modified from UN (2010a).

Due to the financial crisis in 2007-2010 the food prices spiked and brought a worsened situation for all the people who failed in employment and income (UN, 2011). The increase in food price creates additional obstacles in the fight against poverty and hunger, and in 2011, 29 countries required external assistance for food (FAO, 2011a). Accordingly, the Food and Agricultural Organization (FAO) estimated an increase in number of undernourished people from 830 million people in 2008 to more than 1 billion in 2009 (UN, 2010a).

In addition to the financial crisis, the entry to this decade has been influenced by a series of natural disasters. Storms in USA, flooding in Pakistan, landslides in Afghanistan and, earthquakes in New Zealand, Haiti, Chile and Japan (Knabb et al., 2006; Reuters, 2010a; 2010b; BBC, 2011a; 2011b; UN, 2011) have not only inflicted humane disasters, but also caused large problems in the world's food security. Together with the heat wave in

[1] The United Nations Millennium Development Goals: 1. Eradicate extreme poverty and hunger 2. Achieve universal primary education 3. Promote gender equality and empower women 4. Reduce child mortality 5. Improve maternal health 6. Combat HIV/AIDS, Malaria and other diseases 7. Ensure environmental sustainability 8. Develop a global partnership for development

Russia last summer and flooding in Australia, the international wheat market is insecure (FAO, 2011d). Countries that usually hold large food stocks and export crops worldwide are now in a situation were they need their stocks themselves (Bradsher, 2011; Brown, 2011). Currently, the drought in China (Granbo, 2011) last winter adds to the seriousness as previously self-supplied China has now started to import crops, which is forecasted to almost vacuum the international food market (Bradsher, 2011; FAO, 2011a). Hence, the (third) world's need for other food sources has never been more in question!

Why aquaculture?

To eradicate extreme poverty and hunger there is a primal demand for increased food production in the areas of concern. Underweight and malnutrition is a major challenge in the developing world (UNICEF, 2009), so the demand for easy-access nutritious food is substantial. Fish is a major source of proteins, micro-nutrients and essential fatty acids for humans (Roos et al., 2003; Michaelsen et al., 2009). However, so far people in the developing world have mostly been unable to purchase this commodity due to their level of poverty (Wattage, 2009). Aquaculture[2] is one of the world's fastest growing sectors for food production and has since the early 1950s grown more than any other animal food producing sectors. In 2008, aquaculture (excluding plants) supplied the world with 52.5 million tonnes at a value of US$ 98.4 billion (FAO, 2010) and as much as 76 % of the global freshwater finfish was obtained from aquaculture alone (FAO, 2008). The Asia-Pacific region heavily dominates this industry, with 89 % of the world's overall production (2008) (FAO, 2010). Because of the vigorous growth in the aquaculture industry, it has a great potential for food supply, poverty alleviation and enhanced trade and economic benefits, as targeted by the MDGs (Delgado et al., 2003; Wattage, 2009). Hence, fish farming in rural areas can promote socio-economic development for both the local communities and the country itself (Gurung et al., 2005; Gurung et al., 2010). The potential contribution from aquaculture to nutrition, food security and livelihoods can be highly significant, especially in rural areas (Subasinghe, 2005). Within this context Wattage (2009) claims that aquaculture can be developed in any poverty-driven area if an adequate natural resource base exists.

Norwegian development cooperation

Norwegian competence and technology is today at the front line of oil exploration, hydropower development and aquaculture (MPE, 2004; SSB, 2009). The Norwegian Agency for Development Cooperation (NORAD)[3] wants to utilize this capacity to assist in the

[2] Aquaculture is defined as: " the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated." (FAO, 1997)

[3] A directorate under the Norwegian Ministry of Foreign Affairs (MFA).

development of the Third World. Thus, the Norwegian international aid policy is in line with the UN Millennium Development Goals and shall; "Contribute towards permanent improvements in economic, social and political conditions in the developing world, with particular emphasis on benefits for the poorest people of the community" (NORAD, 2011b). Experiences from decades of developmental assistance have shown that the most effective way of reducing poverty is when the recipient country's own development strategies are the basis for the assistance. With contracted obligations for both the donor and the recipient of the financial aid it is more likely to succeed (NORAD, 2011b). Norway has been involved in long-term development cooperation with several countries (NORAD, 2011b) and the total financial aid from Norway in 2011 is estimated to 27.1 billion NOK (equivalent to US$ 4.7 billion) (MFA, 2010; NORAD, 2011a).

Nepal and Norwegian contribution

A connection has existed between Norway and Nepal since Odd Hoftun in 1958 left Norway for a simple life in the Nepalese mountains, where he was the leading light in development of hospitals, power plants as well as foundation of the Butwal Technical School (Svalheim, 2009). During the 70s diplomatic connection was established, and in 1996, the between-government development cooperation was founded (Norwegian Embassy, 2011). Nepal is one of the poorest countries in South Asia (UN, 2010a) with 55 % of the population living on less than US$ 1.25 a day (2005) (WorldBank, 2011) and in 2009, it was among the ten countries that received most financial aid from Norway, with 284.5 million NOK (equivalent to US$ 50 millions) (MFA, 2010; NORAD, 2011a). The Norwegian assistance in Nepal concentrate on primary education, good governance, human rights and energy sector development (with focus on hydropower and electrification) (Norwegian Embassy, 2011). Between 1980 and 2010, Nepal has shown advancement on the 'Human Development Index'[4] (HDI) because of a big progress in health and education, but today's ranking as nr. 138 out of 169 nations still puts Nepal among the countries with low human development (UNDP, 2010).

The hydropower revolution

Climate and resources

Climatically, Nepal lies in a sub-tropical zone, but due to big variations in topography, a wide range of climatic conditions exists (Shrestha et al., 2000). The country is landlocked between India and China, with the northern part covered of the mighty Himalayas. Three parallel geographical zones runs from east to west (Figure 2); the Terai lowland with a sub-tropical climate in the south, the Hills in the middle, and Himalayan mountains in the north with cool summers and severe winters (Shrestha et al., 2000; Petr, 2002; CIA, 2011).

[4] "A composite measure of achievements in three basic dimensions of human development—a long and healthy life, access to education and a decent standard of living" (UNDP, 2010)

Figure 2: Topographic map of Nepal. The experiments for this thesis was executed in Begnas (28°9'54"N, 84°6'34"E), a small village around 15 km east of Pokhara (red dot in the middle of the map). Modified from World Map Finder (2011).

Deforestation, terrace cultivation and road construction in the mountain regions have resulted in heavy soil erosion and landslides during the monsoon season (Shrestha, 1999; Swar, 2002). Two thirds of the Nepalese population relies on farming for their livelihood (CIA, 2011), and loss of crop due to the monsoon creates more work and less income for the farm-workers. Although agriculture is far the most primary source of income and employment (83 % of economically active people) (AQUASTAT, 2010), Nepal is still one of the countries with the lowest farm productivity in South Asia (Pyakuryal et al., 2005; FAO, 2011d). As a result of poor modernization of the agricultural sector, the country relies upon food import and is economically dependent of foreign aid (Jull, 2006; CIA, 2011). The infrastructure needs development and the fact that most of the poor people live in rural terrain limits the connection between rural production areas and urban markets (Jull, 2006; AQUASTAT, 2010).

Hydropower potential

Nepal holds 2.7 % of the world's total freshwater recourses and is the second richest country in the world concerning fresh water availability (Sharma et al., 2005). With over 6000 rivers/tributaries along the vast Himalayan mountains (Shrestha, 1999), the potential for development of hydropower and waterbound food production is great (SPRN, 2008). However, with poorly modernized agriculture, which consumes enormous amounts of water, a better utilization of water resources is necessary. Today's situation is characterized by a

demand for electricity much larger than the power stations are able to supply (Gurung et al., 2010). One option for better exploitation of the water recourses is to combine hydropower and fish farming in rural valleys. In this way, electricity is not the only product, but also fish for human consumption. Fish brings another positive side effect beside nutrients: they save water. Fish do not consume water as plants do; they only "borrow" it on its way back to the river, so the water can still be used for irrigation (SPRN, 2008).

A climate positive SPRN programme

Most of the aquaculture development strategies have until recently concentrated on increased production and not aquaculture as food security. If aquaculture is to become an advantageous strategy for development, opportunities for the poor to engage in aquaculture needs to be a part of the strategy (Friend & Funge-Smith, 2002; Pollock, 2005). Food production can both damage and improve the natural resource base, and the success of aquaculture depends upon its ability to produce fish as well as to maintain the sustainability of its resources (FAO, 1997; Wattage, 2009). SPRN (The Sustainable Poverty Reduction in Nepal) is an initiative to improve the livelihood of people in the rural hilly areas of Nepal, by "Combine fish farming and education with eco-friendly hydropower". Utilization of the hydropower tail-water for mass production of endemic species fingerlings can thereby be carried out without consumption of the water needed for irrigation (SPRN, 2008). The project was initiated summer 2005 by the Norwegian University of Science and Technology (NTNU) with assistance from Sweco-Grøner AS. SPRN is financed by the Norwegian Ministry of Foreign Affairs and is a cooperation between several national and international institutions (SPRN, 2007). The SPRN Programme promotes sustainable poverty reduction by using one of Nepal's major commodities: water for fish farming and hydropower generation. The main objectives for the project are: (1) To develop and improve rural food production and employment; (2) To develop mass production of fish fries in tandem with hydropower stations, and (3) To improve human capacity in aquaculture and freshwater management (SPRN, 2008).

Aquaculture in Nepal: trends and prospects

Today, a total of 183 indigenous and exotic fish species can be found in the rivers and lakes of Nepal (Shrestha, 2002). Capture fisheries is an old tradition in Nepal, but are widely scattered, not organized and very old-fashioned (Shrestha, 1999). The first aquaculture techniques for fish production were tested in the 1950s, together with the introduction of exotic carp species (Swar & Gurung, 1988; Gurung, 2003). Today, the aquaculture production is mainly located in the southern Terai and is dominated by cage culture in lakes and ponds with warm-water carps (Yadav & Bhujel, 1998; Gurung, 2003). The quantity of fish produced by fish farms in Nepal is significantly lower compared to neighbouring countries such as India and Bangladesh (FAO, 2010). In 2008, total aquaculture production was approximately 27 tonnes (Figure 3), with an estimated value of US$ 46.7 million (FAO, 2011c). By comparison, Norwegian aquaculture development started in the 70's and has increased to a

production with sky-high levels in excess of over 1000 tonnes in 2010 (SSB, 2009). The slow growth of Nepalese aquaculture originates from a combination of issues; poor quality of the fish, lack of; production sites, technology interventions, information about local species, fry access, skilled farmers and training programmes (Shrestha, 1999; SPRN, 2007).

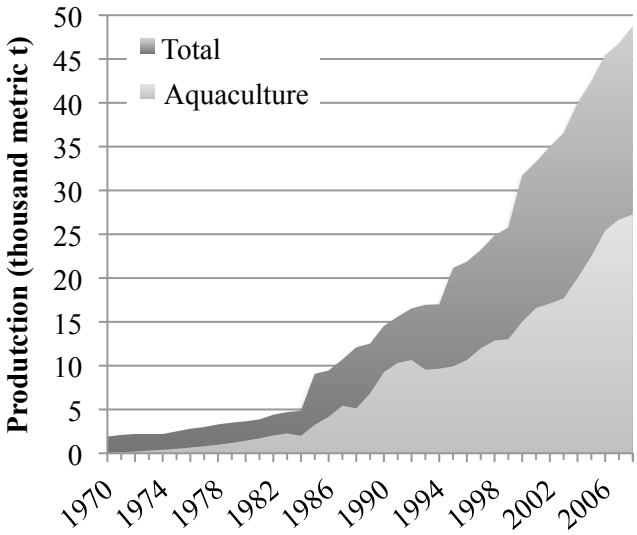

Figure 3: Total Nepalese fish availability and aquaculture share in the period 1970 - 2008.

How can aquaculture in Nepal overcome all these challenges, increase its productivity and obtain sustainability? To ensure a sustainable and economically fish farming, the production depends on rapid growth and good fecundity with a year-round supply of fish fry (Bromage et al., 1992). To obtain such stability it is decisive whether or not it is possible to control the periodicity of maturation and reproduction.

In temperate species it is evident that the duration of the solar day is the most important environmental cue signalling the sexual periodicity. This has been utilized by use of manipulated photoperiods to induce out-of-season spawning (Bromage et al., 1992; Migaud et al., 2010). If fish farms in equatorial areas can utilize the same techniques, it can be an important progress for the aquaculture industry. Besides, by focusing on local endemic species the natural biodiversity will not be affected (Ross et al., 2008; SPRN, 2008). Among the indigenous fish species of Nepal, Katle (*Neolissocheilus hexagonolepis*), Mahseer (*Tor tor* and *Tor putitora*) and Snow trout (*Schizothorax sp*) have been identified as excellent food fish (Swar, 2002). In addition are several induced exotic carp species like Common carp (*Cyprinus carpio*), Rohu (*Labeo rohita*), Silver carp (*Hypophthalmichthys molitrix)*, Grass carp (*Ctenopharyngodon idella*) and Bighead carp (*Hypophthalmichthys nobilis*) proven to be of great use in fish cultivation and consumption (Shrestha, 1999; Gurung, 2003). All these species are herbivorous/omnivorous feeders which make them suitable for low energy input aquaculture (Shrestha, 1999). However, little is known about these species reproduction physiology.

Biorhythms and melatonin

Environmental changes and biorhythms

The physiological state of an animal is endogenously different at different times of day, and in different seasons of the year. All animals possess endogenous physiological timing mechanisms that rhythmically modulate the functioning of cells, tissues and organs (Chowdhury et al., 2008). This control mechanism is termed *the biological clock*, which use environmental info in a feed-forward control to entrain the endogenous rhythms in the organism. The major advantage of biological clocks is that they are predictive: they enable an animal to anticipate and prepare for regular environmental changes so that they are physiological adjusted to the environment at all times (Vivien-Roels & Pévet, 1993; Falcon, 1999; Dunlap et al., 2004).

Photoperiod is the most prominent and reliable external cue and has been established as the main external cue for synchronization of the biological clock (Falcon, 1999; Falcon et al., 2010). Adaptations to the alternations in the 24 h light (L) and darkness (D) cycle gives an organism the capacity to orientate in time on a daily and annual basis (Falcon, 1999; Dunlap et al., 2004). In addition, other external cues like temperature, rainfall, food availability or water salinity may contribute to shape the final biological rhythms (Falcon et al., 2010).

Fish pineal system

Teleosts have photoreceptive cells in the retina, but also in the pineal gland (Ekström & Meissl, 1997), and they have both proven to produce the hormone melatonin (N-acetyl-5-methoxytryptamine), but only the pineal melatonin is thought to have a daily and seasonally effect via endocrine release into the general circulation (Lewy et al., 1980; Iuvone et al., 2005).

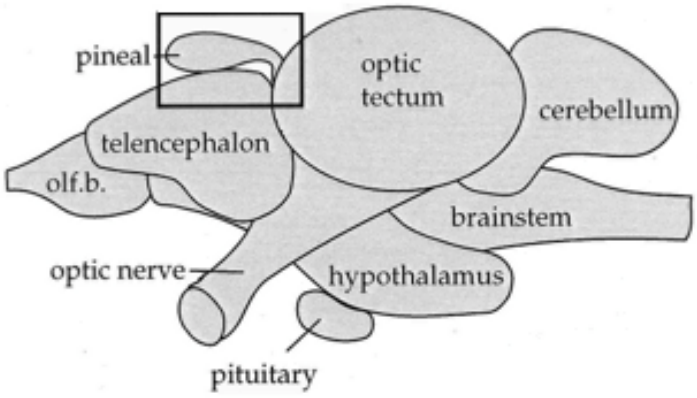

Figure 4: General structure of the brain and location of the pineal gland in teleosts. Modified from Ekström & Meissl (1997).

The pineal gland of teleost fish is a vesicle located dorsal to the diencephalon (Figure 4) and its lumen is an extension of the third ventricle of the brain (Zachmann et al., 1992; Ekström & Meissl, 1997). Usually, light influences the pineal directly through a window in the skull, a spot called the 'pineal window' (Migaud et al., 2006). The active cells of the teleost pineal are specialized cells with both photoreceptive and endocrine functions (Falcon et al., 1992; Ekström & Meissl, 1997; Falcon, 1999). Interestingly, the photoreceptor molecules are shown to be retina-like opsins placed in the outer segment of the receptor (Ekström & Meissl, 1997).

The pineal organ conveys photoperiodic information to the brain via neural pathways and by release of the indoleamine melatonin into the circulation (Ekström & Meissl, 1997). Pineal melatonin is only produced in the dark phase of the LD cycle and the duration of the nocturnal surge in plasma melatonin levels is normally proportional to the length of the night. In this way, melatonin creates a daily and calendar plasma-melatonin cycle in line with the environmental LD cycle (Ekström & Meissl, 1997; Falcon, 1999; Bromage et al., 2001). The pineal glands encoding of seasons therefore involves two processes: (1) night length registration and (2) information processing in the brain. Under natural conditions, the circannual rhythm would be fully synchronized with the ambient light cycle as a result of ongoing re-entrainment by seasonally changing photoperiod (Bromage et al., 2001).

Melatonin synthesis

The general mechanism of melatonin biosynthesis (Figure 5) appears to be identical in all vertebrates (Maitra et al., 2006). First, the melatonin precursor tryptophan is selectively taken up from the circulation and into the photoreceptor cell (Falcon & Collin, 1985), where it is converted to 5-Hydroxytryptophan, by *tryptophan hydroxylase* (TPOH).

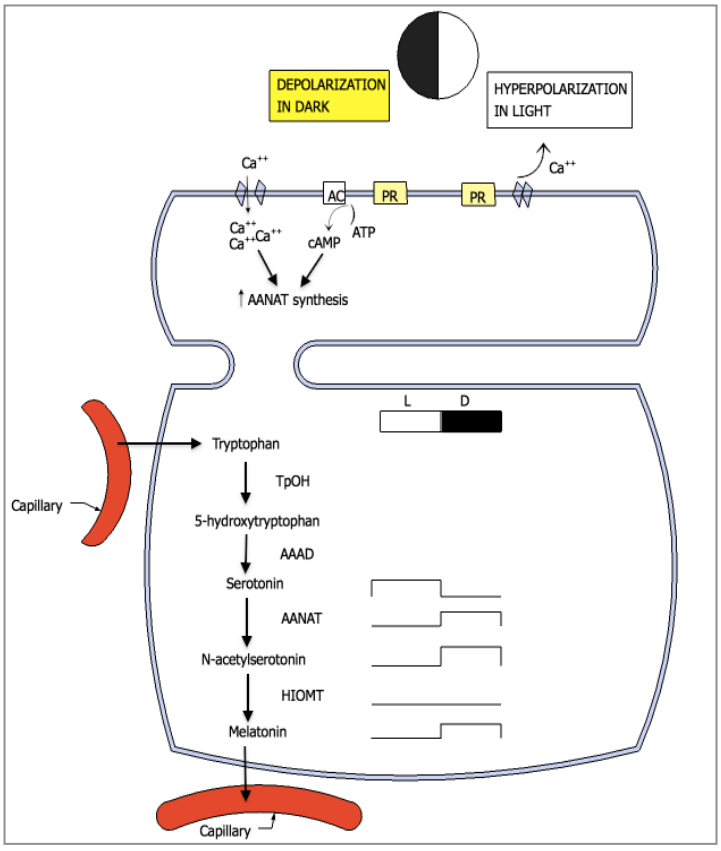

Figure 5: Biosynthesis of melatonin and activation of AANAT by depolarization of the photoreceptor cell in the pineal gland in the dark. On the right, indication of a photoperiodic 24h light (L) and dark (D) cycle with a schematic indication of the daily variations in the corresponding compound or enzyme activity. PR, photoreceptor; AC, adenyl cyclase, TpOH, tryptophane hydroxylase; AAAD, aromatic amino acid decarboxylase; AANAT, arylalkylamine-N-acetyltransferase; HIOMT, hydroxyindole-o-methyl transferase. Developed from Seth & Maitra (2011).

5-Hydroxytryptophan is decarboxylated by *aromatic amino acid decarboxylase* (AAAD) to produce 5-Hydroxytryptamine (Serotonin). Serotonin undergoes N-acetylation by *arylalkylamine-N-acetyltransferase* (AANAT) to produce N-acetyltserotonin, which in turn is methylated by *hydroxyindole-O-methyl transferase* (HIOMT) to produce N-acetyl-5-methoxytryptamine – Melatonin (Falcon & Collin, 1985; Ekström & Meissl, 1997; Klein et al., 1997).

The rhythmic function of melatonin synthesis in the vertebrate pineal is generated by stimulation and degradation of the enzyme AANAT in a rhythmic pattern (Klein et al., 1997). In response to darkness, the fish pineal photoreceptor cell is depolarized, which lead to influx of Ca^{2+} and accumulation of cyclic AMP (cAMP). These intracellular messengers are thought to be involved in the synthesis and regulation of AANAT (Falcon et al., 2001; Iuvone et al., 2005; Seth & Maitra, 2010a). When the photoreceptor cell is illuminated, the Ca^{2+} channels close and the cell hyperpolarize. AANAT is degraded and consequently, pineal melatonin production is inhibited (Falcon & Collin, 1985; Bayarri et al., 2010). Because of this rhythmic stimulation and degradation, AANAT is the rhythm-generating and also the rate-limiting enzyme for production of pineal melatonin (Falcon et al., 1989; Falcon et al., 2001). In addition it is recently revealed that the environmental signal (LD) is not the only regulator, but cholinergic, adrenergic and dopaminergic mechanisms are also involved in the neuronal regulation of the photic signal transduction, but in different ways in different species (Maitra et al., 2006; Seth & Maitra, 2010a; Seth & Maitra, 2011).

Biorhythms and organic control

Melatonin functions as a signal molecule for a great deal of processes in the fish body. This includes: locomotor activity, feeding, daily thermal preference, migration, osmoregulation, smoltification, metabolism, growth and reproduction (Ridha & Cruz, 2000; Boeuf & Falcon, 2001; Almazan-Rueda et al., 2004; Campos-Mendoza et al., 2004; Falcon et al., 2007) Melatonin acts on its target cells through transmembrane G-protein-coupled receptors known as MT1, MT2 and MT3 (Falcon et al., 2007; Falcon et al., 2010). These receptors are found in nervous tissues like the brain (Gaildrat & Falcon, 1999) and retina (Sauzet et al., 2008), but are also identified in peripheral tissues like gills, kidney and gastrointestinal tract (Kulczykowska et al., 2006), which suggest an active uptake or local synthesis of melatonin in these tissues as well.

The rhythmicity of melatonin secretion from the pineal gland is a product of the environmental light-dark cycle, but the pineal gland may contain intra-pineal oscillators, capable of self-sustaining the melatonin rhythm in absence of a photoperiodic cycle. This endogenous melatonin rhythm free-run with a period close to 24 h under constant darkness (Ekström & Meissl, 1997; Falcon, 1999). Such a circadian clock system have been found in numerous species including sea bass (*Dicentrarchus labrax*) (Bayarri et al., 2004), pike (*Exos lucius*) (Falcon et al., 1989), goldfish (*Carassius auratus*) (Kezuka et al., 1992) and zebrafish (*Danio rerio*) (Cahill, 1996). Salmonids, on the other hand, are characterized by a directly

light sensitive pineal, with no pacemaker activity. No melatonin rhythm appears under constant darkness. They have a decentralized system in which the pineal gland responds directly to the environmental light information independently of the eyes. Regardless of time of year or experimental photoperiod, elevated plasma melatonin levels will then accurately reflect the length of the dark period. Hence, the rhythm is created by a passive on/off response to the daily LD cycle and not by an endogenous clock (Max & Menaker, 1992; Randall et al., 1995; Begay et al., 1998; Masuda et al., 2003; Iigo et al., 2007; Migaud et al., 2007). The different mechanisms involved in the perception and transduction of the light-signal through the circadian axis in teleosts has probably changed under evolution as a reflection of the photic environment, in which they have evolved (Migaud et al., 2007).

Biorhythms in aquaculture

Available data indicate that fish reproductive physiology shows a close adaptation to the cyclical variations of photoperiod in the environment by synchronization of their spawning to the period of year when the most appropriate environmental conditions for their offspring are found (Taranger et al., 1998; Bromage et al., 2001; Falcon et al., 2010). Hence, reproduction in fish living at mid-to high-latitudes is naturally restricted to a short annual window. In these areas the reproduction takes place when day length are increasing in the spring (Bromage et al., 2001). This creates a problem for fish farmers that rely on all year supply to meet the increasing demand for fish. Photoperiodic light regimes to manipulate the circannual rhythm of spawning have been a great success in a number of temperate species such as Atlantic salmon (*Salmo salar*) (Thrush et al., 1994; Porter et al., 1999), Rainbow trout (*Oncorhynchus mykiss*) (Whitehead et al., 1978; Davies et al., 1999) Gilthead seabream (*Sparus aurata*) (Kissil et al., 2001), Atlantic cod (*Gadus morhua*) (Hansen et al., 2001) and European sea bass (*Disentrarchus labrax*) (Carrillo et al., 1989).

In contrast to the temperate species, tropical and sub-tropical fish does not experience significant annual changes in photoperiod in their natural habitat (Campos-Mendoza et al., 2004; Maitra & Chattoraj, 2007). Whether photoperiod, temperature or other environmental cues are used in the temporal organization of seasonal breeding remains to be documented. Until now, only a few studies have been carried out on a small number of species. Photoperiod and temperature (Davies & Hanyu, 1986; Davies et al., 1986b) are the most examined cues, but also the effect of lunar cycle (Saavedra & Pousao-Ferreira, 2006; Takemura et al., 2010) and seasonal rainfalls (Reardon & Chapman, 2008) have been investigated. At present, the exact neuroendocrine mechanism for reproductive control are not known, but evidence are suggesting that photoperiod, biological clocks and melatonin release are important in controlling the reproductive rhythms of these fish species as well (Davies et al., 1986a; Campos-Mendoza et al., 2004; Dey et al., 2004; Maitra et al., 2005; Maitra & Chattoraj, 2007).

Aims for this study

Establishment of aquaculture can be of great importance to improve the living conditions for poor people in the rural areas of Nepal. If that is the case, one have to make sure of a secure supply of fish fry both concerning number and seasonal availability. In order to develop out of season production of equatorial carp species, it is crucial to have knowledge of the reproductive cycle. The possible existence of a photoperiodic component that controls reproduction in exotic species must be tested. With such a system, photoperiodic manipulation of maturation can take place.

Based on the preceding introduction this master thesis has developed following scientific questions:

- Does the plasma melatonin levels in Common carp, kept in Nepal, mirror changes in environmental light/darkness?

- How does an extension of the natural dark period influence blood plasma melatonin levels in Common carp in Nepal?

- Can melatonin half-life (t ½) and cortisol impact on Nepalese Common carp be described?

Materials and methods

Study site

The experiments were carried out at NARCs research centre in Begnas, Pokhara Valley, Nepal (Figure 6) during November 2010. Begnas is a small town located about 15 km east from Pokhara in the Kaski district, Gandaki zone in the mid-hills of Nepal.

Figure 6: NARC Fisheries research centre, Begnas, Pokhara Valley.

Nepal Agricultural Research Council (NARC) was established in 1991 and is a semi-autonomous organization under the department of agriculture that conducts agricultural research on a wide spectre of areas like crops, livestock and animal health, fishery and aquaculture to uplift the economic level of the Nepalese people. NARC has fourteen agriculture research stations (NARC, 2007), of which the research centre in Begnas operates with research and development of fisheries and aquaculture technologies to improve production of native and exotic species.

Study animal

Cyprinids are the most cultured freshwater species in the world (71 %), with Asia and especially China as the leading producers (FAO, 2010). Common carp (*Cyprinus carpio*) is a well-known cyprinid in aquaculture of which roughly three million tonnes were produced in 2009 (FAO, 2011b). It was one of the first exotic carp species to be introduced in Nepal in the 1950s (Swar & Gurung, 1988; Penman et al., 2005). It is very suitable for aquaculture due to its high tolerance to a wide variety of conditions in temperature, oxygen, pH and salinity

(Shrestha, 1999; FAO, 2004). In addition it is omnivorous and can thereby utilize artificial feeds with low fishmeal content (Kestemont, 1995; FAO, 2004).

Common carp spawn only once a year in Nepal (Jhingran & Pullin, 1985; Rajbanshi, 1996). The research centre in Begnas keeps Common carp for cultivation and research. The fish used in this study was 7-8 months immature fingerlings held outdoor in an enclosure within a larger pond. Water was supplied from the nearby lake Begnas.

Experiment 1: Melatonin profile in Common carp under indoor simulated natural photoperiod

In this experiment the melatonin profile of Common carp under simulated natural photoperiod (LD-12:12, L=light; D=dark) were investigated. Hundred large sized fingerlings of Common carp were carried in a plastic bag from the outdoor pond to the indoor tanks at the research centre. Indoor, the fish were randomly distributed into seven circular holding tanks (400 L water) with twenty fish in tank 1-3 and ten fish in tank 4-7 (there were only seven tanks available for the experiment). Average length and weight of fish at the time of sampling was 18.5 ± 2.3 cm and 85.3± 34.4 g (n = 60), respectively. Average temperature in the tanks was 24.0 ± 0.6 °C, and average oxygen level was 6.59 ± 0.2 mg/L. Due to poor indoor light, a bulb (60W) was placed over each tank and manually turned on/off at sunrise/sunset in synchronization with the natural photoperiod as long as the experiment was conducted. The fish were acclimated to the tanks for 48 h with a light regime of LD-12:12, before blood sampling started. No food was provided during the experiments. Blood samples were collected from six fish at the following time points: 12:00, 16:00, 18:00, 20:00, 23:00, 02:00, 04:00, 06:00, 08:00 and 12:00.

Experiment 2: Melatonin profile in Common carp under indoor extended darkperiod

Melatonin levels were investigated in Common carp kept indoor and held under extended dark period (LD-6:18). A total of 130 large fingerlings from the pond outside were distributed into the seven tanks indoor. The fish were acclimated to the tanks with a light regime of LD-12:12 for 48 h before the experiment started. No food was provided during the experiment. Average length and weight for fish at the time of sampling was 15.6 ± 2.5 cm and 53.6 ± 30.4 g (n = 60), respectively. Average temperature in the tanks was 23.9 ± 0.9 °C and average oxygen level 6.50 ± 0.20 mg/L. At 15:00, the windows in the research hall were covered with black plastic to create an early scotophase. A light intensity of 0.2 lux was recorded over tank 1 (located in the middle of the room) under this artificial dark phase. The dark phase were completed at 09:00. Blood samples were collected from six fish at each of the following time points: 12:00, 14:00, 16:30, 18:00, 22:00, 02:00, 06:00, 08:00, 10:00 and 12:00.

Experiment 3: Melatonin half-life in Common carp

Calculation of plasma melatonin half-life in Common carp was based on blood sampling at short intervals after onset of light in the middle of a scotophase. Approximately 60 fish were left from experiment 2 and were used in experiment 3. All fish were placed in the same tank inside the research hall. Temperature in the tank was 24.1 °C and oxygen level 6.54 mg/L. For this experiment the simulated natural photoperiod was used with the fish experiencing dark from 18:00 until the light was turned on at 21:00 (three light bulbs over the tank). A total of 1135 lux was recorded by the edge of the tank, under the light bulb, 30 cm above the water surface. After a blow to the head blood samples were taken of as many fish as possible within 41 minutes (with no use of anaesthetics). Average length and weight of sampled fish were 16.0 ± 2.8 cm and 56.0 ± 32.5 g (n = 17), respectively. No food was provided during the experiment.

Blood sampling

Common carp was anesthetized with 50 mg/L MS-222 (Tricaine Methane Sulphonate, Farmaq Ltd, Fordingbridge, UK) and blood collected from caudal vessels by use of heparinised syringes (Heparin LEO 5000 IE/mL). Sampling in dark was executed under dim red light. The fish was killed by a blow to the head immediately after sampling. All fish were body measured (length, weight) and checked for gender and stage of maturity. The blood samples were kept under cover and in ice slush until centrifugation at 4500 rpm for 5 minutes (Heraeus Biofuge® pico, UK). The plasma were collected and kept frozen (at -20 ºC) until analysis. The freezers in Nepal vary in quality and electric rationing occurs daily. Extra ice was made and densely packed around the samples to prevent them the samples from thawing during storage.

Light measurements

Light intensity (lux) were measured (INS DX-200, Digital illumination Meter) outdoor to verify time of light changes (night, dusk, day, dawn) to be simulated during indoor experiment (Figure 7).

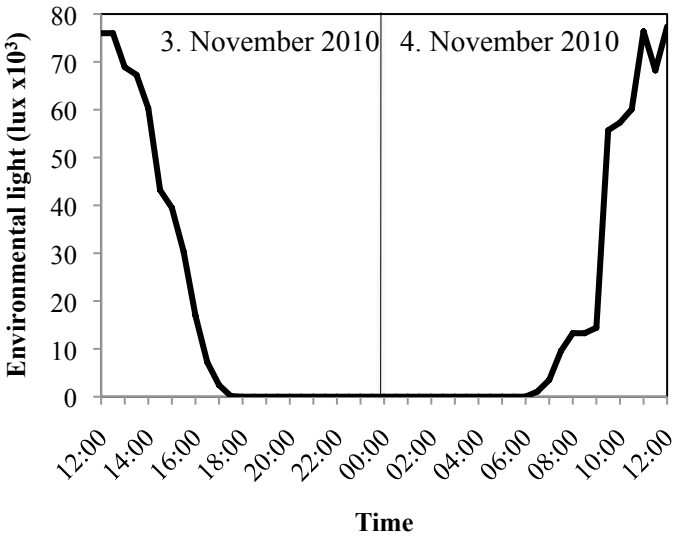

Figure 7: Outdoor light measurements at Begnas research centre, Pokhara, 2010.

Radioimmunoassay principle

The basic principle of radioimmunoassay (RIA) is a competition between a radioactive labelled ("hot") (Ag*) and non-radioactive ("cold") antigen (Ag) for a fixed number of specific antibody (Ab) binding sites:

$$Ag + Ag^* + Ab \Leftrightarrow AgAb + Ag^*Ab + Ag + Ag^*$$

The ratio between bound and free labelled antigen will then reflect the amount of unlabeled antigen in the sample. The amount of Ag is quantified by comparing with a curve simultaneously established from known standards (Figure 8) (Berson & Yalow, 2006).

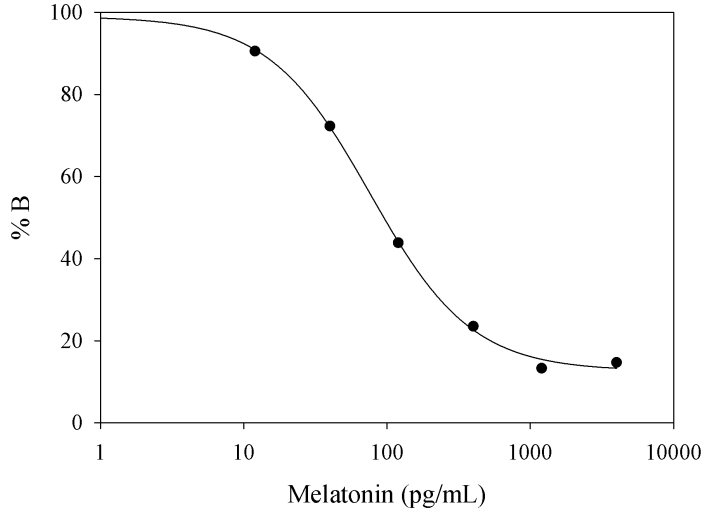

Figure 8: Standard curve from melatonin RIA.

Melatonin RIA

Plasma melatonin concentrations were determined by use of a commercial radioimmunoassay kit (Melatonin Research RIA, Labor Diagnostic Nord GmbH & Co. KG, Nordhorn, Germany). Melatonin in plasma competed with ^{125}I labelled melatonin for the limited number of binding sites on melatonin antiserum. When the system was in equilibrium, the antibody bound radioactivity was precipitated with a second antibody in the presence of polyethylene glycol. The precipitate was counted for one minute in a gammacounter (COBRA II, Packard, USA) and the unknown melatonin levels were calculated by the software included in the gammacounter by comparing their activity against a response curve of known standards (0, 12, 40, 120, 400, 1200 and 4000 pg/mL). The kit was conducted with use of 50 µl plasma and samples were run as singles.

Validation of melatonin assay

The general challenge of immunoassays used for endocrine measurements in biological fluids is the possible influence from other proteins and fats on the assay performance. To minimize such influence, a matrix-specific calibrator ("Equalizing Reagent") was prepared by removal of endogenous melatonin from species specific blood plasma by adsorption to activated charcoal (Welp et al., 2010). The Equalizing Reagent was used to equalize the assay matrix of standards, controls and untreated samples.

The sensitivity (detection limit) for the melatonin RIA kit was determined by solving for the bound complex mean, minus 2 standard deviations (SD) of zero standard replicates (containing no melatonin). All plasma samples measured with melatonin concentration under this limit were set to detection limit: 21.3 pg/mL.

The precision of the kit was tested with use of controls included in the kit with low (L) and medium (M) concentration. Average intra-assay coefficients of variation (CV) were 10.5 % (L) and 9.7 % (M). Inter-assay CV % was 41.6 % (L) and 45.7 % (M). According to Chard (1990) intra- and inter-assay variation should be below 10 % and 20 % respectively.

Conversion factor for melatonin

Melatonin concentrations given in pg/mL can be converted to pmol/L by:

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

Cortisol RIA

Plasma cortisol concentration was quantified by use of a commercial RIA kit (Coat-A-Count Cortisol, Siemens). The kit use the basic principle of RIA by use of ^{125}I labeled cortisol competing with free plasma cortisol for limited binding sites on cortisol antiserum in the pre-coated tubes provided with the kit. Bound fraction was counted for one minute in a gamma counter. All samples were run as singles.

A test run of the assay was conducted with a few randomly chosen plasma samples from experiment 2, and revealed cortisol concentrations in upper part of the standard curve (Figure 9). Consequently, all samples were diluted 1:2 with Standard 0 (containing no cortisol).

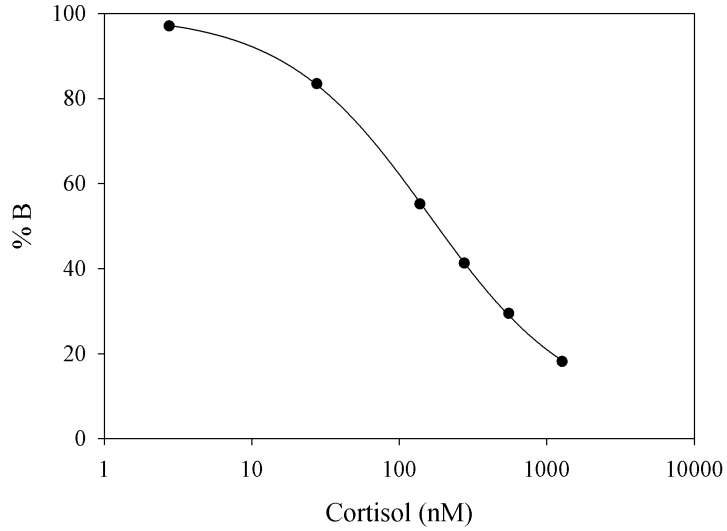

Figure 9: Standardcurve from Cortisol RIA.

Precision of the kit was tested with use of control samples (Lyphochek® Immunoassay Plus Control, BioRad, France) with low (L), medium (M) and high (H) levels. Average intra-assay CV % was 7.8 % (L), 4.5 % (M) and 3.35 (%). Inter-assay CV % was 12.5 % (L), 4.2 % (M) and 3.1 % (H). The detection limit was determined to 2.8 nM.

Conversion factor for cortisol

Cortisol concentrations given in µg/dL can be converted to nmol/L (nM) by:

cortisol (µg/dL) x 27.59 = cortisol (nM)

Statistics and graphics

Google SketchUp 8 for Mac was used for the graphic illustration of a photoreceptor cell and biosynthesis of melatonin. SigmaPlot for Windows Version 11.0 was used for graphical presentation of the data. For statistical analysis, SPSS Statistics Version 19 was used.

One-way ANOVA was used to test the interaction between plasma melatonin level and time of day. ANOVA was followed up by least-significant difference (LSD) pairwise comparison *post hoc* test to analyze differences among groups in each light regime.

For cortisol, one-way ANOVA followed by Bonferroni's *post hoc* test was used to compare plasma cortisol levels and time in the experiment.

Comparing two groups were done using Student's t test. A significance level of $p < 0.05$ was chosen for all tests.

Results

Experiment 1: Melatonin profile in Common carp under indoor simulated natural photoperiod

Plasma melatonin levels in large sized fingerlings of Common carp (*Cyprinus carpio*) held under simulated natural photoperiod (LD-12:12) (Figure 10) have a daytime level below the detection limit of the RIA assay (21.3 pg/mL). Average plasma melatonin right after sunset (18:00) is (77 ± 73 pg/mL), then somewhat plateauing until a maximum of 236 ± 153 pg/mL is reached at 02:00. This peak level is significantly higher than all other levels. Thereafter, average melatonin decreases during the last part of the dark period and is back to 24 ± 7 pg/mL at time of daylight on. All melatonin levels under total darkness (from 20:00 to 04:00) are significantly different from daytime levels (12:00, 16:00, 08:00 and 12:00). Average length and weight of all fish sampled are shown in Table 1.

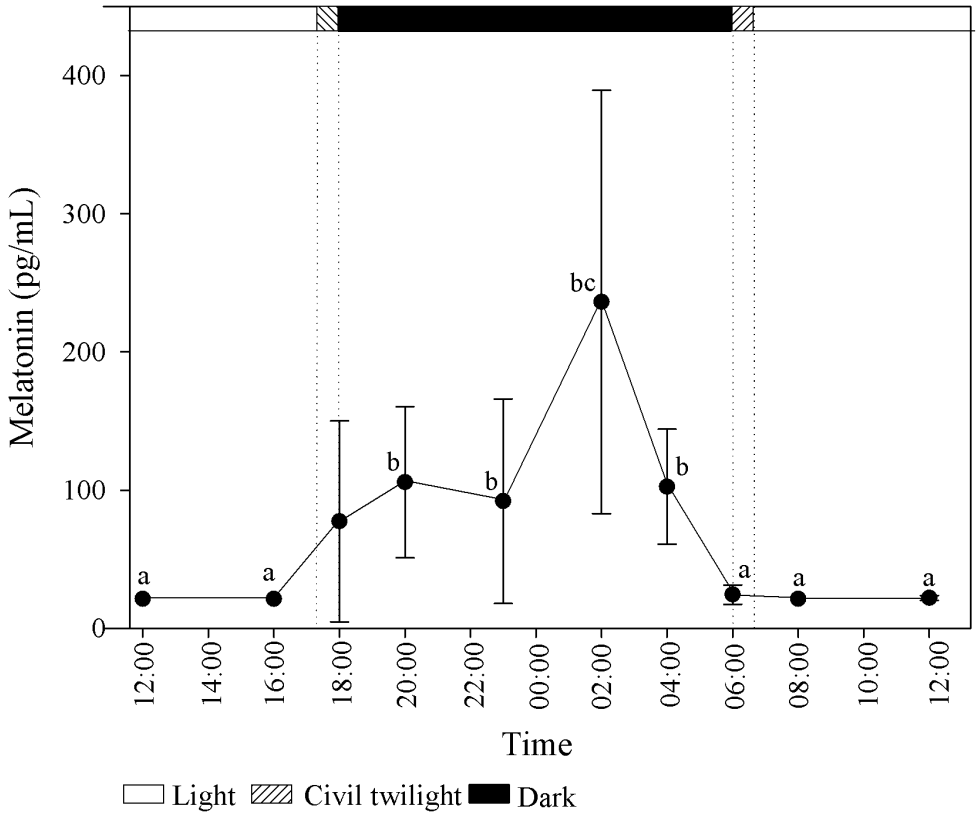

Figure 10: Plasma melatonin profile in Common carp (*Cyprinus Carpio*) maintained indoor (November 2010) under simulated natural photoperiod (LD-12:12) at NARC fisheries research centre, Begnas, Nepal. Values are mean ± SD (n = 6). Different letters denote significant differences.

Table 1. Average length and weight of fish at time of sampling in experiment 1: simulated natural photoperiod. Values are mean ± SD (n = 6).

Time of sampling	Length (cm)	Weight (g)
12.00	17.8 ± 1.1	67.3 ± 10.8
16.00	17.8 ± 2.5	70.3 ± 39.0
18.00	18.5 ± 2.5	88.0 ± 32.5
20.00	17.0 ± 1.4	66.8 ± 20.7
23.00	19.4 ± 3.1	102.3 ± 40.0
02.00	20.1 ± 2.1	108.3 ± 39.6
04.00	20.4 ±1.4	115.5 ± 33.5
06.00	19.1 ± 2.0	97.5 ± 34.7
08.00	16.2 ± 1.8	51.4 ± 14.9
12.00	18.6 ± 1.5	85.0 ± 18.5

Experiment 2: Melatonin profile in Common carp under indoor extended darkperiod

The plasma melatonin profile of large sized fingerlings of Common carp held under extended photoperiod (LD-6:18) (Figure 11) are similar to the profile from natural photoperiod, with levels in light under the detection limit of the RIA assay (21.3 pg/mL). However, no significant differences in melatonin levels are seen until the maximum level of 98 ± 106 pg/mL is reached at 02:00. After this peak, the level decreases and is not significantly different for the rest of the experimental period.

Average length and weight of fish sampled are shown in Table 2. Comparison between experiment 1 and 2 concerning fish size revealed significant lower length and weight of fish sampled in experiment 2.

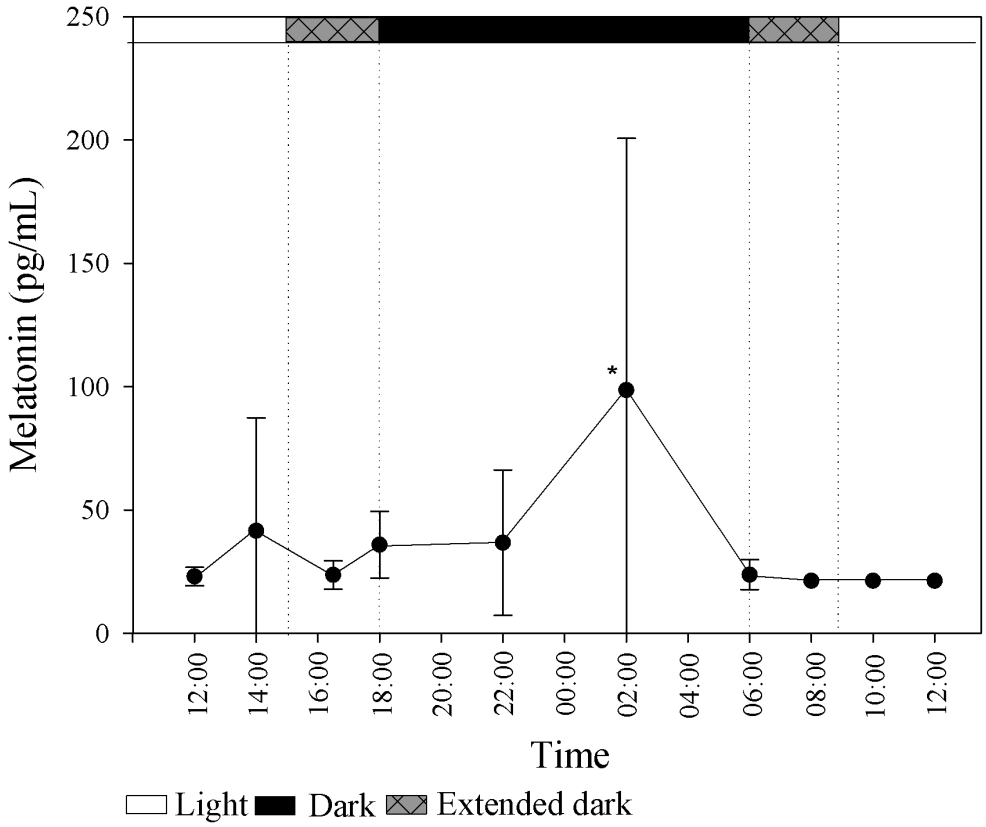

Figure 11: Plasma melatonin profile in Common Carp (*Cyprinus Carpio*) maintained indoor under extended dark period (LD-6:18) at NARC Fisheries Research Centre, Begnas, Nepal. Values are mean ± SD (n = 6). Asterisk denotes significant difference.

Table 2. Length and weight of fish at time of sampling in experiment 2: extended darkperiod. Values are mean ± SD (n = 6).

Time of sampling	Length (cm)	Weight (g)
12.00	18.1 ± 3.2	86.7 ± 39.9
14.00	15.3 ± 1.3	47.2 ± 10.6
16.00	15.3 ± 0.9	50.2 ± 15.0
18.00	14.8 ± 1.6	43.3 ± 13.2
22.00	14.4 ± 0.7	38.4 ± 6.8
02.00	16.5 ± 3.8	68.3 ± 47.6
06.00	14.3 ± 0.8	37.8 ± 4.8
08.00	18.2 ± 3.4	85.0 ± 43.5
10.00	15.2 ± 1.8	46.5 ± 20.9
12.00	13.4 ± 0.8	31.1 ± 6.0

Experiment 3: Melatonin half-life in Common carp

All melatonin levels from the experiment were under the detection limit of the radioimmunoassay (Figure 12). The first sample was taken 2.54 minutes after onset of light.

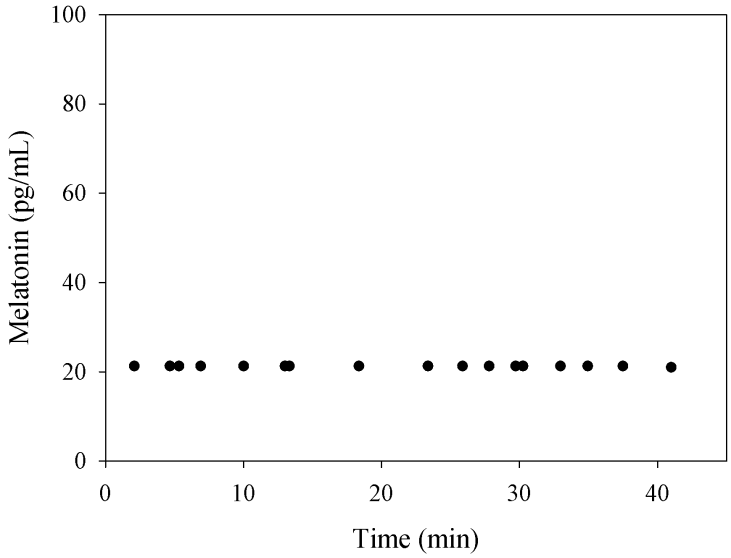

Figure 12: Plasma melatonin concentration in Common carp (*Cyprinus carpio*) after onset of light three hours into natural scotophase Blood was sampled at short intervals after abruption of scotophase (n =17).

Variation in plasma cortisol levels under photoperiod experiments

Plasma cortisol levels under experiment 1

The highest mean level of cortisol from experiment 1, under natural photoperiod, was found at the beginning of the experiment at 12:00 (856 ± 705 nM) (Figure 13). No significant changes in plasma cortisol were found throughout the experiment.

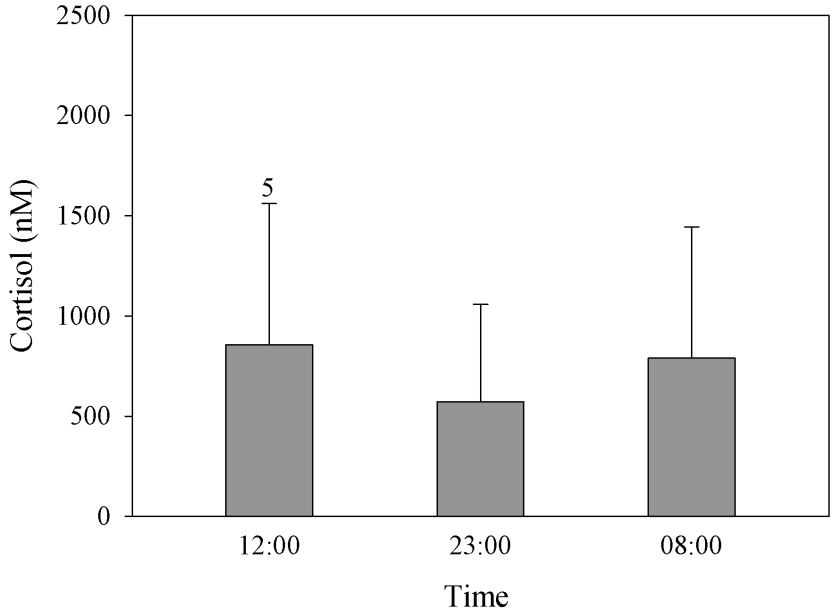

Figure 13: Plasma cortisol levels in Common carp (*Cyprinus Carpio*) under experiment 1, maintained under simulated natural photoperiod (LD-12:12). Values are mean ± SD (n = 6, otherwise denoted).

The individual cortisol levels in fish were plotted for each of the three sampling points tested (Figure 14). The trendline show only weak increase ($r^2 = 0.23$) from fish 1 to 6 sampled.

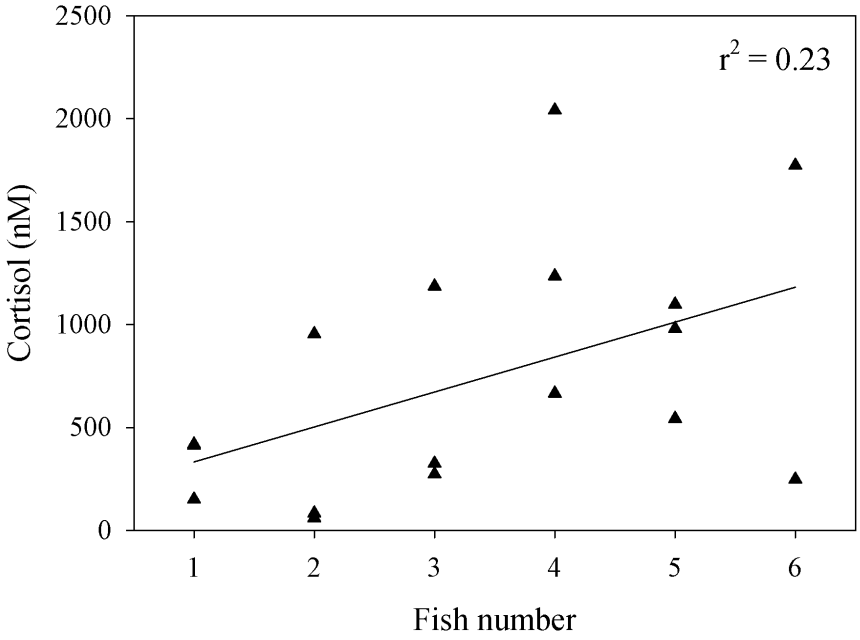

Figure 14. Cortisol levels in individual fish of Common carp (*Cyprinus carpio*) within three blood sampling time points (12:00; 23:00; 08:00) in experiment 1.

Plasma cortisol levels under experiment 2

Under experiment 2, extended dark period, the highest mean cortisol level (Figure 15) was found at the beginning of the experiment, at 12:00 (887 ± 421 nmol/L). Cortisol levels did not differ significantly throughout the experiment.

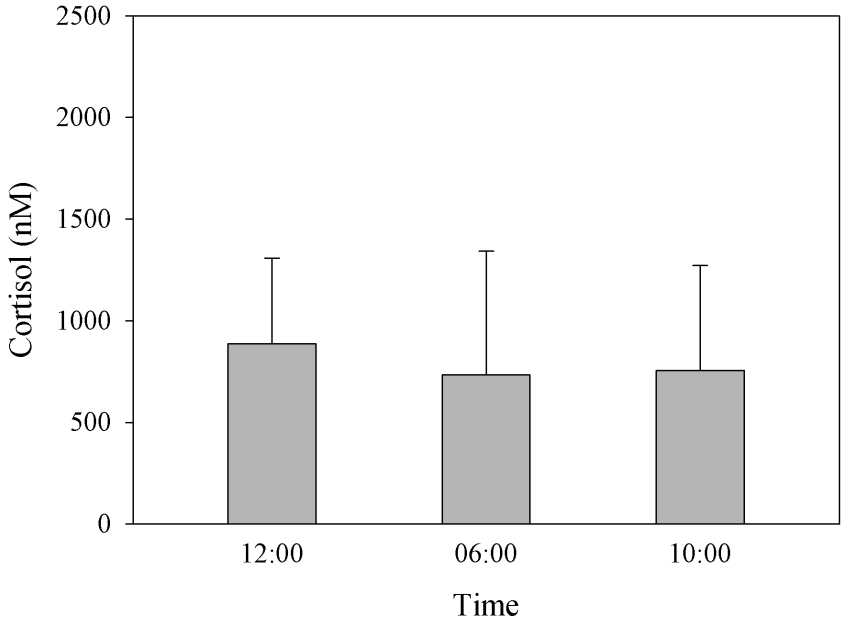

Figure 15: Plasma cortisol levels in Common carp (*Cyprinus carpio*) under experiment 2, kept under extended dark period (LD-6:18). Values are mean ± SD (n = 6).

The individual cortisol levels in fish were plotted for each of the three sampling points tested (Figure 16). The trendline show an increase ($r^2 = 0.56$) from fish 1 to 6 sampled.

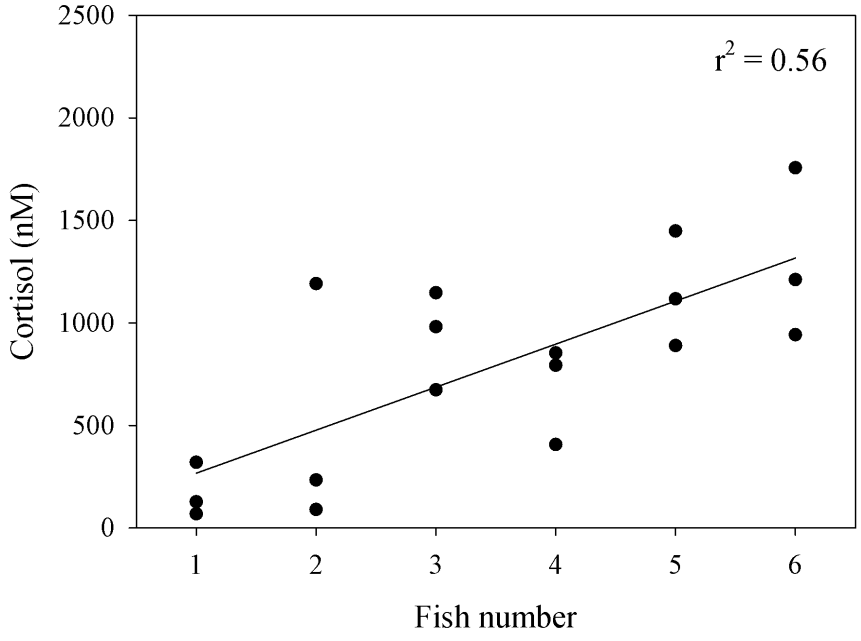

Figure 16: Cortisol levels in individual fish of Common carp (*Cyprinus carpio*), within three blood sampling time points (12:00; 06:00; 10:00) in experiment 2.

Discussion

The aquaculture industry in temperate areas has greatly improved the production efficiency by controlling biorhythms and reproductive physiology of fish through controlled photoperiodic exposure. Light regimes are used to suppress the rhythmic melatonin signal or change the season with intention to manipulate the timing of broodstock spawning, smoltification, and early maturation in a number of commercially important species (salmon, trout, sea bass, cod, etc) (Taranger et al., 1998; Bromage et al., 2001; Hansen et al., 2001). While these techniques are mostly based on knowledge from studies of temperate species, little is known about the rhythmic control systems in lower latitude species. If Nepalese aquaculture industry could manage to develop the same knowledge and facilities, a sustainable year-round fish supply could dramatically improve the livelihood of Nepals large poor people population.

The production and release of melatonin from the pineal gland are found in all vertebrates, including fish (Ekström & Meissl, 1997). Since secretory pattern of melatonin reflects changes in LD cycles, plasma melatonin levels may provide the animal with information about the time of day and anticipate upcoming season (Reiter, 1993). Salmonids are found to have a passive response to the environmental LD signal where the elevated melatonin levels accurately reflect the length of the darkphase (Randall et al., 1995). As a consequence, continuous light (LL) suppresses pineal melatonin production, whereas constant darkness (DD) results in high levels of melatonin secretion. In the majority of teleost species, however, the response to LD is not passive. In these species the control of melatonin secretion involves a circadian clock system located within the photoreceptor part of the pineal cells (Bolliet et al., 1996a; Bolliet et al., 1996b). Existing knowledge on melatonin patterns in fish is primarily based on studies on species from the temperate zones, were the environmental LD cycle vary significant between seasons. In tropical and sub-tropical areas, on the other hand, the daily light/dark cycles show only small variations throughout a year, so substantial circannual variations in the melatonin profile is not expected in fish from these areas. The current study was executed to describe the natural pineal produced plasma melatonin profile in Common carp and also to investigate how an extended dark period would influence this profile. This is a part of the SPRN programme goal of manipulating the reproductive timing in Asian carps.

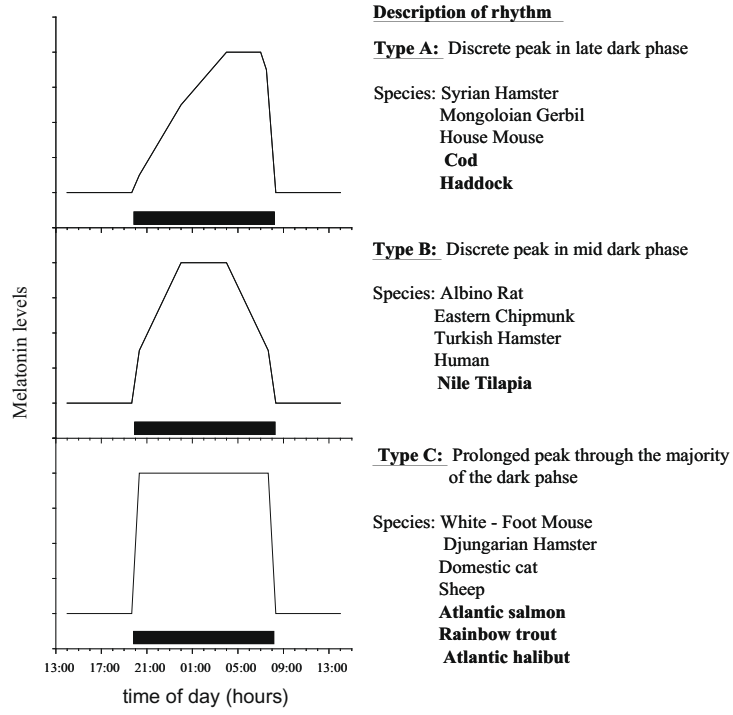

Figure 17: Diagrammatic representation of the different melatonin profiles recorded in vertebrates. Examples of species which express such patterns of plasma melatonin for each profile is listed. Horizontal black bar denotes subjective dark period. Reproduced from Falcon et al. (2010)

Although the melatonin secretion during dark phase is believed to be species specific, attempts to find similarities between the different rhythms have occurred. Reiter and co-workers (1987; 1991) have proposed that animals belong to one of three different melatonin secretory patterns (Figure 17). Animals with the type A profile does not show an immediate increase in melatonin after light offset, and have a short duration peak in late scotophase before it decreases in synchrony with onset of light. In type B animals the pineal melatonin production increase from onset of darkness and peaks near mid-dark, after which the levels begins to drop and reach low daytime levels at about the onset of daylight. In animals with the type C profile the pineal melatonin level increases soon after lights off to reach a plateau, which is maintained for the duration of the scotophase until the time of lights on. As emphasized earlier the salmonids fall into the category C that expresses a nocturnal increase in circulating melatonin levels that accurately reflect the duration of the dark phase.

Simulated natural photoperiod

Common carp kept under simulated natural photoperiod in the present study showed an evident rise in plasma melatonin during the darkphase. This is in accordance with findings in other vertebrates, where low levels during photophase and high levels during scotophase are found (Reiter, 1993; Ekström & Meissl, 1997). Right after offset of light the melatonin level increases to a moderate plateau until it peaks in the second half of the darkphase and decreases to low daytime levels before the onset of light. These findings suggest the melatonin profile of Common carp to be of type B.

An *in vitro* study of the melatonin release from the pineal gland of Common carp originating from France, found a similar profile to the current study. The melatonin level showed a clear similarity to the type B, with melatonin release from the pineal that increased gradually until a

peak around mid-dark phase (Bolliet et al., 1996a). The profile observed in these studies are in contrast to other findings in Common carp. Kezuka and coworkers (1988) found a profile more similar to type C, with a rapid increase in plasma melatonin after lights off, and the high levels were maintained during the darkphase. After the lights on the level returned to daytime levels. Kezuka and coworkers (1988) performed the experiments in Tokyo, Japan, where the alterations in length of daylight are more noticeably between seasons than in Nepal. The apparently more passive rhythm found in the Japanese carps might be a result from adaptation to an area with more distinct differences in circannual LD cycles. On the contrary, Bolliet (1996a) performed the experiment with carps from France, which lies at an even higher latitude than Japan, and here the plasma melatonin profile was more similar to the results from this study.

To explain these contrary results one can look closer to the fact that Common carp is one of the most cultured species in the world. Domestication has occurred over centuries and over 30 strains are found in Europe (FAO, 2011b). Development of different strains all over the world may have led to adaptation to the environment the fish have been cultured in. Further studies in Common carp from different strains and from different latitudes should be conducted to find out if the environment have led to differentiation of the perception and exploitation of the photoperiodic signal within the same species.

Experiments in Copper mahseer (*Neolossocheilus hexagonoleipis*), a species native to Nepal, found a profile with characteristics close to both type B and C. The level increases rapidly after onset of dark and remain at a plateau, but decreases to low daytime levels right before onset of light (Guttu, pers. com.). Interestingly, the same decrease in plasma melatonin levels during late darkphase was found in this study, and the levels reached daytime levels before the start of a new photophase. Resembling results with decreasing melatonin concentrations prior to the photophase has been found in several tropical and sub-tropical species, e.g. the Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*) (Martinez-Chavez et al., 2008) and Goldfish (*Carassius auratus*) (Iigo et al., 1994). The rhythm of melatonin secretion from the pineal gland may partly be a product of the environmental light-dark cycle, were light will suppress the expression and activity of the rate-limiting enzyme AANAT and as a consequence, the melatonin production is inhibited (Falcon et al., 2001). However, if the pineal gland contain intra-pineal oscillators, it can be capable of self-sustaining the melatonin rhythm in absence of a photoperiodic cycle. The decrease in plasma melatonin before onset of lights in this study may suggest the involvement of a clock-controlled system of melatonin secretion that is capable of anticipating the next photoperiod.

Extended darkperiod

To establish if the brain of Common carp contains a functional pineal that has the ability to mirror the environmental LD information, an experiment with extended dark period was conducted. The plasma melatonin profile under extended dark showed some surprisingly

results. Daytime scotophase levels did not differ from levels in light. This cannot be due to photoinhibition, as indoor environmental light was measured to less than 0.2 lux. The only increase in plasma melatonin level was found at 02:00, although slightly higher levels are observed between 18:00 and 06:00 (corresponding with the hours of darkness outdoor). The peak at 02:00 is in correspondence with the previous findings in natural photoperiod. Under extended dark, the profile appear to be of type B, with a the latency before an increase can be seen, and a single peak in late dark phase. The small increase found between 18:00 and 06:00 may indicate that the pineal have not been influenced by the new light regime and, hence, might be an indication of a clock system in this species. The clock may have anticipated the rhythm by estimations from the previous circadian rhythm. These results correlates with findings from a study with similar methodology as current, were an increase in plasma melatonin was detected three hours after onset of darkness, in Copper mahseer (Guttu, pers. com). Guttu also found a drop in plasma melatonin levels at the time of natural sunrise (but the fish still experienced dark for four hours before lights on). Similar results was found in the pike (*Exos lucius*), where the superfused pineal glands subjected to advanced dark period did not show an increase in melatonin secretion until six hours after onset of dark (Falcon et al., 1989). On the other side, in the preparations for this study, no acclimation was made to the extended dark period. Further studies should therefore verify if acclimation time could affect the ability to adapt to a new photoperiodic regime. Average length and weight of fish was found to be significant higher in the experiment with simulated natural photoperiod than extended darkperiod. However, all fish was found to be immature and would therefore not express elevated sex hormones that possibly could influence melatonin levels.

If Common carp contain a intra-pineal clock, the pineal gland will continue to produce melatonin in a rhythmic manner under constant darkness, with a circadian periodicity as found in most other fish species investigated (Falcon et al., 1989; Zachmann et al., 1992; Bolliet et al., 1996a; Cahill, 1996; Okimoto & Stetson, 1999). Bolliet and coworkers (1996a) carried out an *in vitro* study on superfused pineal glands from Common carp. The rhythm sustained under DD, but showed a marked drop in peaks and tended to dampen out after a few cycles. Application of a 24 h LD cycle at the end of the DD period re-established rhythmicity, but the final cycle did not restore nocturnal levels of melatonin secretion as high as before continuous darkness. The experiment conditions under the *in vitro* studies may have affected the organ (via removal from the skullcap and under renewing of the culture medium). Hence, further studies to confirm if an intra-pineal clock controls the melatonin rhythm in Common carp should be conducted with plasma samples *in vivo*.

Melatonin half-life

Blood plasma melatonin levels are the result of two actions: the release of melatonin into plasma, and removal of melatonin from the plasma. Change in melatonin levels at night are more likely to be a result of increased release of melatonin from the pineal gland and not by a decrease in rate of degradation in the liver. This t ½ experiment was designed to utilize

melatonin release under natural photoperiod three hours after onset of dark (at 21:00). The results from the experiment to find half-life of endogenous produced melatonin in Common carp showed no plasma melatonin at all from the first to the last sample after abruption of darkphase. Fish from the experiment with extended darkperiod was used, however, as results show, at 21:00 in the profile under extended dark period, almost no plasma melatonin was found (Figure 11). It was therefore no melatonin to find under t ½ experiment. New experiments would have to take a new group of fish from the outdoor pond and acclimate them to the indoor tanks as done in the earlier studies, with no change in light regime.

Few studies have been done to determine melatonin half-life in teleosts, but in Rainbow trout (*Oncorhynchus mykiss*) a half-life of 20 minutes was found (Max & Menaker, 1992). Skjulsvik (2005) found a half-life of melatonin in Atlantic salmon (*Salmo salar*) of about 10 minutes, both in darkness and in light. However, these studies are conducted on salmonid fish, which is known to not contain a circadian clock system. Guttu (pers. com.) found a half-life of 10-12 minutes in Copper mahseer. Based on the preceding findings in teleost species one could speculate that t ½ of plasma melatonin in Common carp would be 10-20 minutes.

Possible influence on plasma melatonin

Several studies have shown that the plasma melatonin profile expressed in the specific species may relate to a number of factors such as the genetic make-up of the animal, the endocrine state of the animal and the previous photoperiodic history. The environmental light may vary in terms of quantity (intensity), quality (spectral content) and duration (photoperiod) (Reiter, 1987; Sumpter, 1992; Bayarri et al., 2002; Migaud et al., 2006). All these factors highlight the difficulties generally encountered when attempting to compare with existing literature for fish.

Temperature

Fish are ectotherms and their metabolic activity is directly influenced by the external water temperature. Common carp used in present study were kept at 24 °C, a temperature found suitable for cultivation of freshwater teleosts in sub-tropical areas and corresponds to temperature in the ponds at the NARC research centre during November. Studies in different teleosts suggest that temperature has an effect on melatonin production, and the effect is proposed to be mediated through modifications in AANAT kinetic (Falcon et al., 1996; Falcon, 1999; Seth & Maitra, 2010b). However, contradictory results are found between species. In pike, increasing temperature led to increased melatonin production (Falcon et al., 1996). In contrast, the data reported in the pineal of *Catla catla* exhibited diurnal expression of AANAT, which closely matched the reported diurnal profile of circulating melatonin. Under spawning, the melatonin amplitude was lower, while temperature was at its highest (Maitra et al., 2005; Chattoraj et al., 2009; Seth & Maitra, 2010b). In addition, the rhythmic release of melatonin synchronized with a temperature cycle under constant darkness was no longer observed after removal of the external photoperiod (Falcon et al., 1994). Thus, the

environmental info from photoperiod determines the duration of the pineal melatonin signal, while temperature appear to determine its amplitude. Together, the photo-thermal conditions may provide the animal with accurate information of both the daily and annual cycles. Change in temperature related to husbandry, may thus have consequences for the time-keeping system in fish and must therefore be documented in the animal at its location. These environmental changes may, however, be transformed into melatonin signals depending on the latitude location of the animal.

Reproduction

One of the major functions assigned to melatonin in fish is the regulation of reproduction, and this topic has been investigated using photoperiod manipulations and/or melatonin administrations (Ekström & Meissl, 1997). The majority of teleosts show peak reproductive activity or spawning for a short seasonal period, which is preceded by a long and complicated process of preparation resulting from an interplay between several hormones of the hypothalamo-pituitary-gonadal (HPG) axis (Bromage et al., 2001; Maitra et al., 2005). Thus, an examination of this complexity is demonstrated within a series of studies in one of the Indian major carps, *Catla catla.* The results revealed a pineal melatonin profile with a diurnal peak that varied from mid-scotophase (during the pre-spawning, spawning and post-spawning phases) to late-scotophase (in preparatory phase) during different parts of the annual cycle. Since the natural daylength during the preparatory phase and post-spawning phase does not exhibit remarkable variations (about LD-12:12), the study propose a possible relationship between functions of the pineal organ and reproduction, rather than a function of photoperiod (Bhattacharya et al., 2007). Seth and Maitra (2010b) recently found evidence for a temporal organization and correlation between photoreceptor proteins, AANAT and the environmental light cycle. Expression of AANAT had a maximum during post-spawning phase, when the nocturnal peak in melatonin plasma concentration was at its highest (Maitra et al., 2005; Seth & Maitra, 2010b). Other studies in *Catla catla* indicate an inverse relationship with gonadal hormones and melatonin. Estradiol 17-ß was found to increase gradually from preparatory phase to reach a seasonal peak in the spawning phase. Exogenous administration of melatonin had a stimulatory effect on the testis during the preparatory phase, inhibitory effect during the pre-spawning and spawning phases and no effect at all during the post-spawning phase (Bhattacharya et al., 2003; Maitra et al., 2005; Chattoraj et al., 2009). Incubation of isolated oocytes with melatonin and maturation inducing hormone (MIH) resulted in acceleration of the oocyte maturation in Common carp (Chattoraj et al., 2005).

Extra-pineal melatonin production

Since the discovery of melatonin in the bovine pineal gland (Lerner et al., 1958), and in most other animals studied, other organ sites of melatonin production have been revealed (Chowdhury et al., 2008; Falcon et al., 2010). Melatonin is a highly lipophilic molecule and can easily penetrate any membrane (Pardridge & Mietus, 1980) and thereby offer an additional receptor-independent non-hormonal role (Chowdhury et al., 2008). By this mean,

other production sites could contribute to the rhythmic plasma melatonin concentration. For interpretation of measured plasma melatonin changes it is essential to understand if the melatonin from these production sites interferes with the pineal signal.

Retina

The retina is one of the best-known extra-pineal melatonin production sites. Daily oscillations in melatonin levels have been reported for retinas in fish, amphibians, birds, and mammals such as rat, guinea pig and rabbit (Yu et al., 1981; Cahill & Besharse, 1995; Cahill, 1996). The production of retinal melatonin is regulated by the environmental LD cycle in the same way as pineal melatonin, and the presence of a circadian oscillator is diverse among fish species. In zebra fish (*Danio rerio*) the retina appeared to contain a circadian oscillator, but the rhythm sustained only for one full cycle and then damped out (Cahill, 1996). *Zacco platypus* was found not to contain a retinal oscillator rhythm, and the retinal melatonin was regulated by the LD cycle (Iigo et al., 1997b). These results are in contrast to findings in sea bass, where high ocular levels were found in light and low levels in dark during the LD cycle (Iigo et al., 1997a). Rhythm is found to persist following pinealectomy and is hence not regulated by the pineal gland (Cahill, 1996). On the other hand, retinal melatonin acts primarily within the eye where it is thought to have an autocrine function on rhythmic processes such as retinomotor movements, dopamine synthesis, release and metabolism and phagocytosis (Zawilska & Nowak, 1992; Cahill & Besharse, 1995; Zawilska et al., 2003). Studies performed on cultured eye cups of lower vertebrates indicate that retina, unlike the pineal gland, has the capacity for rapid metabolism of melatonin (Cahill & Besharse, 1989). These results indicate that retinal melatonin is degraded within the retina and does not contribute to the plasma melatonin levels.

Gastrointestinal tract

Another interesting discovery among the extra-pineal melatonin production sites are the gastrointestinal tract (GIT). Melatonin has been detected in the mucus membrane of the GIT in all vertebrate classes (Huether, 1993; Bubenik & Pang, 1997) and the concentration surpass by far the levels of pineal gland (x400) and blood (x10-100) (Huether et al., 1992; Huether, 1993). Unlike the photoperiodically regulated production of melatonin in the pineal, the release of gastrointestinal melatonin seems to be related to the periodicity of food intake. Food intake and, paradoxically, also food deprivation resulted in an increase of tissue and plasma concentrations of melatonin (Huether et al., 1992; Bubenik, 2002). Tryptophan administration was found to increase the circulating level in melatonin in chicks and rat, and the same results were obtained after pinealectomy, indicating that GIT itself is the major source of circulating melatonin after tryptophan intake (Huether et al., 1992). Melatonin has been found to relax smooth muscle cells in the GIT (Bubenik, 2008). It is also a remarkably good antioxidant, in fact, melatonin is a scavenger more effective than vitamin E (Pieri et al., 1994). By relieving the gut from serotonin-induced contractions, more time to utilize nutrients and accomplish reabsorption of food are obtained. However, if melatonin in the large amounts reported to be produced in the gut are let into the circulation, it could destroy the time-

keeping system provided by the pineal gland. Melatonin from the gut is forwarded to the liver via the portal vein, but only tiny amounts are found to survive the first liver passage, after which is excreted through the bile and back to the gut (Bubenik et al., 2000; Bubenik, 2008). In this way, the possible counteracting effect between melatonin from pineal and GIT is avoided. In Common carp, melatonin was detected in the stomach, proximal gut and distal gut (Bubenik & Pang, 1997), however, since no food was provided during the current experiment and very low daytime levels of plasma melatonin were detected in the study, it most likely indicates that GIT melatonin did not contribute to blood plasma levels.

Melatonin and stress influence

In teleosts, cortisol is the main glucocorticoid released during stress and plasma cortisol concentrations can be used as indicator of hypothalamic-pituitary-interrenal (HPI) axis activation. Cortisol is synthesized in the interrenal tissue of fish and activation of the stress response is considered to be of positive character when the duration of the exposure to stress is only briefly. However, daily exposure to stress can lead to lower immune capacity and increase disease susceptibility (Saeij et al., 2003). If the stress response sustain over time due to prolonged or repeated exposure, the costs associated with the response may outweigh its benefits (Barton & Iwama, 1991; Korte et al., 2005). By this means, it is important to monitor the stress level in the animal used in research and find out whether a deviation in cortisol levels can have an impact on the experimental results.

Mean plasma cortisol levels in this study (850-890 nM) correlates with results found in a previous study on Common carp, where resting plasma levels was 1242 nM (Svobodova et al., 1999). On the contrary, other studies on the same species have found unstressed Common carp to have a plasma cortisol level of 7.2 nM and fish exposed to stress by confinement had a maximum plasma cortisol level of 389 nM one hour after the stressor (Pottinger, 2010). Ruane and co-workers (2001) reported basal cortisol levels in Common carp fingerlings maintained at 25 ºC to be 82.77 nM. Under stress exposure, the level raised to between 662-883 nM. For Mosambique tilapia (*Oreochromis mossambicus*) basal levels have been found to range between 27-70 nM (Foo & Lam, 1993; Vijayan et al., 1997). Similar resting levels are found in the Nile tilapia (30-80 nM) (Barcellos et al., 1999). Bunes (2010) measured cortisol in Rohu (Labeo rohita) fingerlings maintained at the same place as this study and found similar levels with a resting level ranging between 376-1094 nM. Another similar study on Copper mahseer showed concentrations that ranged from 600-1400 nM (Guttu, pers. com.). These similar findings may be a result of the methodology used in the experiments of these Nepalese species, with short acclimation time of only 48 h. In addition, the carp species used are progeny from captured wild fish in Nepal and possible have naturally higher cortisol levels than found in other studies with domesticated species.

Plasma cortisol concentrations found in this study appear to deviate from findings in other experiments with Common carp. However, resting levels of cortisol is difficult to determine,

due to the consequence of transport and acclimation to a new environment, and blood sampling. The highest levels were found at the first time point in each experiment, when procedures lasted up to 25 minutes for sampling of six fish. Sampling itself may therefore have contributed to the increase in cortisol. When testing, however, only low correlation was found between cortisol concentration and the fish number sampled.

Water temperature also presents a potential source of variation in plasma cortisol levels. Arends et al. (1998) found elevated plasma cortisol levels in Common carp acclimated to 22 ºC, compared to acclimated to 15 ºC. In the current study, water temperatures were approximately constant at 24 ºC and could therefore be a possible source for the constant high cortisol levels found.

Daily and seasonal variations in plasma cortisol have been reported in Brown trout (*Salmo trutta*) (Pickering & Pottinger, 1983) and a potential effect of stress on pineal melatonin secretion has been proposed (Nikaido et al., 2010). In vivo experiments on chicks revealed that prolonged treatment with glucocorticoid dexamethasone reduced the amplitude of the circadian melatonin rhythm in the pineal gland (Zawilska & Sadowska, 2002). Receptors for glucocorticoids have been found in the teleost pineal and treatment with cortisol was found to inhibit the activity of AANAT (Benyassi et al., 2001; Yanthan & Gupta, 2007). A direct effect of stress on melatonin production has been suggested via *in vitro* studies in the Mozambique tilapia (Nikaido et al., 2010), where melatonin levels in dark decreased significantly with increased cortisol treatment. As a consequence, fish subjected to stress may therefore show lower melatonin levels.

Melatonin and anaesthetics

Handling of blood-sampling may be a stressful experience for fish, and anaesthesia is always required for invasive procedures (Burka et al., 1997). Anaesthesia will pharmacologically depress the central nerve system (CNS), usually by blocking receptor sites, which prevent the initiation and conduction of nerve impulses to the sensory centres in brain cortex, and simultaneous suppression of the somatic motor system (Ross & Ross, 1999). Blood sampling in the current study was executed on fish anaesthetized with MS-222. That is known to be have high anaesthetic efficacy on Common carp (Mohamed, 1999). However, Kezuka and coworkers (1988) reported a two-fold increase in melatonin levels with use of tricaine methanesulfonate (MS-222) under blood sampling in Common carp. It was therefore a possibility of anaesthetic influence in the present study, but the low plasma melatonin levels found during daytime indicate that the system was not affected.

Conclusions

- Blood plasma melatonin levels in Common carp exhibit a clear profile with high levels in dark and low levels in light under simulated natural photoperiod.

- The blood plasma profile for melatonin under extended darkperiod seem to repeat the profile found under simulated natural LD cycle, which may indicate the possibility of a circadian clock in Common carp.

- Blood plasma cortisol levels were high during experiments in Common carp, but sampling may not have influenced the melatonin levels.

Perspectives

To reach the long-term goal of SPRN to reduce poverty and increase food availability in rural hilly areas of Nepal with aquaculture in tandem with rural electrification, further development of Asian carp species in aquaculture should focus on the photoperiodic control of reproduction. Manipulation of the natural circannual rhythm of spawning has been developed with great success in many temperate species and it is now known that also sub-tropical fish species possess a melatonin production in the pineal gland. In this study of a species experiencing sub-tropical LD-cycles, the pineal system has been found to reflect environmental changes in LD, which may involve a circadian clock that can anticipate the next LD-cycle. To test whether Common carp contain a circadian clock that can be manipulated, experiments can be conducted with blood sampling under repeated DD cycles. To further decide if this possible clock is intra-pineal, *in vitro* experiments with the pineal gland under DD should be conducted.

If one accepts that endogenous endocrine mechanisms underlie the timing of reproduction in fish, then environmental photomanipulation may also influence the timing of reproduction of these low latitude species. In order to reach the goal of year-round supply of inexpensive carp fries to rural fishponds, further work should test the temporal pattern of the melatonin profile with simultaneously measurements of the response in the HPG axis and the gonadal status. Experiments should thereafter be carried out with attempts to manipulate time of maturation.

It is also important to test if temperature and food availability have an effect on the manipulation of time of spawning.

To secure a sustainable food production in rural areas one must educate local labour so the people in these areas can operate their own fish-production. This can employ poor people in addition to secure them with healthy food.

References

Almazan-Rueda, P., Schrama, J. W., Verreth, J. A. J. (2004). Behavioural responses under different feeding methods and light regimes of the African catfish (*Clarias gariepinus*) juveniles. *Aquaculture*, 231 (1-4)**:** 347-359.

AQUASTAT (2010). FAO's information system on water and agriculture. Nepal [Online]. Food and Agriculture Organization of the UN. Available from: http://www.fao.org/nr/water/aquastat/countries_regions/nepal/index.stm [Accessed: 19.05.2011].

Arends, R. J., van der Gaag, R., Martens, G. J. M., Bonga, S. E. W., Flik, G. (1998). Differential expression of two pro-opiomelanocortin mRNAs during temperature stress in common carp (*Cyprinus carpio L.*). *Journal of Endocrinology*, 159 (1)**:** 85-91.

Barcellos, L. J. G., Nicolaiewsky, S., de Souza, S. M. G., Lulhier, F. (1999). Plasmatic levels of cortisol in the response to acute stress in Nile tilapia (*Oreochromis niloticus L.*), previously exposed to chronic stress. *Aquaculture Research*, 30 (6)**:** 437-444.

Barton, B. A., Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, 1**:** 3-26.

Bayarri, M. J., Falcon, J., Zanuy, S., Carrillo, M. (2010). Continuous light and melatonin: daily and seasonal variations of brain binding sites and plasma concentration during the first reproductive cycle of sea bass. *General and Comparative Endocrinology*, 169 (1)**:** 58-64.

Bayarri, M. J., Garcia-Allegue, R., Lopez-Olmeda, J., Madrid, J., Sanchez-Vazquez, F. (2004). Circadian melatonin release in vitro by European sea bass pineal. *Fish Physiology and Biochemistry*, 30 (1)**:** 87-89.

Bayarri, M. J., Madrid, J. A., Sanchez-Vazquez, F. J. (2002). Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. *Journal of Pineal Research*, 32 (1)**:** 34-40.

BBC (2011a). Japan earthquake: Tsunami hits north-east [Online]. BBC News. Available from: http://www.bbc.co.uk/news/world-asia-pacific-12709598 [Accessed: 04.05.2011].

BBC (2011b). US tornado outbreak was 'biggest ever' [Online]. BBC News. Available from: http://www.bbc.co.uk/news/world-us-canada-13262644 [Accessed: 04.05.2011].

Begay, V., Falcon, J., Cahill, G. M., Klein, D. C., Coon, S. L. (1998). Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: Circadian regulation in pike and Zebrafish, but not in trout. *Endocrinology*, 139 (3)**:** 905-912.

Benyassi, A., Schwartz, C., Ducouret, B., Falcon, J. (2001). Glucocorticoid receptors and serotonin N-acetyltransferase activity in the fish pineal organ. *Neuroreport*, 12 (5)**:** 889-892.

Berson, S. A., Yalow, R. S. (2006). General principles of radioimmunoassay (Reprinted from Clinica Chimica Acta, vol 22, pg 51-69, 1968). *Clinica Chimica Acta*, 369 (2)**:** 125-143.

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 (4)**:** 629-650.

Bhattacharya, S., Dey, R., Basu, A., Maitra, S. K., Banerji, T. K. (2003). The structure of the pineal complex in a common Indian teleost, *Catla catla*: Evidence for pineal-induced inhibition of testicular function within an annual reproductive cycle. *Endocrine Research*, 29 (2)**:** 141-156.

Boeuf, G., Falcon, J. (2001). Photoperiod and growth in fish. *Vie Et Milieu*, 51 (4)**:** 247-266.

Bolliet, V., Ali, M. A., Lapointe, F. J., Falcon, J. (1996a). Rhythmic melatonin secretion in different teleost species: An *in vitro* study. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology*, 165 (8)**:** 677-683.

Bolliet, V., Begay, V., Taragnat, C., Ravault, J. P., Collin, J. P., Falcon, J. (1996b). Photoreceptor cells of the pike pineal organ as cellular circadian oscillators. *European Journal of Neuroscience*, 9 (4)**:** 643-653.

Bradsher, K. (2011). U.N. Food Agency Issues Warning on China Drought [Online]. The New York Times. Available from: http://www.nytimes.com/2011/02/09/business/global/09food.html [Accessed: 25.03.11].

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 (1-3)**:** 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 (1-4)**:** 63-98.

Brown, L. (2011). This will be the Arab world´s next battle [Online]. The Guardian. Available from: http://www.guardian.co.uk/commentisfree/2011/apr/22/water-the-next-arab-battle [Accessed: 25.04.11].

Bubenik, G. A. (2002). Gastrointestinal melatonin: Localization, function, and clinical relevance. *Digestive Diseases and Sciences*, 47 (10)**:** 2336-2348.

Bubenik, G. A. (2008). Thirty four years since the discovery of gastrointestinal melatonin. *Journal of Physiology and Pharmacology*, 59**:** 33-51.

Bubenik, G. A., Pang, S. F. (1997). Melatonin levels in the gastrointestinal tissues of fish, amphibians, and a reptile. *General and Comparative Endocrinology*, 106 (3)**:** 415-419.

Bubenik, G. A., Pang, S. F., Cockshut, J. R., Smith, P. S., Grovum, L. W., Friendship, R. M., Hacker, R. R. (2000). Circadian variation of portal, arterial and venous blood levels of melatonin in pigs and its relationship to food intake and sleep. *Journal of Pineal Research*, 28 (1)**:** 9-15.

Bunes, H. (2010). Virkeevne og stressreduserende effekt til tre typer anestesimidler på rohu (*Labeo rohita*). Thesis (M.Sc). Norwegian University of Science and Technology (NTNU), Trondheim. 61 p

Burka, J. F., Hammell, K. L., Horsberg, T. E., Johnson, G. R., Rainnie, D. J., Speare, D. J. (1997). Drugs in salmonid aquaculture - A review. *Journal of Veterinary Pharmacology and Therapeutics*, 20 (5)**:** 333-349.

Cahill, G. M. (1996). Circadian regulation of melatonin production in cultured zebrafish pineal and retina. *Brain Research*, 708 (1-2)**:** 177-181.

Cahill, G. M., Besharse, J. C. (1989). Retinal melatonin is metabolized within the eye of *Xenopus laevis*. *Proceedings of the National Academy of Sciences of the United States of America*, 86 (3)**:** 1098-1102.

Cahill, G. M., Besharse, J. C. (1995). Circadian rhythmicity in vertebrate retinas: regulation by a photoreceptor oscillator. *Progress in Retinal and Eye Research*, 14 (1)**:** 267-291.

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 (1-4)**:** 299-314.

Carrillo, M., Bromage, N., Zanuy, S., Serrano, R., Prat, F. (1989). The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in sea bass (*Dicentrarchus-Labrax L.*) *Aquaculture*, 81 (3-4)**:** 351-365.

Chard, T. (1990). An introduction to radioimmunoassay and related techniques. Elsevier, Amsterdam. 290 p.

Chattoraj, A., Bhattacharyya, S., Basu, D., Bhattacharya, S., Maitra, S. K. (2005). Melatonin accelerates maturation inducing hormone (MIH): induced oocyte maturation in carps. *General and Comparative Endocrinology*, 140 (3)**:** 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 (4)**:** 347-359.

Chen, S. H., Ravallion, M. (2010). The developing world is poorer than we thought, but no less successful in the fight against poverty. *Quarterly Journal of Economics*, 125 (4)**:** 1577-1625.

Chowdhury, I., Sengupta, A., Maitra, S. K. (2008). Melatonin: fifty years of scientific journey from the discovery in bovine pineal gland to delineation of functions in human. *Indian Journal of Biochemistry & Biophysics*, 45 (5)**:** 289-304.

CIA (2011). The World Fact Book, Nepal [Online]. Central Intelligence Agency (CIA). Available from: https://www.cia.gov/library/publications/the-world-factbook/index.html [Accessed: 18.01.2011].

Davies, B., Bromage, N., Swanson, P. (1999). The brain-pituitary-gonadal axis of female rainbow trout, *Oncorhynchus mykiss*: Effects of photoperiod manipulation. *General and Comparative Endocrinology*, 115 (1)**:** 155-166.

Davies, P. R., Hanyu, I. (1986). Effect of temperature and photoperiod on sexual maturation and spawning of the common carp I. Under conditions of high temperature. *Aquaculture*, 51 (3-4)**:** 277-288.

Davies, P. R., Hanyu, I., Furukawa, K., Nomura, M. (1986a). Effect of temperature and photoperiod on sexual maturation and spawning of the common carp II. Under conditions of low temperature *Aquaculture*, 52 (1)**:** 51-58.

Davies, P. R., Hanyu, I., Furukawa, K., Nomura, M. (1986b). 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 (2)**:** 137-144.

Delgado, C. L., Wada, N., Rosengrant, M. W., Meijer, S., Ahmed, M. (2003). Fish to 2020. Supply and Demand in Changing Global Market. International Food Policy Research Institute & WorldFish Center, Washington D.C. 226 p.

Dey, R., Bhattacharya, S., Maitra, S. K. (2004). Temporal pattern of ovarian activity in a major carp *Catla catla* and its possible environmental correlate in an annual cycle. *Biological Rhythm Research*, 35 (4-5)**:** 329-353.

Dunlap, J. C., Loros, J. J., DeCoursey, P. J. (Ed.). (2004). Chronobiology: Biological timekeeping. Sinauer Associates. Sunderland, MA. 406 p.

Ekström, P., Meissl, H. (1997). The pineal organ of teleost fishes. *Reviews in Fish Biology and Fisheries*, 7 (2)**:** 199-284.

Falcon, J. (1999). Cellular circadian clocks in the pineal. *Progress in Neurobiology*, 58 (2)**:** 121-162.

Falcon, J., Besseau, L., Sauzet, S., Boeuf, G. (2007). Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends in Endocrinology and Metabolism*, 18 (2)**:** 81-88.

Falcon, J., Bolliet, V., Collin, J. P. (1996). Partial characterization of serotonin N-acetyltransferases from northern pike (*Esox lucius, L*) pineal organ and retina: Effects of temperature. *Pflugers Archiv-European Journal of Physiology*, 432 (3)**:** 386-393.

Falcon, J., Bolliet, V., Ravault, J. P., Chesneau, D., Ali, M. A., Collin, J. P. (1994). Rhythmic secretion of melatonin by the superfused pineal organ - thermoperiod and photoperiod interaction. *Neuroendocrinology*, 60 (5)**:** 535-543.

Falcon, J., Collin, J. P. (1985). In vitro uptake and metabolism of [H-3] indole compounds in the pineal organ of the pike. II. A radioautographic study. *Journal of Pineal Research*, 2 (4)**:** 357-373.

Falcon, 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 (5)**:** 1804-1813.

Falcon, 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 (6)**:** 1943-1950.

Falcon, J., Migaud, H., Munoz-Cueto, J. A., Carrillo, M. (2010). Current knowledge on the melatonin system in teleost fish. *General and Comparative Endocrinology*, 165 (3)**:** 469-482.

Falcon, J., Thibault, C., Begay, V., Zachmann, A., Collin, J.-P. (1992). Regulation of the rhythmic melatonin secretion by fish pineal photoreceptor cells. In: M. A. Ali, (Ed.), Rhythms in fishes. Plenum Press. New York. pp. 167-198.

FAO (1997). FAO Technical guidelines for responsible fisheries. No. 5. FAO Fisheries Department, Rome. 40 p.

FAO (2004). Cultured aquatic species information programme. Cyprinus carpio. [Online]. FAO Fisheries and Aquaculture Department. Available from: http://www.fao.org/fishery/culturedspecies/Cyprinus_carpio/en [Accessed: 19.05.2011].

FAO (2008). The state of world fisheries and aquaculture 2008. FAO Fisheries and Aquaculture Department, Rome. 176 p.

FAO (2010). The state of world fisheries and aquaculture 2010. FAO Fisheries and Aquaculture Department, Rome. 197 p.

FAO (2011a). Crop prospects and food situation. FAO Global Information and Early Warning Service, Rome. 31 p.

FAO (2011b). Cultured aquatic species information programme. *Cyprinus carpio*. [Online]. FAO Fisheries and Aquaculture Department. Available from: http://www.fao.org/fishery/culturedspecies/Cyprinus_carpio/en [Accessed: 19.05.2011].

FAO (2011c). Fishery and aquaculture country profiles, Nepal. [Online]. FAO Fishery and Aquaculture Department. Available from: http://www.fao.org/fishery/countrysector/FI-CP_NP/3/en [Accessed: 09.02.2011].

FAO (2011d). The state of food and agriculture 2010-2011. Food and agriculture organization of the UN, Rome. 147 p.

Foo, J. T. W., Lam, T. J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. *Aquaculture*, 115 (1-2)**:** 145-158.

Friend, R., Funge-Smith, S. J. (2002). Focusing on small-scale aquaculture and aquatic resource management on poverty alleviation. FAO, Network of Aquaculture Centres in Asia-Pacific (NACA), Bangkok. 24 p.

Gaildrat, P., Falcon, J. (1999). Expression of melatonin receptors and 2-[I-125]iodomelatonin binding sites in the pituitary of a teleost fish. In: J. Olcese, (Ed.), Melatonin after Four Decades - an Assessment of Its Potential. Kluwer Academic / Plenum Publ. New York. pp. 61-72.

Granbo, K. (2011). Nå venter kineserne på regnet [Online]. NRK. Available from: http://www.nrk.no/nyheter/verden/1.7648847 [Accessed: 26.05.2011].

Gurung, T. B. (2003). Fisheries and Aquaculture Activities in Nepal. *Aquaculture Asia*, 8**:** 14-19.

Gurung, T. B., Mulmi, R. M., Kalyan, K. C., Wagle, G., Pradhan, G. B., Upadhayaya, K., Rai, A. K. (2010). Cage Fish Culture: An Alternative Livelihood Option for Communities Displaced by Reservoir Impoundment in Kulekhani, Nepal. In: S. S. De Silva, F. B. Davy, (Ed.), Success Stories in Asian Aquaculture. Springer. Rome. pp. 85-102.

Gurung, T. B., Wagle, S. K., Bista, J. D., Dhakal, R. P., Joshi, P. L., Batajoo, R., Adhikari, P., Rai, A. K. (2005). Participatory fisheries management for livelihood improvement of fishers in Phewa Lake, Pokhara, Nepal. *Himalayan Journal of Sciences*, 3 (5)**:** 47-52.

Hansen, T., Karlsen, O., Taranger, G. L., Hemre, G. I., Holm, J. C., Kjesbu, O. S. (2001). Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. *Aquaculture*, 203 (1-2)**:** 51-67.

Huether, G. (1993). The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. *Experientia*, 49 (8)**:** 665-670.

Huether, G., Poeggeler, B., Reimer, A., George, A. (1992). Effect of tryptophan administration on circulating melatonin levels in chicks and rats - evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. *Life Sciences*, 51 (12)**:** 945-953.

Iigo, M., Abe, T., Kambayashi, S., Oikawa, K., Masuda, T., Mizusawa, K., Kitamura, S., Azuma, T., Takagi, Y., Aida, K., Yanagisawa, T. (2007). Lack of circadian regulation of in vitro melatonin release from the pineal organ of salmonid teleosts. *General and Comparative Endocrinology*, 154 (1-3)**:** 91-97.

Iigo, M., Kezuka, H., Suzuki, T., Tabata, M., Aida, K. (1994). Melatonin signal-transduction in the goldfish, *Carassius auratus*. *Neuroscience and Biobehavioral Reviews*, 18 (4)**:** 563-569.

Iigo, M., SanchezVazquez, F. J., Madrid, J. A., Zamora, S., Tabata, M. (1997a). Unusual responses to light and darkness of ocular melatonin in European sea bass. *Neuroreport*, 8 (7)**:** 1631-1635.

Iigo, M., Tabata, M., Aida, K. (1997b). Ocular melatonin rhythms in a cyprinid teleost, oikawa *Zacco platypus*, are driven by light-dark cycles. *Zoological Science*, 14 (2)**:** 243-248.

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 (4)**:** 433-456.

Jhingran, V. G., Pullin, R. S. V. (1985). A hatchery manual for the Common, Chinese and Indian major carps. Asian Development Bank, Manila. 191 p.

Jull, C. (2006). The impact of agriculture-related WTO agreements on the domestic legal framework in the Kingdom of Nepal. FAO legal papers online #58, FAO. Rome. 27 p.

Kestemont, P. (1995). Different systems of carp production and their impacts on the environment. *Aquaculture*, 129 (1-4)**:** 347-372.

Kezuka, H., Furukawa, K., Aida, K., Hanyu, I. (1988). Daily cycles in plasma melatonin levels under long or short photoperiod in the Common carp, *Cyprinus carpio General and Comparative Endocrinology*, 72 (2)**:** 296-302.

Kezuka, H., Iigo, M., Furukawa, K., Aida, K., Hanyu, I. (1992). Effects of photoperiod, pinealectomy and opthalmectomy on circulating melatonin rhythms in the goldfish, *Carassius auratus*. *Zoological Science*, 9 (5)**:** 1047-1053.

Kissil, G. W., Lupatsch, I., Elizur, A., Zohar, Y. (2001). Long photoperiod delayed spawning and increased somatic growth in gilthead seabream (*Sparus aurata*). *Aquaculture*, 200 (3-4)**:** 363-379.

Klein, D. C., Coon, S. L., Roseboom, P. H., Weller, J. L., Bernard, M., Gastel, J. A., Zatz, M., Iuvone, P. M., Rodriguez, I. R., Begay, V., Falcon, J., Cahill, G. M., Cassone, V. M., Baler, R. (1997). The melatonin rhythm-generating enzyme: Molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Progress in Hormone Research, Proceedings of the 1996 Conference*, 52**:** 307-358.

Knabb, R. D., Rhome, J. R., Brown, D. P. (2006). Tropical Cyclone Report. National Hurricane Center, Miami. 43 p.

Korte, S. M., Koolhaas, J. M., Wingfield, J. C., McEwen, B. S. (2005). The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neuroscience and Biobehavioral Reviews*, 29 (1)**:** 3-38.

Kulczykowska, E., Kalamarz, H., Warne, J., Balment, R. (2006). Day–night specific binding of 2-[125I]Iodomelatonin and melatonin content in gill, small intestine and kidney of three fish species. *Journal of Comparative Physiology B*, 176 (4)**:** 277-285.

Lerner, A. B., Case, J. D., Takahashi, Y., Lee, T. H., Mori, W. (1958). Isolation of melatonin, the pineal gland factor that lightens melatonocytes. *Journal of the American Chemical Society*, 80 (10)**:** 2587-2587.

Lewy, A. J., Tetsuo, M., Markey, S. P., Goodwin, F. K., Kopin, I. J. (1980). Pinealectomy abolishes plasma melatonin in the rat. *Journal of Clinical Endocrinology & Metabolism*, 50 (1)**:** 204-205.

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.

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 (2-3)**:** 201-207.

Maitra, S. K., Seth, M., Chattoraj, A. (2006). Photoperiod, pineal photoreceptors and melatonin as the signal of photoperiod in the regulation of reproduction in fish. *Journal of Endocrinology and Reproduction*, 10 (2)**:** 73-87.

Martinez-Chavez, C. C., Al-Khamees, S., Campos-Mendoza, A., Penman, D. J., Migaud, H. (2008). Clock-controlled endogenous melatonin rhythms in nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*). *Chronobiology International*, 25 (1)**:** 31-49.

Masuda, T., Iigo, M., Mizusawa, K., Naruse, M., Oishi, T., Aida, K., Tabata, M. (2003). Variations in plasma melatonin levels of the rainbow trout (*Oncorhynchus mykiss*) under various light and temperature conditions. *Zoological Science*, 20 (8)**:** 1011-1016.

Max, M., Menaker, M. (1992). Regulation of melatonin production by light, darkness and temperature in the trout pineal. *Journal of Comparative Physiology A- Sensory Neural and Behavioral Physiology*, 170 (4)**:** 479-489.

MFA (2010). Bilateral bistand i 2009 [Online]. The Norwegian Ministry of Foreign Affairs. Available from: http://www.regjeringen.no/nb/dep/ud/dok/regpubl/prop/2010-2011/prop-1-s-20102011-/16.html?id=616458# [Accessed: 01.02.2011].

Michaelsen, K. F., Hoppe, C., Roos, N., Kaestel, P., Stougaard, M., Lauritzen, L., Mølgaard, C., Girma, T., Friis, H. (2009). Choice of foods and ingredients for moderately malnourished children 6 months to 5 years of age. *Food and nutrition bulletin*, 30 (3)**:** 343-404.

Migaud, H., Davie, A., Chavez, C. C. M., Al-Khamees, S. (2007). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. *Journal of Pineal Research*, 43 (4)**:** 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 (1)**:** 27-68.

Migaud, H., Taylor, J. F., Taranger, G. L., Davie, A., Cerda-Reverter, J. M., Carrillo, M., Hansen, T., Bromage, N. R. (2006). A comparative *ex vivo* and *in vivo* study of day and night perception in teleosts species using the melatonin rhythm. *Journal of Pineal Research*, 41 (1)**:** 42-52.

Mohamed, S. J. (1999). Comparative efficacy of four anesthetics on Common carp (*Cyprinus carpio L.*). *Acta Ichthyologica et Piscatoria*, 29 (2)**:** 91-97.

MPE (2004). Norges Offentlige Utredninger: Hydrogen som fremtidens energibærer. The Norwegian Ministry of Petroleum and Energy, Oslo. 91 p.

NARC (2007). Nepal Agricultural Research Council [Online]. NARC. Available from: http://narc.gov.np/narc/index.php [Accessed: 28.07.2011].

Nikaido, Y., Aluru, N., McGuire, A., Park, Y. J., Vijayan, M. M., Takemura, A. (2010). Effect of cortisol on melatonin production by the pineal organ of tilapia, *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 155 (1)**:** 84-90.

NORAD (2011a). Norsk bistand i tall [Online]. The Norwegian Agency for Development Cooperation (NORAD). Available from: http://www.norad.no/Norskbistanditall [Accessed: 01.02.2010].

NORAD (2011b). Norsk utviklingspolitikk [Online]. The Norwegian Agency for Development Cooperation (NORAD). Available from: http://www.norad.no/Om+bistand/Norsk+utviklingspolitikk [Accessed: 31.01.2011].

NorwegianEmbassy (2011). Norwegian Development Cooperation with Nepal [Online]. The Royal Norwegian Embassy in Kathmandu. Available from: http://www.norway.org.np/Embassy/Development-cooperation/DevCoop/ [Accessed: 31.01.2011].

Okimoto, D. K., Stetson, M. H. (1999). Presence of an intrapineal circadian oscillator in the teleostean family *Poeciliidae*. *General and Comparative Endocrinology*, 114 (2)**:** 304-312.

Pardridge, W. M., Mietus, L. J. (1980). Transport of albumin-bound melatonin through the blood-brain barrier. *Journal of Neurochemistry*, 34 (6)**:** 1761-1763.

Penman, D. J., Gupta, M. V., Dey, M. M. (2005). Carp genetic resources for aquaculture in Asia. WorldFish Center, Penang. 152 p.

Petr, T. (2002). Cold water fish and fisheries in countries of the high mountain arc of Asia. In: T. Petr, D. B. Swar (Ed.), Cold water fisheries in the trans-Himalayan countries. FAO Fisheries Technical Paper. FAO, Rome. pp. 1-38.

Pickering, A. D., Pottinger, T. G. (1983). Seasonal and diel changes in plasma cortisol levels of the Brown trout, *Salmo trutta*. *General and Comparative Endocrinology*, 49 (2)**:** 232-239.

Pieri, C., Marra, M., Moroni, F., Recchioni, R., Marcheselli, F. (1994). Melatonin: A peroxyl radical scavenger more effective than vitamin E *Life Sciences*, 55 (15)**:** 271-276.

Pollock, L. J. (2005). Integration of aquaculture within irrigation systems: A poverty-focused approach. Thesis (Ph.D). University of Sterling, Stirling. 402 p

Porter, M. J. R., Duncan, N. J., Mitchell, D., Bromagea, N. R. (1999). The use of cage lighting to reduce plasma melatonin in Atlantic salmon (Salmo salar) and its effects on the inhibition of grilsing. *Aquaculture*, 176 (3-4)**:** 237-244.

Pottinger, T. G. (2010). A multivariate comparison of the stress response in three salmonid and three cyprinid species: evidence for inter-family differences. *Journal of Fish Biology*, 76 (3)**:** 601-621.

PRB (2011). World Population Data Sheet 2010. Populaton Reference Bureau, Washington D.C. 19 p.

Pyakuryal, B., Thapa, Y. B., Roy, D. (2005). Trade liberalization and food security in Nepal. International Food Policy Research Institute, Washington DC. 72 p.

Rajbanshi, K. G. (1996). Fisheries development in Azad Jammu and Kashmir. FAO Fisheries and Aquaculture Department, Rome. 153 p.

Randall, C. F., Bromage, N. R., Thorpe, J. E., Miles, M. S., Muir, J. S. (1995). Melatonin rhythms in Atlantic Salmon (*Salmo Salar*) maintained under natural and out-of-phase photoperiods. *General and Comparative Endocrinology*, 98 (1)**:** 73-86.

Reardon, E. E., Chapman, L. J. (2008). Reproductive seasonality in a swamp-locked African cichlid. *Ecology of Freshwater Fish*, 17 (1)**:** 20-29.

Reiter, R. J. (1987). The melatonin message: duration versus coincidence hypotheses. *Life Sciences*, 40 (22)**:** 2119-2131.

Reiter, R. J. (1991). Neuroendocrine effects of light. *International Journal of Biometeorology*, 35 (3)**:** 169-175.

Reiter, R. J. (1993). The Melatonin rhythm - both a clock and a calendar. *Experientia*, 49 (8)**:** 654-664.

Reuters (2010a). Major magnitude 7.3 quake hits Haiti [Online]. Thomson Reuters. Available from: http://www.reuters.com/article/2010/01/12/quake-haiti-idUSN1220889720100112 [Accessed: 04.05.2011].

Reuters (2010b). Strong quake hits New Zealand near Christchurch [Online]. Thomson Reuters. Available from: http://www.reuters.com/article/2010/09/03/quake-newzealand-idUSN0311581920100903 [Accessed: 04.05.2011].

Ridha, M. T., Cruz, E. M. (2000). Effect of light intensity and photoperiod on Nile tilapia (*Oreochromis niloticus L.*) seed production. *Aquaculture Research*, 31 (7)**:** 609-617.

Roos, N., Islam, M. M., Thilsted, S. H. (2003). Small indigenous fish species in Bangladesh: Contribution to vitamin A, calcium and iron intakes. *The Journal of Nutrition*, 133 (11)**:** 4021S-4026S.

Ross, L. G., Martinez Palacios, C. A., Morales, E. J. (2008). Developing native fish species for aquaculture: the interacting demands of biodiversity, sustainable aquaculture and livelihoods. *Aquaculture Research*, 39 (7)**:** 675-683.

Ross, L. G., Ross, B. (1999). Anaesthetic and sedative techniques for aquatic animals, (2.ed). Blackwell Science Ltd., Oxford. 157 p.

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)**:** 1-12.

Saavedra, M., Pousao-Ferreira, P. (2006). A preliminary study on the effect of lunar cycles on the spawning behaviour of the gilt-head sea bream, *Sparus aurata*. *Journal of the Marine Biological Association of the United Kingdom*, 86 (4)**:** 899-901.

Saeij, J. P. J., Verburg-van Kemenade, L. B. M., van Muiswinkel, W. B., Wiegertjes, G. F. (2003). Daily handling stress reduces resistance of carp to *Trypanoplasma borreli*: *in vitro* modulatory effects of cortisol on leukocyte function and apoptosis. *Developmental and Comparative Immunology*, 27 (3)**:** 233-245.

Sauzet, S., Besseau, L., Perez, P. H., Coves, D., Chatain, B., Peyric, E., Boeuf, G., Munoz-Cueto, J. A., Falcon, J. (2008). Cloning and retinal expression of melatonin receptors in the European sea bass, *Dicentrarchus labrax*. *General and Comparative Endocrinology*, 157 (2)**:** 186-195.

Seth, M., Maitra, S. K. (2010a). Neuronal regulation of photo-induced pineal photoreceptor proteins in carp *Catla catla*. *Journal of Neurochemistry*, 114 (4)**:** 1049-1062.

Seth, M., Maitra, S. K. (2010b). 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 (1)**:** 21-28.

Seth, M., Maitra, S. K. (2011). Neural regulation of dark-induced abundance of Arylalkylamine N-acetyltransferase (AANAT) and nelatonin in the carp (*Catla catla*) pineal: An *in vitro* study. *Chronobiology International*, 0 (0)**:** 1-14.

Sharma, S., Bajracharya, R. M., Sitaula, B. K., Merz, J. (2005). Water quality in the Central Himalaya. *Current Science*, 89 (5)**:** 774-786.

Shrestha, A. B., Wake, C. P., Dibb, J. E., Mayewski, P. A. (2000). Precipitation fluctuations in the Nepal Himalaya and its vicinity and relationship with some large scale climatological parameters. *International Journal of Climatology*, 20 (3)**:** 317-327.

Shrestha, J. (1999). Cold water fish and fisheries in Nepal. In: T. Petr (Ed.), Fish and fisheries at higher altitudes: Asia - Technical Paper no. 385. FAO Fisheries Department, Rome. pp. 304-325.

Shrestha, J. (2002). Taxonomic revision of cold water fishes of Nepal. In: T. Petr, D. B. Swar (Ed.), Cold water fisheries in the trans-Himalayan countries. FAO Fisheries Technical Paper. FAO, Rome. pp. 273-288.

Skjulsvik, J. E. (2005). Plasma melatonin profiles in Atlantic salmon (Salmo salar) subjected to natural and artificial light-regimes. Thesis (Cand. Scient.). Norwegian University of Science and technology (NTNU), Trondheim. 51 p

SPRN (2007). The SPRN programme report I (April 2006- August 2007). Sustainable Poverty Reduction in Nepal (SPRN), Norwegian University of Science and Technology (NTNU), Sweco Grøner A.S., Trondheim/Lysaker. 70 p.

SPRN (2008). Sustainable poverty reduction in Nepal by combining fish farming and education with eco-friendly hydropower (2009-2012). Sustainable Poverty Reduction in Nepal (SPRN), Kathmandu. 91 p.

SSB (2009). Fiskeri og havbruk [Online]. Statistics Norway (SSB). Available from: http://www.ssb.no/emner/10/05/fiskeri_havbruk/ [Accessed: 04.05.2010].

Subasinghe, R. P. (2005). Epidemiological approach to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. *Preventive Veterinary Medicine*, 67 (2-3)**:** 117-124.

Sumpter, J. P. (1992). Control of growth of Rainbow trout (*Oncorhynchus mykiss*) *Aquaculture*, 100 (1-3)**:** 299-320.

Svalheim, P. (2009). Kraftverket. Odd Hoftun: portrett av et livsverk. Luther Forlag, Oslo. 415 p.

Svobodova, Z., Kalab, P., Dusek, L., Vykusova, B., Kolarova, J., Janouskova, D. (1999). The effect of handling and transport on the concentration of glucose and cortisol in blood plasma of Common carp. *Acta Veterinaria Brno*, 68 (4)**:** 265-274.

Swar, D. B. (2002). The status of cold water fish and fisheries in Nepal and prospects of their utilization for poverty reduction. In: T. Petr, D. B. Swar (Ed.), Cold water fisheries in the trans-Himalayan countries. FAO Fisheries Technical Paper. 431. FAO, Rome. pp. 79-96.

Swar, D. B., Gurung, T. B. (1988). Introduction and cage culture of exotic carps and their impact on fish harvested in Lake Begnas, Nepal. *Hydrobiologia*, 166 (3)**:** 277-283.

Takemura, A., Rahman, M. S., Park, Y. J. (2010). External and internal controls of lunar-related reproductive rhythms in fishes. *Journal of Fish Biology*, 76 (1)**:** 7-26.

Taranger, G. L., Haux, C., Stefansson, S. O., Bjornsson, B. T., Walther, B. T., Hansen, T. (1998). Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and estradiol-17 beta profiles in Atlantic salmon, *Salmo salar*. *Aquaculture*, 162 (1-2)**:** 85-98.

Thrush, M. A., Duncan, N. J., Bromage, N. R. (1994). The use of photoperiod in the production of out-of-season Atlantic salmon (Salmo salar) smolts. *Aquaculture*, 121 (1-3)**:** 29-44.

UN (2010a). The Millennium Development Goals Report 2010. UN Department of Economic and Social Affairs, New York. 76 p.

UN (2010b). United Nations Development Goals [Online]. UN Department of Public Information. Available from: http://www.un.org/millenniumgoals/ [Accessed: 14.01.2011].

UN (2011). World Economic Situation and Prospects 2011. United Nations, New York. 204 p.

UNDP (2010). Human Development Report 2010, The Real Wealth of Nations: Pathways to Human Development. United Nations Development Programme, New York. 227 p.

UNICEF (2009). Tracking Progress on Child and Maternal Nutrition: A survival and development priority. UNICEF. New York. 119 p.

Vijayan, M. M., Pereira, C., Grau, E. G., Iwama, G. K. (1997). Metabolic responses associated with confinement stress in tilapia: The role of cortisol. *Comparative Biochemistry and Physiology - Part C: Pharmacology Toxicology & Endocrinology*, 116 (1)**:** 89-95.

Vivien-Roels, B., Pévet, P. (1993). Melatonin: presence and formation in invertebrates. *Cellular and Molecular Life Sciences*, 49 (8)**:** 642-647.

Wattage, P. (2009). Millennium Development Goals and aquaculture: indicators to evaluate the conservation of the resource base for poverty reduction. *FAO Fisheries and Aquaculture Technical Paper*, 534**:** 59-72.

Welp, A., Manz, B., Peschke, E. (2010). Development and validation of a high throughput direct radioimmunoassay for the quantitative determination of serum and plasma melatonin (N-acetyl-5-methoxytryptamine) in mice. *Journal of Immunological Methods*, 358 (1-2)**:** 1-8.

Whitehead, C., Bromage, N. R., Forster, J. R. M., Matty, A. J. (1978). Effects of alterations in photoperiod on ovarian development and spawning time in rainbow- trout (*Salmo-gairdneri*). *Annales De Biologie Animale Biochimie Biophysique*, 18 (4)**:** 1035-1043.

WorldBank (2011). Countries and Economies: Nepal [Online]. The World Bank Group. Available from: http://data.worldbank.org/country/nepal [Accessed: 31.01.2011].

WorldMapFinder (2011). Map of Nepal [Online]. Available from: http://www.worldmapfinder.com/Map_Search.php?ID=/En/Asia/Nepal&PG=2 [Accessed: 11.04.2011].

Yadav, C. N. R., Bhujel, R. C. (1998). Water resource and its utilization for aquaculture in Nepal. *Journal of Institute of Agriculture and Animal Science*, 19-20**:** 193-197.

Yanthan, L., Gupta, B. B. P. (2007). In vitro effects of steroid hormones on arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of fish, *Clarias gariepinus* (Burchell, 1822) during different phases of breeding cycle. *Indian Journal of Experimental Biology*, 45 (8)**:** 676-682.

Yu, H. S., Pang, S. F., Tang, P. L. (1981). Increase in the level of retinal melatonin and persistence of its diurnal rhythm in rats after pinealectomy. *Journal of Endocrinology*, 91 (3)**:** 477-481.

Zachmann, A., Ali, M. A., Falcon, J. (1992). Melatonin and its effects in fishes - An overview. In: M. A. Ali, (Ed.), Rhythms in Fishes. Plenum Press. New York. pp. 149-165.

Zawilska, J. B., Berezinska, M., Rosiak, J., Vivien-Roels, B., Skene, D. J., Pèvet, P., Nowak, J. Z. (2003). Daily variation in the concentration of melatonin and 5-methoxytryptophol in the goose pineal gland, retina, and plasma. *General and Comparative Endocrinology*, 134 (3)**:** 296-302.

Zawilska, J. B., Nowak, J. Z. (1992). Regulatory mechanisms in melatonin biosynthesis in retina. *Neurochemistry International*, 20 (1)**:** 23-36.

Zawilska, J. B., Sadowska, M. (2002). Prolonged treatment with glucocorticoid dexamethasone suppresses melatonin production by the chick pineal gland and retina. *Polish Journal of Pharmacology*, 54 (1)**:** 61-66.