“The saddest aspect of life right now is
that science gathers knowledge faster
than society gathers wisdom.“

Isaac Asimov

Spotlight on fish:
The biological impacts of artificial light at night

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy of Freie Universität Berlin

by

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From Berlin, Germany

2016

This thesis comprises work from 8/2010 to 3/2016.

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Date of Defense:	11.07.2016

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Summary

Light is fundamental for the existence of flora and fauna on earth. It serves not only as a source of energy but also as a source of information to organisms that drives daily and seasonal biological cycles. The natural alteration and length of day and night sets the internal clock of organisms and ensures that behavioural patterns and physical processes are synchronized with time of day and season. Recent decades have seen a profound transformation of nightscapes, with an increasing proportion of the Earth‘s surface being illuminated at night. This substantial transformation demonstrates a pressing need to understand the negative effects of artificial light at night (ALAN), commonly referred to as “light pollution”, on biological processes. However, major knowledge gaps remain, in particular the ecological impact of light pollution, targeting animal populations or even whole ecosystems. Historically, research on this topic focused on humans and terrestrial organisms. But also aquatic ecosystems can be strongly influenced by light pollution. In fish, many traits underlie either daily or seasonal rhythms. ALAN can interfere with all these rhythmical, light controlled processes by diminishing the distinction between day and night. In fish both, the retina and the pineal organ possess circadian oscillators that integrate photoperiodic information and translate it into hormonal signals. However, for most fish it seems to be mainly the pineal organ that receives light signals, translate it to melatonin signals in a rhythmical manner and can therefore act as messenger for other parts of the organism. The melatonin output is high at night and low during the day with intermediate levels during twilight, but also season modulates this rhythm. Hence the melatonin rhythm reflects the current photoperiod and provides the fish with information about daily and calendar time. Light at night can suppress the nocturnal rise in melatonin and consequently may influence processes that are based on this rhythm including seasonal processes like reproduction.

The aim of this study was to evaluate the effect of low intensity ALAN on the melatonin rhythm, stress response (cortisol) and reproductive parameters of two of the most abundant fish species in European freshwaters. In laboratory experiments European perch (*Perca fluviatilis)* and roach (*Rutilus rutilus)* were exposed to four different light intensities during the night, 0 lx (control), 1 lx (potential light level in urban waters), 10 lx (street lighting at night) and 100 lx (positive control, strong streetlight). Melatonin and cortisol concentrations were measured from water samples of the fish tanks every three hours during a 24 hour period. Gene expression of gonadotropins luteinizing hormone and follicle stimulating hormone, important reproductive hormones, were determined from pituitary tissue excised after water sampling. In a second experiment the influence of different light qualities (blue,

green, red) on melatonin rhythm and gene expression of gonadotropins was studied, to examine the spectral sensitivity of the circadian axis in both species.

The laboratory studies revealed that the melatonin rhythm is suppressed in both species already by the lowest intensity of white light. In the control treatment the expected rhythm of high levels in the night and low levels during the day could be observed. At all tested light intensities nocturnal melatonin concentrations were significantly lowered. However, whereas in roach all treatments lowered the concentrations similarly, with no differences between night and day levels, melatonin levels of perch in the 1 lx treatment exhibited a noticeable increase during the night. These results suggest, that for perch, the threshold light intensity for melatonin suppression must lower than 1 lx. Roach are presumably even more sensitive. Concerning different light colours at night, results were similar. In perch and roach, all three light colours suppressed nocturnal melatonin concentration in comparison with the control treatment. In roach no differences in the effect of colour treatments were found. In perch however, blue light was less effective in suppressing nocturnal melatonin levels. The assumption, that roach is more sensitive to nocturnal light appears to be confirmed by the results.

Cortisol levels, on the other hand, were not affected by any of the treatments in perch, suggesting, that light pollution represents no additional stressor.

Gene expression of gonadotropins was only effected in perch in the experiment with different light intensities. This experiment was conducted under simulated photoperiod resembling October light conditions. The experiment with different light colours and perch was conducted under September light conditions and the experiments with roach were carried out under December conditions. ALAN during September and December had no effect on the reproduction of the experimental fish, suggesting the existence of a photo-labile phase, where reproduction of fish is particularly sensitive to ALAN. However, laboratory experiments cannot fully display natural environments. To attribute above mentioned impacts to the presence of ALAN, temperature was kept constant in all experiment. However, temperature is an important cue for the timing of reproductive events.

Therefore an additional experiment was conducted under natural conditions in the field. Perch and roach were exposed to street light or natural (control) conditions in a net cage for four weeks in August/September. Concentrations of melatonin and sex steroids (17ß-estradiol and 11-ketotestosterone) were determined from blood samples and gene expression of gonadotropins was measured from pituitary tissue. Levels of sex steroids and gene expression of gonadotropins was significantly reduced in the light treatment. The onset of the reproductive phase in perch as well as in roach is around August, when the experiment took place. This is in accordance to studies in other species, where a photo-labile phase was found around the onset of the reproductive phase. Thus it is most likely, that discrepancies in the effects of ALAN

on reproduction between laboratory and field experiment are related to temperature and season.

In conclusion, light pollution has a great potential to impact biological rhythms of fish. The suppression of the melatonin rhythm can yield several adverse effects that are not studied so far. Impacts on the immune system, growth and development as well as behavioural traits can influence not only individuals but whole populations. Impacts on reproduction and species specific differences in sensitivitiy to ALAN may cause unnatural shifts of biological niches, affect predator prey relationships and influence species communities and whole ecosystems.

Zusammenfassung

Licht ist eine elementare Voraussetzung für die Existenz des Lebens. Es dient nicht nur als Energiequelle, sondern stellt auch eine wichtige Information für Lebewesen dar, mit der circadiane und saisonale Rhythmen gesteuert werden. Der natürliche Wechsel und die Länge von Tag und Nacht stellt die innere Uhr und sorgt dafür, dass Verhaltensweisen und physiologische Prozesse mit der Tages- und Jahreszeit synchronisiert werden. Die letzten Jahrzehnte zeichneten sich durch eine tiefgreifende Veränderung der Nachtlandschaft aus, da ein zunehmender Teil der Erdoberfläche in der Nacht künstlich beleuchtet wird. Wissensdefizite gibt es vor allem bezüglich der ökologischen Auswirkungen auf aquatische Ökosysteme, die oft stark von künstlichem Licht beeinflusst sind. Bei Fischen unterliegen die meisten Vorgänge einer tages- oder jahreszeitlichen Rhythmik. Künstliches Licht in der Nacht kann in diese Vorgänge eingreifen, indem es die Grenze zwischen Tag und Nacht verwischt. Bei Fischen ist es hauptsächlich das Pinealorgan im Gehirn, das Lichtsignale in rhythmische Hormonsignale umwandelt und sie dem Blutkreislauf zuführt. Das betreffende Hormon, Melatonin, wird hauptsächlich in der Nacht produziert, am Tage sind die Konzentrationen gering. Auf diese Weise reflektiert der Melatoninrhythmus immer die vorherrschende Photoperiode und versorgt den Fisch konstant mit Informationen über die Tages- und Jahreszeit. Licht in der Nacht unterdrückt den nächtlichen Anstieg des Melatoninspiegels und kann somit Prozesse beeinflussen, die diesem Rhythmus folgen, unter anderem auch saisonale Prozesse wie die Fortpflanzung.

Ziel dieser Arbeit war es, die Auswirkungen von künstlichem Licht in der Nacht auf den Melatoninrhythmus, die Stressantwort (Cortisol) und Parameter der Fortpflanzung von zwei in europäischen Gewässern weit verbreiteten Fischarten zu bewerten. In Laborversuchen wurden hierzu Flussbarsche (*Perca fluviatilis*) und Plötzen (*Rutilus rutilus*) vier verschiedenen nächtlichen Lichtintensitäten ausgesetzt, 0 lx (Kontrolle), 1 lx (potenzielle Lichtverschmutzung in urbanen Gewässern), 10 lx (nächtliche Straßenbeleuchtung) und 100 lx (Positivkontrolle, starke Straßenbeleuchtung). Die Melatonin- und Cortisolkonzentrationen wurden aus Wasserproben bestimmt, die über einen Zeitraum von 24 Stunden alle drei Stunden aus den Fischbecken entnommen wurden. Die Genexpressionsraten zweier wichtiger Fortpflanzungshormone, der sogenannten Gonadotropine (follikelstimulierendes Hormon und luteinisierendes Hormon), wurden mittels qPCR aus dem Hypophysengewebe der Fische bestimmt. In einem weiteren Experiment wurde der Einfluss von verschiedenen Lichtfarben (blau, grün, rot) geringer Lichtintensitäten in der Nacht auf den Melatonin- und Cortisolrhythmus und die Genexpressionsrate der

Gonadotropine untersucht. Dies hatte zum Ziel, die spektrale Empfindlichkeit der biologischen Rhythmen für beide Arten zu ermitteln.
Die Laborstudien ergaben, dass der Melatoninrhythmus bei Barsch und Plötze bereits bei der geringsten Intensität weißen Lichts unterdrückt wird. Bei allen getesteten Lichtintensitäten war die nächtliche Melatoninkonzentration im Haltungswasser der Fische signifikant reduziert. Bei Barschen und Plötzen der 1 lux-Behandlungsgruppe konnte jedoch ein sichtbarer Anstieg der nächtlichen Melatoninkonzentration beobachtet werden. Diese Ergebnisse weisen darauf hin, dass der Schwellenwert der Melatoninsuppression für diese Spezies unter einem Lux liegen muss. Die nächtliche Beleuchtung mit Licht unterschiedlicher Wellenlängen führte zu ähnlichen Ergebnissen. Sowohl beim Barsch, als auch bei der Plötze wurde die Melatoninproduktion von allen drei Lichtfarben im Vergleich zur Kontrollgruppe unterdrückt. Bei der Plötze konnte dabei kein Einfluss der Lichtfarbe festgestellt werden, beim Barsch hingegen war die nächtliche Melatoninsuppression bei blauem Licht signifikant geringer ausgeprägt. Demnach scheint der biologische Rhythmus von Plötzen empfindlicher auf künstliches Licht in der Nacht zu reagieren, als der von Barschen. Die Cortisolkonzentrationen wurden bei Barschen von keiner der nächtlichen Lichtbehandlungen beeinflusst. Lichtverschmutzung stellt demnach keinen messbaren Stressor dar.
Die Genexpressionsraten der Gonadotropine wurden nur beim Barsch im Lichtintensitäts-Experiment unterdrückt. Dieses Experiment wurde mit einer Photoperiode durchgeführt, die die natürlichen Lichtbedingungen im Oktober simuliert. Künstliches Licht in der Nacht im simulierten September und Dezember hatte keinen Einfluss auf die getesteten Reproduktionsparameter. Diese Ergebnisse legen die Existenz einer photolabilen Phase nahe, in der die Reproduktion von Fischen besonders sensitiv auf Licht in der Nacht reagiert. Laborexperimente bilden die natürliche Umgebung jedoch meist nicht ausreichend ab. Um die oben genannten Effekte auf das Licht zurückzuführen, wurde zum Beispiel die Temperatur in diesen Experimenten konstant gehalten. Die Temperatur ist jedoch ebenfalls ein wichtiger Parameter für die zeitliche Regulierung von Fortpflanzungsereignissen.
Aus diesem Grund wurden weitere Untersuchungen in Form von Freilandexperimenten durchgeführt, bei denen Barsche und Plötzen 4 Wochen lang während der Monate August und September in Netzkäfigen gehalten wurden. Ein Teil der Versuchsfische wurde dabei nachts jeweils künstlicher Straßenbeleuchtung ausgesetzt, die Kontrollgruppe hingegen unter natürlichen Verhältnissen gehalten. Die Konzentrationen von Melatonin und den Sexualsteroiden 17ß-Estradiol und 11-Ketotestosteron wurden aus dem Blut und die Genexpressionrate der Gonadotropine aus dem Hypophysengewebe der Fische bestimmt. Die Sexualsteroid-Konzentrationen und die Expression der Gonadotropine waren bei den in der Nacht künstlich beleuchteten Fischen signifikant herabgesetzt. Bei Barsch und Plötze

beginnt die Vorbereitungsphase der Reproduktion üblicherweise im August. Die Ergebnisse dieses Experiments entsprechen Studien mit anderen Fischarten, bei denen eine photolabile Phase zu Beginn der Fortpflanzungsperiode nachgewiesen wurde. Daher ist es sehr wahrscheinlich, dass Diskrepanzen zwischen Labor- und Feldexperimenten mit den unterschiedlichen Temperaturbedingungen während der Versuchszeit zusammenhängen.

Zusammenfassend ist festzustellen, dass Lichtverschmutzung sehr wahrscheinlich biologische Rhythmen von Fischen beeinflusst. Die Unterdrückung des Melatoninrhythmus kann dabei verschiedenste negative Auswirkungen haben, die bis jetzt noch nicht ausreichend untersucht sind. So könnten Auswirkungen auf Immunsystem, Wachstum und Entwicklung, aber auch auf Verhaltensweisen, nicht nur einzelne Fische, sondern ganze Populationen beeinflussen. Potentielle artspezifische Unterschiede in der Empfindlichkeit gegenüber künstlichem Licht in der Nacht und die damit einhergehenden Auswirkungen auf die Reproduktionsbiologie der Fische könnten zu Verlagerungen von biologischen Nischen führen, Räuber-Beute-Beziehungen beeinflussen und sich auf Artgemeinschaften und ganze Ökosysteme auswirken.

Introduction

Light is an essential prerequisite for the existence of flora and fauna on earth, since it determines the rhythm of life for most organisms. The natural alternation of light and dark sets the biological clock and synchronises physiological processes and behaviours with daytime and season.

Light is, for instance, highly important in reproduction to synchronise gametogenesis, courtship behaviour, but also to coordinate mating and rearing of the offspring with the provision of natural resources and thereby optimise survival chances. However, nowadays natural light regimes are altered by artificial light, especially in urban areas, where the night can become so bright that a vast majority of stars are not visible anymore (Cinzano et al. 2001, Kyba et al. 2015). The reason for this is the enormous increase in artificial light sources since the invention of electric light (Smith 2009, Hölker et al. 2010), which was always regarded as a sign of wealth and aesthetics. With increasing technical progress also the efficiency of electric light sources increased and costs were lowered. Artificial light transformed natural nightscapes and enabled manhood to shift activities to the night time.

But also for remote areas that usually lack extensive artificial lighting, urban light pollution can have considerable impacts. Airborne aerosols, dirt particles and other atmospheric molecules scatter artificial light, creating a sky glow that also affects areas outside of the city. As a result, nocturnal skies in rural areas can become brighter as during a full moon night (Kyba et al. 2011). Especially clouds affect the night sky brightness; so that a cloud covered sky can be 18 times brighter than a clear sky at the same spot, with clouds acting as amplifier.

Implications for astronomers are well known, but artificial light at night (ALAN) can also impact human health. In shift workers the natural rhythm is desynchronised. As a result they suffer from cardiovascular diseases more often and are more likely to become obese (Wyse et al. 2011, Reiter et al. 2012, Ramin et al. 2014). Moreover, the cancer risk in shift workers is significantly higher than in others (Pauley 2004, Haim and Portnov 2013).

However, ALAN has an effect on most living creatures since light regulates the biological rhythm which influences a lot of natural behaviours like foraging, courtship and mating, migration or hibernation and physiological processes like reproduction, growth, development and metabolism. When a dark phase is missing, or the duration is altered by ALAN, it is conceivable that diurnal or seasonal processes are affected. Many nocturnal mammals respond to increasing moonlight (full moon) with restricted foraging behaviour (Beier 2006), a strategy to avoid visual predators. The impact of ALAN on birds is well studied. Migratory birds are highly attracted to artificial light and can even get trapped and lose their energy reserves

(Gauthreaux Jr. and Belser 2006). A similar behaviour is known for insects that die on or around street lamps as a result of exhaustion, burning or predation (Eisenbeis 2006). Also growth and flowering of plants is dependent on light and hence can be influenced by light pollution (Briggs 2006, Bennie et al. 2015).

Light in aquatic ecosystems

The consequences of ALAN are not only relevant for terrestrial habitats; also aquatic ecosystems can be strongly influenced by light pollution, as humans settle in the proximity of water ever since. To date, approximately 50% of the world's population lives within 3 km distance to freshwater and 90% of the population lives not further than 10 km away (Kummu et al. 2011). The rationale is that access to freshwater is essential for life, but water bodies are also used for transport, e.g. of trading goods, and serve as locations for recreation and tourism. Therefore it is obvious that ALAN plays a huge role, especially on the shores of streams, rivers, channels or lakes, but also on the coast of oceans and seas. Consequently this can lead to a great light pressure in the vicinity of water bodies. Nocturnal light levels ranging from 4 to 17 lx were already found at the surface of waters (Meyer and Sullivan 2013, Perkin et al. 2014a, Hölker et al. 2015). Bearing in mind that the brightest natural light at night, the full moon, has an intensity of only 0.3 lx maximum, one realises that urban waters are often many times brighter than under natural conditions.

The majority of aquatic organisms possess internal genetically encoded clocks. With these clocks physiological processes and behaviours are adjusted to environmental conditions throughout the day. However, the internal rhythm covers only approximately one day and different cells and systems may have their own internal rhythms that are synchronized with each other and the environment via master clocks. The most important mechanism to set the clock is the daily alternation of day and night which reliably controls e.g. the sleep-wake cycle, the hormonal balance, but also active patterns like foraging or vertical migration. Also seasonal patterns are synchronised since the day length is a clear indicator of the time of the year. Thus, seasonal processes like reproduction can be optimally controlled. In aquatic ecosystems many species use different habitats throughout the course of the year to have e.g. optimal food resources and reproduction conditions. ALAN can interfere with all these rhythmical, light controlled processes by diminishing the distinction between day and night and thus influence physiological processes and behavioural aspects. Hence, ALAN has to be regarded as a serious threat for aquatic ecosystems, especially since numerous organisms that lives in aquatic habitats are considered to be endangered and are undoubtedly highly susceptible to ALAN.

Physical basics of light in water

To fathom the possible threat of ALAN on aquatic organisms, physical properties of light in water have to be considered. Light behaves differently in aqueous media than it does in air. Water has a different refractive index than air and the absorption of light in water is much higher. Different wavelengths penetrate into the water to different depths. Shorter wavelengths are higher in energy than longer wavelengths and thus reach deeper into the water column. Within the visible spectrum red is least energetic and is absorbed first, after about 3 m, followed by orange (10 m), yellow (30 m) and green light (40 m). Blue and violet can travel deepest, up to 150 m. However, these data are only valid for distilled water. Natural waters, e.g. lakes contain a number of different characteristic organic particles and other suspended substances. Light is scattered by these particles and thus the water acts as a filter. The more particles are suspended in water the less deeply light can penetrate or the higher is the absorption. Oceans and seas with little suspended matter and also very clear lakes often appear blue, since shorter wavelengths are absorbed last. Depending on the properties of suspended particles longer wavelengths may be reflected and shorter wavelengths are absorbed. The colour of most lakes originates from dissolved organic matter and its selective absorption of shorter wavelengths of the visible spectrum. As a result most lakes appear to be greenish (algae). With increasing concentration of e.g. humic substances emitted scattered light dominates more in the yellow and red part of the spectrum. Therefore, every water body has its characteristic colour (Wetzel 2001).

Implication of ALAN on aquatic ecosystems

Light pollution modifies the intensity, the spectrum, the frequency and the duration of nocturnal light that impinges on the water surface and enters the water. Consequently, biological rhythms of aquatic organisms can be disturbed by light at unnatural times, with unnatural spectral composition and elevated intensity. Processes that are normally synchronised can become imbalanced. The resulting disturbance of the physiology of affectedorganisms, the interaction among organisms and between organisms and their environment can cause severe alterations in the aquatic ecosystem and can make it more susceptible for other stressors like water pollution or temperature changes. Especially for nocturnal aquatic species this can become a problem. Similar to terrestrial ecosystems, the activity periods of diurnal or crepuscular predators can be shifted into the night if the water is brighter than normal, which can result in a distortion of food webs (Becker et al. 2013). Stream invertebrates like insect larvae (e.g. mayfly nymphs, blackfly larvae, chironomid

larvae, caddisfly larvae) are known to alter their drift behaviour in response to ALAN (Moore et al. 2006, Perkin et al. 2014b). As a predator avoiding strategy invertebrates usually drift at night to reach new feeding grounds. If the drift is interrupted, food competition is higher, potentially resulting in reduced growth rates of the invertebrates and consequently of their predators, fish. A very good example is also the diel vertical migration of zooplankton like *Daphnia* that feed on phytoplankton. Under natural conditions they spend the day in deeper water layers to avoid diurnal predators. In the night they migrate towards the water surface to feed. Artificial light can alter this migration in amplitude and amount of migrating individuals (Moore et al. 2001, Moore et al. 2006). Accordingly, a reduction of the vertical migration can implicate a dramatic increase of algae biomass that is possibly enhanced by the higher reproduction rate of algae caused by artificial light. As a result, the water quality may severely decrease. Some fish also perform a vertical migration; they follow their zooplankton prey to forage under the cover of darkness. However, in light polluted waters the predation risk by diurnal predators is higher for nocturnal fish leading to an unnatural top-down regulation (Becker et al. 2013).

ALAN is further known to affect several other behavioural patterns in fish. A prominent example is the spawning migration of fish (e.g. eel *Anguilla anguilla* or Atlantic salmon *Salmo salar*). In fall, eel migrate via streams and rivers back into the Sargasso Sea to mate and spawn. As a predator protection strategy, this migration predominantly takes place at night. Already low light intensities are sufficient to disturb this migration (Lowe 1952, Vøllestad et al. 1986, Hadderingh et al. 1999, Cullen and McCarthy 2000), as eels interrupt their movement even at clear full moon nights to seek shelter. Atlantic salmon also perform migrations, e.g. the seaward migration of young smolts, leaving their rearing tributaries to reach the feeding grounds in the ocean. As in eel, this migration takes place mostly during night (McCormick et al. 1998) and can be interrupted by ALAN (Hansen and Jonsson 1985, Greenstreet 1992).

The above mentioned examples stress the impact ALAN can have on migratory fish species. Apart from other obstacles like hydropower plants, lights along the bank or extensively illuminated bridges can represent a strong migration barrier for fish, making the migration more time and energy consuming.

Moreover, also physiological rhythms are often light dependent, since they are based on the circadian system.

The relevance of light for biological rhythms in fish

The circadian axis in fish

In all vertebrates the circadian axis is based on circadian pacemaker mechanisms. It translates light signals perceived by photoreceptors into neuroendocrine signals that transport this information to target tissues and elicit a physiological response.
In humans visual photoreceptors in the eye send signals to the suprachiasmatic nucleus, which in turn controls the chemical output by the pineal gland (Dibner et al. 2010).

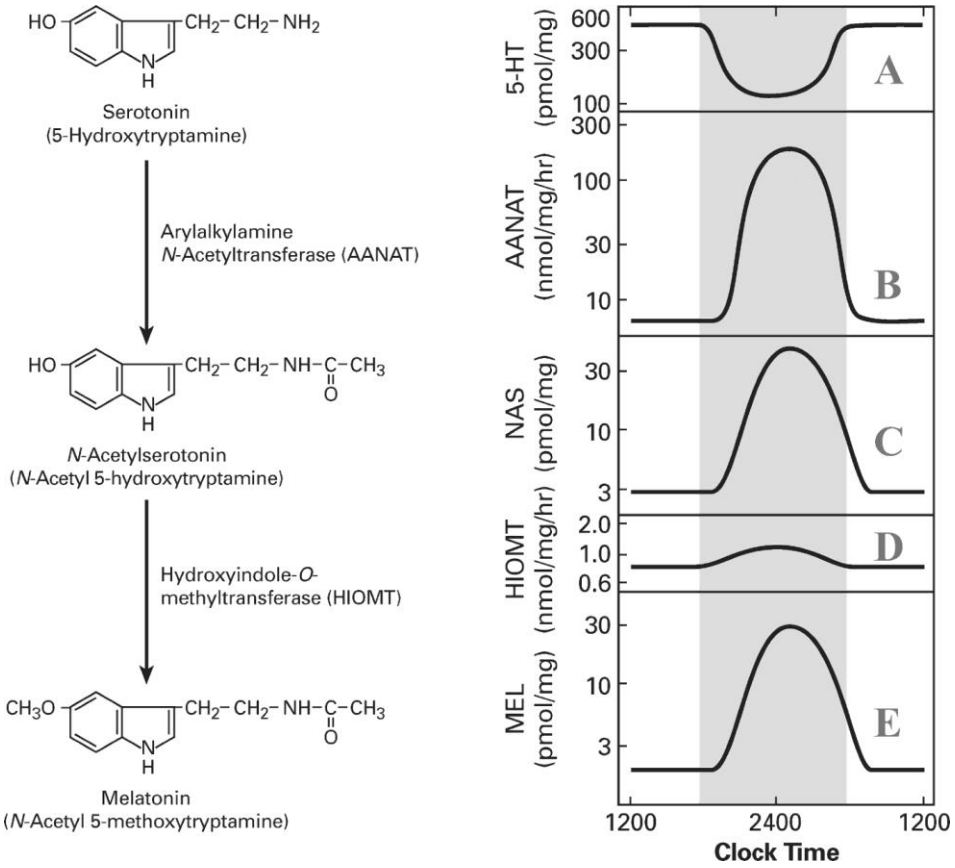

Figure 0.1 Daily changes in the conversion of serotonin to melatonin in the pineal gland. (modified from Klein 2006).

Teleosts, however, possess a pineal organ that is light sensitive itself (Bolliet et al. 1996, Migaud et al. 2006) and is therefore able to directly transduce light signals that reach the pineal through a translucent window in the skull, into hormonal signals. Pineal photoreceptors resemble retinal cone cells, structurally and functionally. They possess the machinery for the phototransduction cascade (chromophores, opsins etc.)

and furthermore they have the ability to rhythmically convert serotonin to melatonin through the action of the enzyme arylalkylamine N-acetyltransferase (AANAT) (Fig. 0.1).

In fish both, the retina and the pineal organ possess circadian oscillators that integrate photoperiodic information and translate it into hormonal signals, but mostly only pineal melatonin is secreted into the blood and cerebrospinal fluid and can therefore act as messenger for other parts of the organism. Retinal melatonin is considered to act mostly autocrine and paracrine (Falcón et al. 2011).

In some fish retinal melatonin output shows, contrary to the pineal output, a reversed rhythm with high concentrations during daytime and low concentrations in the night. Retinal melatonin is probably involved in adaption of the retina or acting as local antioxidant (Migaud et al. 2007b). This study indicates the existence of 3 different circadian systems in fish. In salmonids and goldfish, circadian melatonin rhythm seems to be controlled exclusively by the pineal organ. In cod and sea bass both, pineal and retinal inputs appear to contribute to the circadian melatonin rhythm. For Nile tilapia and African catfish (*Clarius gariepinus*) a third circadian system is suggested, where the pineal is not or only little light sensitive. This was not finally proven but the authors state that in the latter four species retinal and/or deep brain photoreception may contribute. However, Bolliet et al. (1996) could show rhythmical melatonin secretion from isolated pineals of different families of fish, including catfish, cyprinids and percids.

In contrast to mammals that possess only ocular photoreceptors (Peirson et al. 2009) the circadian rhythm of fish may be controlled by multiple tissues and receptors beside retinal and pineal photoreceptors. Deep brain photoreceptors – photo-responsive molecules in the brain for example may also respond to photic stimuli (Kojima et al. 2000, Philp et al. 2000, Fernandes et al. 2012). The *saccus vasculosus* on the other hand may be an additional sensor for seasonal information and modulates the thyroid system in some fish species (Tsuneki 1992, Nakane et al. 2013). Non-image forming photoreceptors such as melanopsin in the retinal ganglion cells and horizontal and amacrine cells that can possess vertebrate ancient opsin may also signal environmental irradiance (Kojima et al. 2000, Philp et al. 2000, Peirson et al. 2009).

However, for most fish it seems to be the pineal organ that receives light signals and translate it to melatonin signals in a rhythmical manner. The output is high at night and low during the day with intermediate levels during twilight. Also the season modulates this rhythm, namely the amplitude of the fluctuation (Fig. 0.2).

Under short day photoperiods the amplitude is usually flat and wide, under long day photoperiods it is narrow and high (Masuda et al. 2003, Falcón et al. 2011). Hence the melatonin rhythm reflects the current photoperiod and provides the fish with information about daily and calendar time.

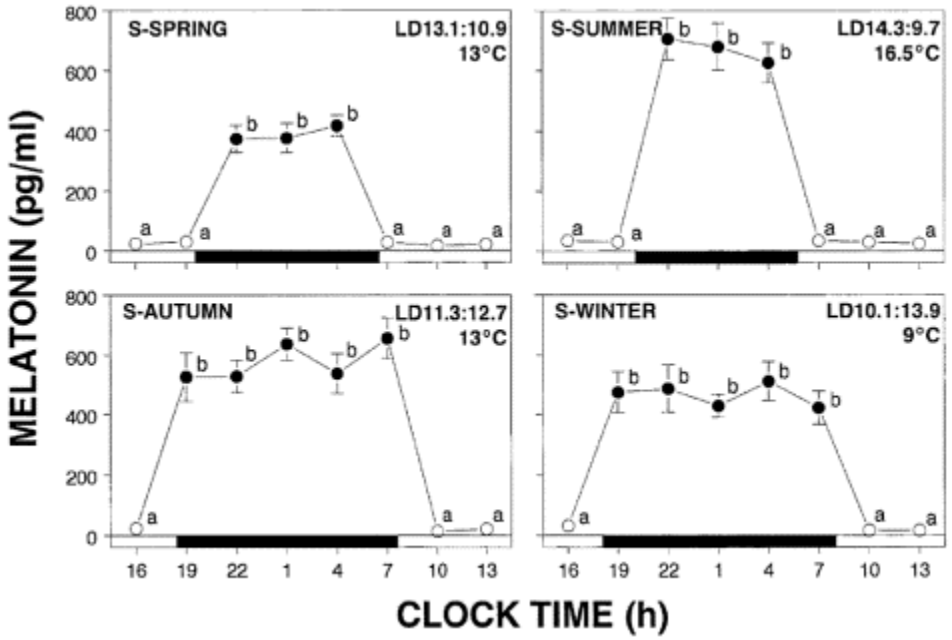

Figure 0.2 Seasonal differences of the daily variations in plasma melatonin levels of the rainbow trout (*Oncorhynchus mykiss*). Solid and open bars along the X-axis represent the dark and light phases, respectively (from Masuda et al. (2003)).

Cortisol rhythms in fish

Most environmental stressors activate the hypothalamus-pituitary-interrenal axis of fish. The resulting elevation of plasma cortisol is the best established indicator for stress in fish. The cortisol response is likely linked to its energy mobilising and osmoregulatory properties.

The control of corticosteroid secretion involves corticotropin-releasing hormone (CRH) secreted by the hypothalamus and adrenocorticotropic hormone (ACTH) secreted by the pituitary gland as the most important substances that stimulate cortisol production as the main adrenocortical end product of the axis (Wendelaar Bonga 1997). Cortisol in turn has negative feedback on hypothalamus and pituitary. The corticosteroid production in teleost fish is located in the interrenal cells, scattered clumps of cells distributed around the venous vessels within the lymphoid head kidney (Norris and Hobbs 2006).

Main actions of cortisol in fish are related to the regulation of hydromineral balance (e.g. seawater adaption in migratory fish) and energy metabolism.

Plasma cortisol levels typically start to rise within minutes as an answer to stressors and decrease a few hours later, although chronic stress may cause constantly elevated concentrations. In most fish species, blood levels of cortisol also exhibit daily and

seasonal rhythms, but are also influenced by other external cues. In goldfish for instance, the schedule is entrained by the photoperiod. Cortisol concentrations peak around light onset, minimum concentrations occur at light offset (Noeske and Spieler 1983). This so called cortisol awakening response was also found in Nile tilapia (Binuramesh and Michael 2011). Seasonal cortisol rhythms exist as well, e.g. in migratory salmonids, where smoltification in spring is associated with elevated plasma cortisol levels, most likely a preparation for the osmoregulatory challenges of entering sea water (Young et al. 1989). However, cortisol elevations have also been found as response to feeding (Pickering and Pottinger 1983, Laidley and Leatherland 1988). However, until now several studies could not find an impact of ALAN on cortisol rhythms (Biswas et al. 2006, Biswas et al. 2008, Honryo et al. 2013). But these studies used invasive sampling methods that may be stressful *per se*, whereas non-invasive methods could reveal possible effects.

The hypothalamus-pituitary-gonad (HPG) axis in fish

Since the melatonin rhythm provides information about the season, obviously seasonal rhythms are also light dependent. In fish species of temperate waters reproduction is a highly seasonal process that takes place mostly during one distinct period in the year. Photoperiod and temperature are the most important cues that entrain and synchronize the reproductive process (Bromage et al. 2001). However, photoperiod represents the most reliable, precise and noise-free signal to entrain reproductive processes. Because reproduction is a very energy and resource consuming process, gonadal recruitment and development have to be timed precisely. The precision of these cues also ensures that offspring experience the most beneficial conditions for their survival. The seasonal changing photoperiod, together with the occurrence of optimal temperatures, is the cue that triggers the initiation and progression of gonadal development (Pankhurst and Porter 2003).

Environmental and social information is transmitted to several centres in the fish brain and integrated in the hypothalamus. Hypothalamic neurosecretory cells send projections to the pituitary and regulate the production and secretion of the gonadotropin releasing hormone (GnRH). GnRHs act on pituitary cells (gonadotrophs) to regulate synthesis of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH and FSH are released into the circulation and bind to gonadotropin receptors in the gonad membrane (Zohar et al. 2010).

Gonadotropins play central roles in regulating gametogenesis and production of gonadal hormones (sex steroids) required for the development of sexual behaviour and secondary sex characteristics (Swanson et al. 2003).

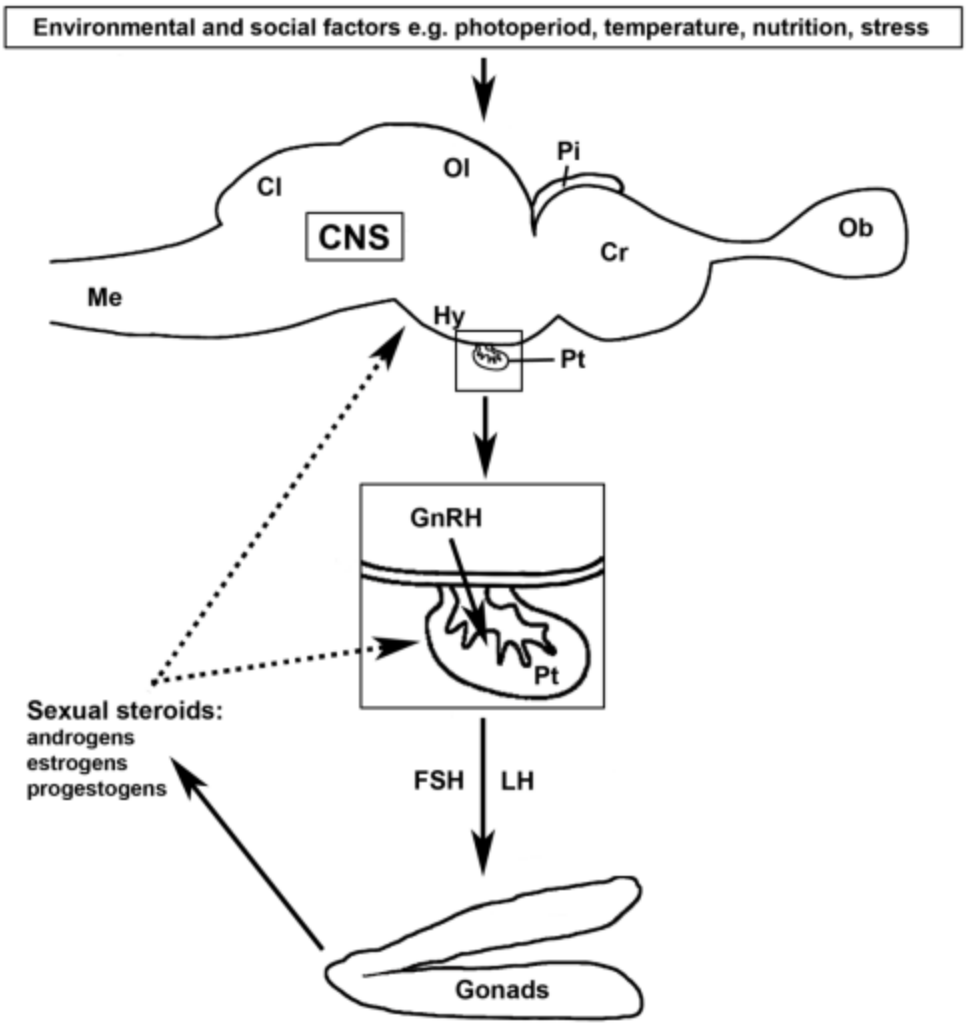

Figure 0.3 Schematic figure of the HPG-axis in fish. Environmental and social factors stimulate the hypothalamus to secrete gonadotropin releasing hormone that acts on the pituitary to regulate production of gonadotropins. In response to gonadotropins (follicle stimulating hormone (FSH), luteinizing hormone (LH)) the gonads synthesize and secrete sex steroids. Sex steroids in turn cause feedback on pituitary and hypothalamus. **Cl**: cerebellum; **CNS**: central nervous system; **Cr**: cerebrum; **FSH**: follicle stimulating hormone; **GnRH**: gonadotropin releasing hormone; **Hy**: hypothalamus; **LH**: luteinizing hormone; **Me**: medulla; **Ob**: olfactory bulb; **Ol**: optic lobes; **Pi**: pineal organ; **Pt**: pituitary

11-ketotesosterone (11-KT), 17β-estradiol (E2) and 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) are the predominant sexual steroids produced by the gonads of fish. Sex steroids act on target cells and cause feedback (positive and negative) on hypothalamus and pituitary for regulating homeostasis (Kloas et al. 2009, Zohar et al. 2010).

Impact of ALAN on fish

The influence of ALAN on the circadian system has been proven for a variety of fish species. In European sea bass *Dicentrarchus labrax* (Bayarri et al. 2002), Atlantic salmon *Salmo salar* of different developmental stages (Porter et al. 1999, Porter et al. 2001, Migaud et al. 2007a), rainbow trout *Salmo gairdneri* (Max and Menaker 1992), brook trout *Salvelinus fontinalis* (Zachmann et al. 1992), Senegal sole *Solea senegalensis* (Oliveira et al. 2007), tench *Tinca tinca* (Vera et al. 2005), to name only a few, nocturnal artificial light of varying intensities significantly decreased plasma melatonin concentrations.

Melatonin is suggested to act in several physiological processes in fish, mainly by indicating the prevailing photoperiod. Melatonin for example appears to directly or indirectly regulate the immune system of fish (Leonardi and Klempau 2003, Cuesta et al. 2007, Cuesta et al. 2008, Esteban et al. 2013, Ren et al. 2015). A suppression of melatonin by ALAN might therefore have consequences for the health and well-being of fish, e.g. increase the susceptibility to diseases and parasites or to other stressors like water pollutants. Melatonin plays a role in daily locomotor activity patterns. Melatonin administration e.g. decreased locomotor activity and food intake in diurnal fish, probably by simulating night-time melatonin levels (Lopez-Olmeda et al. 2006). Consequently, ALAN and thus melatonin suppression might cause an extension of locomotor activity in diurnal fish into the night, affecting nocturnal fish species (e.g. increased predation, increased food competition).

Seasonal rhythms depend on photoperiod as reliable indicators of time of the year as well and are therefore prone to light induced alterations. The aquaculture industry is taking advantage of this fact. The reproductive period of most aquacultural important temperate fish species is restricted to a few months of the year, which is a problem for fish farmers who have to satisfy an all-year-round demand for a regular supply of fish. Another problem for commercial fisheries is early maturation, leading to poorer growth and decreased quality. Consequently mechanisms have been developed to solve these problems. Based on the knowledge of photoperiodical entrainment of reproductive events, manipulated photoperiods are used to control reproduction. Prolonged or compressed photoperiods can be used to direct spawning events, allowing the production of fish even off-season (Macquarrie et al. 1979, Bromage et al. 1982, Kolkovski and Dabrowski 1998, Bromage et al. 2001). Also sexual maturation can be controlled by photoperiod manipulations (Imsland et al. 2003, Begtashi et al. 2004, Rodríguez et al. 2005, Taranger et al. 2006, Felip et al. 2008), which is mostly done to prevent precocity and improve growth and thereby flesh quality. Mainly by continuous photoperiod reproduction can even be fully inhibited, allowing the fish to invest resources in growth rather than in gonado- and

gametogenesis. This is seen for example in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) reproduction (Davie et al. 2007a, Davie et al. 2007b).
Melatonin is known to be able to alter several components of the hypothalamus-pituitary-gonadal (HPG) axis such as gonadotropins, sex steroids or gonadal maturation (Amano et al., 2000; Bhattacharya et al., 2007; Carnevali et al., 2011; Chattoraj et al., 2005; Khan and Thomas, 1996; Sébert et al., 2008). However, the role of melatonin in reproduction is not yet fully clarified and results appear contradictory and species specific (Falcón et al. 2007, Maitra et al. 2013). Nevertheless it is doubtless and generally accepted that it is light, i.e. the photoperiod, which entrains and synchronizes the reproductive process in most temperate fish species (Bromage et al. 2001) and that seasonal variations in the photoperiod play a crucial role in timing of reproduction (Migaud et al. 2004, Falcón et al. 2010).
Therefore ALAN most likely to interferes with daily, but also with seasonal rhythms of fish. For some species the time around the onset of gonadogenesis was found to be a photo-labile phase, were ALAN, i.e. continuous photoperiod, is able to influence reproductive parameters, e.g. in European sea bass (Felip et al. 2008, Rodríguez et al. 2012) and Atlantic cod (Davie et al. 2007b). However, studies about the possible ecological effect of environmentally relevant intensities of ALAN on reproduction of non-commercial fish species are missing to date.

Experimental animals

To investigate the impact of light pollution on fish, two of the most abundant fish species in European freshwater systems were chosen. European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) inhabit a wide range of habitats, including most types of lakes and streams (Kottelat and Freyhof, 2007). In both species, onset of gonadogenesis occurs in late summer and spawning takes place during April and May (Treasurer 1988, Wang and Eckmann 1994, Sulistyo et al. 1998, Sulistyo et al. 2000, Trubiroha et al. 2012). However perch and roach have diverging life history traits. European perch are diurnal and crepuscular consumers that feed on zooplankton when young, but undergo an ontogenetic niche shift and feed on benthic organisms and fish when older (Persson 1986). Roach are diurnal and crepuscular as well but nocturnal feeding was observed as well (Weatherley 1987, Okun and Mehner 2005). However, they are also found to show antipredator behaviour towards nocturnal predators by reduced activity and habitat shifts (Hölker et al. 2007). Both species therefore represent different lifestyles. In both species the effect of ALAN on daily and seasonal rhythms is not studied so far and sensitivities and spectral action curves for nocturnal melatonin suppression do not exist yet.

Objectives and aims of this study

Despite of general knowledge of the circadian axis of fish and of photoperiod manipulations and its usages in aquaculture, little has been elaborated about the influence of ALAN in nature. Light pollution is a phenomenon that is not restricted to urban areas. Also in periurban and rural habitats the night is more and more influenced by artificial light. ALAN might be beneficial in aquaculture of commercial fish species but might be deleterious for fish in nature. Thus it is essential to enhance the understanding of the impacts of ALAN on the physiology of fish to determine possible consequences for fish communities and implications for whole ecosystems.
The aims and objectives of this thesis are listed below.

1. The first aim is to establish an experimental setup that effectively resembles natural light conditions. Preliminary tests should reveal biological action of light on the melatonin rhythm of European perch. Together with mathematical modelling approaches, test conditions ought to be found that enable the measurement of melatonin from samples of the holding water.
2. In a first approach the effects of different intensities of white ALAN on melatonin and cortisol time course patterns should be determined in perch. ALAN is expected to alter the rhythms of melatonin and cortisol. The goal was to find the effective threshold of light intensity, above which an alteration occurs.
3. To determine the spectral sensitivity of the melatonin rhythm in perch, the effects of different low intensity light colours are examined. Longer wavelengths are expected to have a greater effect on melatonin suppression. Additionally, the impacts of different intensities of white ALAN and different colours of ALAN on gene expression of gonadotropins were investigated. The hypothesis is that ALAN can impair reproduction of perch by impairing the gene expression of LH and FSH.
4. In a second approach, the experiments performed in perch are applied to another species with a different life history and biology, the roach. It is expected that ALAN impacts melatonin rhythm and reproduction of roach as well but potentially with different sensitivities.
5. The third objective is to examine the impact of ALAN on perch and roach in a realistic scenario in a naturally dark environment. As in the laboratory experiments, the aim is to identify impacts of ALAN on melatonin levels and reproductive traits under realistic environmental influences in order to get a proof of principle for wildlife.

List of Publications

This thesis was part of the interdisciplinary research Project “Verlust der NACHT” (http://www.verlustdernacht.de), funded by the Federal Ministry of Education and Research, Germany (grant number BMBF-033L038A).
The thesis is structured according to five research papers (1-5), covering the corresponding above mentioned working hypotheses. Each of the papers forms one chapter containing introduction, methods, results, discussion and references. The papers are either reprinted with permission of the publisher (Paper 1 - 3) or under review in a peer-reviewed journal (Paper 4 and 5).
To yield a consistent layout, the text has been formatted and figures and tables were renumbered continuously. The papers are followed by a general discussion, connecting and comparing the findings of the single papers and evaluating them in a broader context

1 Franke, S., A. Brüning, F. Hölker and W. Kloas (2013). "Study of biological action of light on fish." Journal of Light & Visual Environment **37**(4).

2 Brüning, A., F. Hölker, S. Franke, T. Preuer and W. Kloas (2015). "Spotlight on fish: Light pollution affects circadian rhythms of European perch but does not cause stress." Science of The Total Environment **511**(0): 516-522.

3 Brüning, A., F. Hölker, S. Franke, W. Kleiner and W. Kloas (2016). "Impact of different colours of artificial light at night on melatonin rhythm and gene expression of gonadotropins in European perch." Science of The Total Environment **543**, Part A: 214-222.

4 Brüning, A., F. Hölker, S. Franke, W. Kleiner and W. Kloas (submitted). "Influence of light intensity and spectral composition of artificial light at night on melatonin rhythm and expression of gonadotropins in roach Rutilus rutilus."

5 Brüning, A., F. Hölker, T. Preuer and W. Kloas (submitted). "Influence of artificially induced light pollution on the hormone system of perch and roach in a rural habitat."

Additional publications that are not part of this thesis are listed below.

- Brüning, A., F. Hölker and C. Wolter (2011). "Artificial light at night: implications for early life stages development in four temperate freshwater fish species." Aquatic Sciences 73(1): 143-152.
- Holzhauer, S. I., S. Franke, C. Kyba, A. Manfrin, R. Klenke, C. C. Voigt, D. Lewanzik, M. Oehlert, M. T. Monaghan, S. Schneider, S. Heller, H. U. Kuechly, A. Brüning, A.-C. Honnen and F. Hölker (2015). "Out of the Dark: Establishing a Large-Scale Field Experiment to Assess the Effects of Artificial Light at Night on Species and Food Webs." Sustainability 7(11): 15593-15616.
- Brüning,A. and F. Hölker (2013). "Lichtverschmutzung und die Folgen für Fische." *BfN-Skripten*, 336: 69-72.
- Brüning, A. and F. Hölker (2015)."Künstliches Licht an Gewässern – Auswirkungen und Lösungsansätze.", Wildtier Schweiz, FaunaFocus Kunstlicht & Gewässer 24: 1-12.

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Chapter 1

Study of Biological Action of Light on Fish

Study of Biological Action of Light on Fish

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Journal of Light & Visual Environment.
http://doi.org/10.2150/jlve.IEIJ130000518

Abstract

Nocturnal outdoor illumination is mainly designed aiming at human needs. However, side effects on other organisms and ecosystems have to be expected, for example for fish populations. Basic knowledge about biological action of light on fish could be gained from laboratory experiments. This work presents an experimental strategy to study the biological action of light on fish. It is found that the melatonin expression of European perch (*Perca fluviatilis*) is significantly suppressed at an illuminance level of 100 lx throughout the night. The experimental approach is described in detail including biological methods, experimental illumination design and mathematical modeling for reconstruction of melatonin release rates from water melatonin concentrations.

Keywords: biological action of light, fish, light pollution, street lighting, melatonin suppression, European perch

1 Introduction

Nocturnal outdoor illumination is mainly aimed at human needs so far, where illumination of public areas has primarily to ensure a reliable recognition of objects. In order to avoid unacceptable light levels there are already guidelines to limit illuminance levels in the planning of outdoor scenes. However, they are focused mainly on security issues and the recognition of humans. Less attention is paid on the biological action of artificial light on ecosystems. Over the last decade, a novel non-image forming photoreceptor system in the vertebrate retina has been discovered and characterized. Intrinsically photosensitive retinal ganglion cells were found to play a key role for the human circadian clock and melatonin expression, where the action curve of nocturnal melatonin expression exhibits a maximum in the blue spectral range. Hence, the linear dose response relation according to Gall and Lapuente (2002) introduced a metric allowing characterization of light sources with respect to their biological action. As a consequence spectra can be optimized not only for their visual perception but also for their action on nocturnal melatonin expression (Kozakov et al.

2008). But again, these considerations are aimed at light sources optimized for human physiology. The last years, the biological action of artificial light not only on humans but also on animals and whole ecosystems came into focus (Hölker et al. 2010, Zubidat et al. 2011). In particular outdoor illumination is a critical issue with respect to ecosystems. Street lights attract insects, where lots of them die, due to exhaustion. The concentration of insects to artificial light consequently attracts predators such as spiders. This may serve as an illustrative example that nocturnal illumination in principle is capable to impact food webs. Thus, the action of light is not isolated on selected species. Possible consequences are shifts in population densities and loss of biodiversity (Hölker et al. 2010). There are already some studies that document the collateral effects of increasing outdoor illumination and light pollution, e.g. on fish (Hadderingh et al. 1999), turtles (Bourgeois et al. 2009), birds (Gauthreaux Jr. and Belser 2006) or rodents (Zubidat et al. 2011).However, a more detailed understanding about the action of artificial light on animals and their circadian clock is still needed, although there is already a tremendous knowledge about biological clocks in biosciences. For instance it is known that circadian clock in fish is controlled by the pineal gland (Kulczykowska et al. 2010). The pineal organ of fish is an evagination of the forebrain and connected to the diencephalon. The upper part is located directly below or in the roof of the skull. This area is often more transparent than the rest of the skull to allow the light to penetrate the skull and reach the pineal organ. In contrast to humans and other mammals, the pineal organ of fish is directly photosensitive. Every photoreceptor cell of the pineal organ is a circadian system containing the clock, a phototransduction unit to entrain the clock (input pathway) and the apparatus to produce melatonin as hormonal output (Falcón 1999). With this circadian system the fish is able to coordinate circadian patterns like food consumption or regenerating processes and circannual patterns like reproduction, growth and development or migration with the time of the day and the year. This is where light pollution may intervene. Artificial light at night may blur the change of day to night. If this photoperiodical cue is missing, circadian and circannual processes can be impaired. Even low light intensities can block melatonin synthesis partially and a modified level of melatonin can influence the secretion of other hormones. A good example is the migratory behaviour of the silver eel (*Anguilla anguilla*). The silver eel migrates in the autumn via the rivers to his spawning area, the Sargasso Sea. This spawning migration appears almost exclusively during the night and can be interrupted by artificial light, i.e. at illuminated bridges (Lowe 1952, Hadderingh et al. 1999). For other migratory fish such as salmons light can also be a barrier resulting in a more time and energy consuming migration, which can finally influence the reproductive success (Riley et al. 2012). In aquaculture light, especially shifted photoperiods or continuous lighting is used to control reproduction or even prevent maturation (Porter et al. 1999, Davie et al. 2007). This is useful to produce eggs apart

from the spawning season or to improve growth by lengthening the feeding periods. The energy that is normally used for reproduction can now be used for faster growth (Falcón et al. 2010). But what might be beneficial in aquaculture can be deleterious in nature. Further, open questions are remaining: What is the threshold light intensity for interrupting melatonin production and thereby impairing related biological rhythms? What is the most invasive light colour? The influence of artificial light on fish is often studied but mostly in an aquaculture context, e.g. to find the optimal photoperiod for growth or to control reproduction. This study is the first approach to examine the impact of light pollution on biological rhythms in fish, because research is needed to find out how an ecosystem reacts on urban lighting and how outdoor lighting might be improved to find a compromise between the different requirements. The focus of this work is on methods to study the biological action of light on fish and brings together the expertise of lighting engineering, biology and the mathematical modeling of dynamic systems. Experimental results of nocturnal melatonin suppression in fish are presented for 100 lx nocturnal illumination with white light. In detail this work describes an experimental strategy how to find the onset of nocturnal melatonin suppression and the most effective spectral region for melatonin suppression in fish with laboratory experiments. In section 2 the study design of laboratory experiments with fish is introduced. Section 3 describes the requirements on the experimental illumination, where section 4 describes the biological methods to obtain melatonin profiles and section 5 presents basics of the mathematical modeling for reconstruction of melatonin release rates from concentration of melatonin in aquarium water. Exemplarily in section 6 results on nocturnal melatonin suppression in European perch (*Perca fluviatilis*) at a nocturnal illuminance level of 100 lx are reported and discussed.

2 Study design

Two questions are addressed with respect to the action of nocturnal outdoor illumination on fish.

(A) Melatonin suppression onset: To which extend is the melatonin synthesis of fish suppressed by different illuminance levels of white light?

(B) Most invasive light colour: Which spectral range of light is most effective with respect to its biological action on the melatonin synthesis of fish?

To answer these questions it is necessary to create a proper experimental setup. This section describes the design of animal experiments including a partial characterization of the biology of European perch (*Perca fluviatilis*) as study organism.

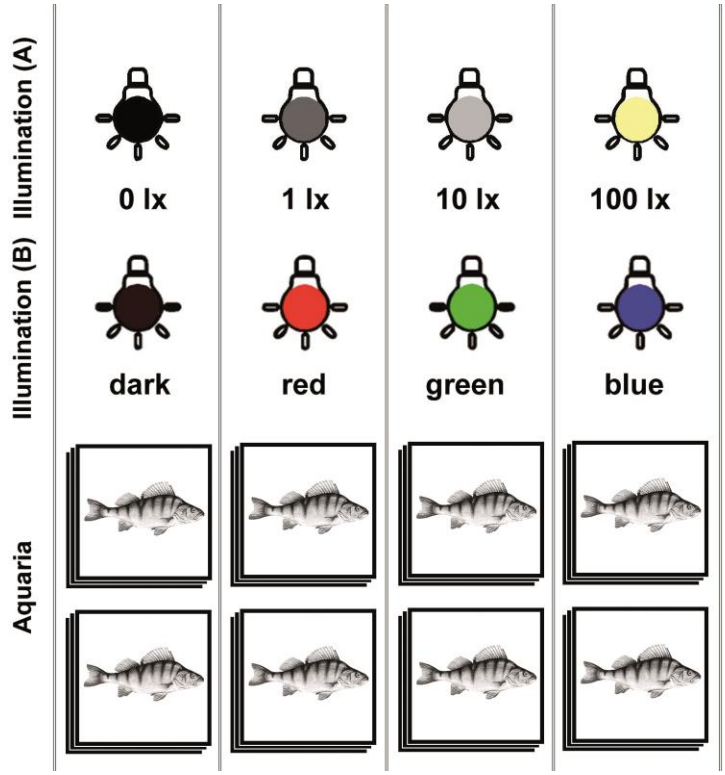

Figure 1.1 White light nocturnal illumination of 1 lx, 10 lx and 100 lx (illumination A) can be realized as well as coloured nocturnal illumination with red, green and blue light (illumination B), where each 6 of 24 aquaria are equipped with identical illumination scenarios. One group of 6 aquaria serves as reference group without nocturnal illumination.

2.1 Experiments with fish

The experimental set-up consists of 24 aquaria with a water volume of 84 l that are inserted in a climate chamber at 16°C. The aquaria are taped with black foil from all sides to make them lightproof. The experimental aquarium illumination, housed in the aquarium covers, is described in detail in section 3. Every 6th aquarium is equipped with identical nocturnal illumination scenarios. For experiments asking for melatonin suppression onset a nocturnal illuminance level of 1 lx, 10 lx and 100 lx can be applied to the aquaria, where 6 aquaria serve as reference group with 0 lx nocturnal illumination (Fig. 1.1). In order to determine the most invasive light colour

aquaria are equipped with red, green, blue and amber nocturnal illumination, where three colours can be investigated simultaneously in one experiment, because 6 aquaria have again to serve as reference group without nocturnal illumination (Fig. 1.1). Each aquarium is stocked with 30 juvenile perch of comparable age and size, which undergo three phases of the experiment.

Phase A: Acclimation
Acclimation phase takes 14 days. Perch is placed in aquaria with a flow-through of approximately 10 l per hour with aerated tap water. The photoperiod is set to 12 hours/12 hours with daylight from 7 a.m. to 7 p.m. and a dusk and dawn of 3 hours. Onset of dawn is at 7 a.m. and onset of dusk at 4 p.m. The night is kept dark in all aquaria and the fish is fed with red bloodworms twice a day.

Phase B: Treatment
It follows a treatment phase of 8 days, where the nocturnal illumination is activated. Additionally the water flow is reduced to 4.1±0.2 l/h and the fish is not fed anymore to maintain good water quality.

Phase C: Probing
The probing phase takes 24 hours. Water samples are taken with Tygon tubing (Idex Health and Sciences) that reached from every aquarium to a 24-channel peristaltic pump (BVP, Idex Health and Sciences) outside of the climate-chamber. In this way, water samples can be taken without disturbing the fish, even without entering the climate chamber. Water was pumped via 1–2 μm fiberglass-syringe filters (Roth) to remove particular substances. 1000 ml of water is collected from each tank at each sampling point into 1 l-glass bottles. Sampling times are 11 a.m., 2 p.m., 5 p.m., 8 p.m., 11 p.m., 2 a.m., 5 a.m., 8 a.m. and 11 a.m. Water samples are stored dark at 4°C until processed.

2.2 Biology of the study organism, European perch

The European perch (*Perca fluviatilis*) is a predatory species and distributed throughout Europe and Asia. Perch inhabits a wide range of habitats, including all types of lakes and most streams. They prefer still or slow flowing waters and areas that provide shelters like submerged dead wood or vegetation. Perch larvae live in open water and feed on small zooplankton and return to the shore during the first summer to feed on benthic prey like insect larvae. Adults are opportunistic, highly diurnal feeders, using all available prey, from benthic invertebrates to smaller fishes. Perch are preying mainly at sunrise and sunset, but also during the day (Kottelat and

Freyhof 2007). In the presence of nocturnal predators they become completely inactive during night (Hölker et al. 2007). Perches are dichromatic, i.e. their retina is, in addition to rods, composed of two kinds of cone-like photoreceptors. The maximum sensitivity of these cones is around 540 nm and 650 nm, thus in the green and red part of the spectrum (Cameron 1982). This may partly be explained by their way of life. Perch are found in deeper regions of the water, whereas roach, a tetrachromate (cones with four sensitivity maxima) live in shallower regions (Loew and Lythgoe 1985). The pineal epithelium consists of three main cell types: photoreceptor cells, neurons and glial cells. The photoreceptor cells are structurally analog to the retinal cones. There is only scarce information about the visual pigments in the pineal photoreceptors. In some fish species the photopigments seem to be identical to the pigments of the retina (i.e. goldfish (Peirson and Bowmaker 1999)), in other species they are spectrally different to the retinal pigments. However, it is assumed, that the photoreceptors of the teleost pineal have at least two or more pigments (Ekström and Meissl 1997) but the nature of these pigments seems to be species specific (Herrera-Pérez et al. 2011). The phototransduction from photons to the nervous message is very similar to that of the retina, with some differences in latency and recovery (Meissl and Ekström 1988). The pineal photoreceptors cannot distinguish between rapid light changes but are ideal in connecting the gradually changing light of the environment to the daily and seasonal organization of fish physiology and behaviour (Ekström and Meissl 1997). Because melatonin is produced in the photoreceptor cells, the rhythmical output is directly regulated by the light-dark cycle. Melatonin production is suppressed by light, thus is high at night and low at daytime, always reflecting the current photoperiod. The European perch is chosen because it is one of the most abundant species in European freshwaters (Kottelat and Freyhof 2007). They are known to be quite tolerant towards changing environmental conditions. Therefore, if light pollution impacts on perch physiology, it is most likely, that more sensitive species will encounter serious problems.

3 Experimental illumination

As detailed in the previous section two lighting scenarios are required, one with white light throughout the night to find an onset of melatonin suppression and one with coloured light to determine spectral sensitivity of melatonin suppression. The requirements on the simulated daylight can be summarized as follows: white light with illuminance levels comparable to daylight including simulation of sunrise and sunset. These requirements have to be discussed in detail, because experimental illumination for fish differs significantly from typical experiments designed to investigate biological action of light on humans.

3.1 White light

First of all one has to recognize that obviously visual perception of light may differ for fish compared to humans. This is illustrated by the fact that perch owns a dichromatic vision (Cameron 1982) whereas as a cyprinid such as roach (*Rutilus rutilus*) is a tetrachromate (Downing et al. 1986), to give two examples. Hence white light with photometric measures like the general colour rendering index $Ra > 80$ and the CIE 1960 UCS colour difference $DC = 0$ may not be perceived as white light by fish. Furthermore no spectral action curves for nocturnal melatonin suppression exist for fish species of interest. To avoid any effects due to an unbalanced spectral distribution it is preferable to present almost continuous emission to fish. Although high intensity discharge lamps are capable providing superior spectral white light characteristics, their applicability is limited because the study design requires a number of single aquaria with dimming characteristics. LEDs would be of interest, if white light and colour light could be accomplished by the same light source at a sufficient illuminance level. But this technical approach was found to be too expensive for this kind of studies. Moreover, realization of a continuous spectrum and the targeted illuminance levels would be questionable. Consequently, fluorescent lamps are preferred because they can provide a spatial homogenous illumination at different colour temperatures and they allow easy dimming. Typical colour temperatures of natural daylight are around 6000 K, providing almost equal spectral irradiance in the visible spectral range (Fig. 1.2). All spectra in this work are obtained with fiber optic spectrometers (Avantes AvaSpec-2048-USB2) supplied with cosine corrected entrance optics at a spectral resolution of around 1.4 nm. Calibration in units of spectral radiance is accomplished by calibrated incandescent lamp standards (Avantes AvaLight-DH-S-BAL or Gigahertz-Optik FEL 100 W). The daylight spectrum in Figure 1.2 was acquired at 14.12° E and 52.25° N on October 20th, ck UTC. Geometrical constraints are given by the aquaria to be illuminated, which are 800 mm×350 mm×400 mm in length, width and height, respectively. Finally, simulated daylight is realized by fluorescent lamps with best available continuous spectrum at a colour temperature around 6,000 K (OSRAM Biolux T8/18W). As Figure 1.2 reveals, there are still spectral line peaks in the spectrum of fluorescent lamps, but these spectral lines cannot be resolved by fish eye due to the broad spectral sensitivity of fish cones (Cameron 1982, Downing et al. 1986), comparable to those found in the human eye. Furthermore it is obvious that in contrast to the daylight spectrum the fluorescent lamp does not provide significant radiative power below 400 nm and above 700 nm. This might be potentially critical for some cyprinids such as roach that owns a cone with maximum spectral sensitivity in the near UV (Downing et al. 1986), but it is expected that UV and NIR will not affect the circadian rhythm significantly (Kulczykowska et al. 2010). It can be summarized that the spectrum of

the fluorescent lamp is considered to be an appropriate fit to natural daylight spectrum.

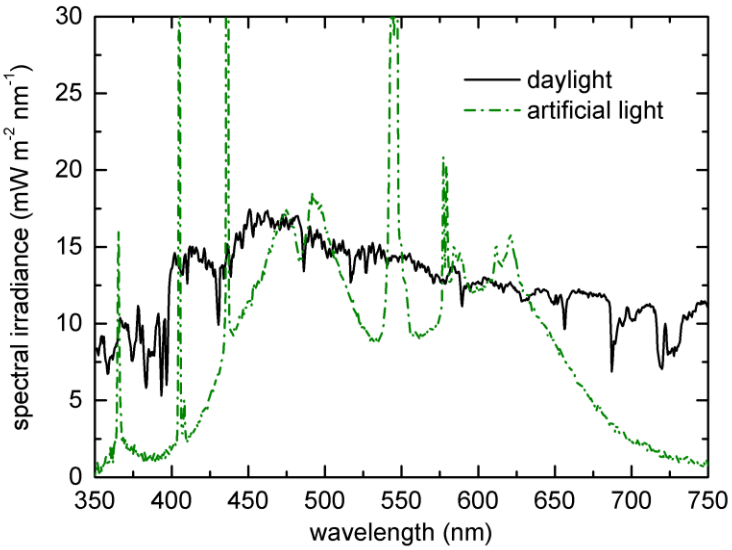

Figure 1.2 Comparison of artificial light applied to aquaria (dash-dotted line, CCT=6100 K) with natural daylight normalized to 1000 lx (solid line, CCT=6500 K)

3.2 Illuminance levels

Illuminance levels of natural daylight typically go up to 200,000 lx, depending on geographical region and weather conditions. Even at cloudy days 6,000 lx can be achieved at 52° northern latitude (Berlin) throughout the year. Illuminance values, presented in Figure 3, are obtained from spectral irradiance and from luxmeters (International Light Technologies ILT1700), respectively. As Fig. 1.3 reveals, even at cloudy days there is a natural variance of the illuminance as well as of the correlated colour temperature of daylight spectra. The spectrum was measured in October 2010 close to Berlin, where the typical average daily temperature is around 10°C in this season. For better comparison with artificial sunrise the illuminance is multiplied by a factor of 0.63 in Fig. 1.3. For experimental illumination design a fixed illuminance level is chosen as well as a fixed correlated colourcolour temperature. It is decided to realize an illuminance of 6,000 lx at water level for aquarium illumination. Although this is a quite high value compared to typical aquarium illumination, it is not high with respect to natural daylight. One may argue that fish can hide in their natural environment, avoiding extreme illuminance levels. However, it is expected that photohistory affects the biological clock (Vera et al. 2010) and therefore illuminance

levels should not be too low. Hence, the selected illuminance is a compromise between technical capabilities, natural conditions and laboratory constraints. To achieve an illuminance of 6,000 lx three fluorescent lamps per aquarium are installed.

3.3 Simulated sunrise and sunset

Sunrise and sunset trigger not only the biological clock, but also give rise to seasonal variations of fish physiology, like reproduction phases. Simulated sunrise and sunset are therefore realized by a programmable time switch, where the illuminance level is gradually increased by linear power variation during artificial sunrise reaching maximum illuminance within 3 hours as found with natural conditions referring to the example in Fig. 1.3. Due to technical constraints light on and light off is realized with a minimum illuminance of 100 lx. Simulated sunrise and sunset fulfil two purposes. On the one hand the moment of light on/off is hard to define under natural conditions, because there is a gradual transition from the dark to the light that is simulated by the described scheme. On the other hand a sharp onset of light is avoided to minimize stress on fish by abrupt exposure to light. Again the optimum illumination is not to have a perfect fit to natural daylight conditions, but to create defined conditions that are comparable to natural ones.

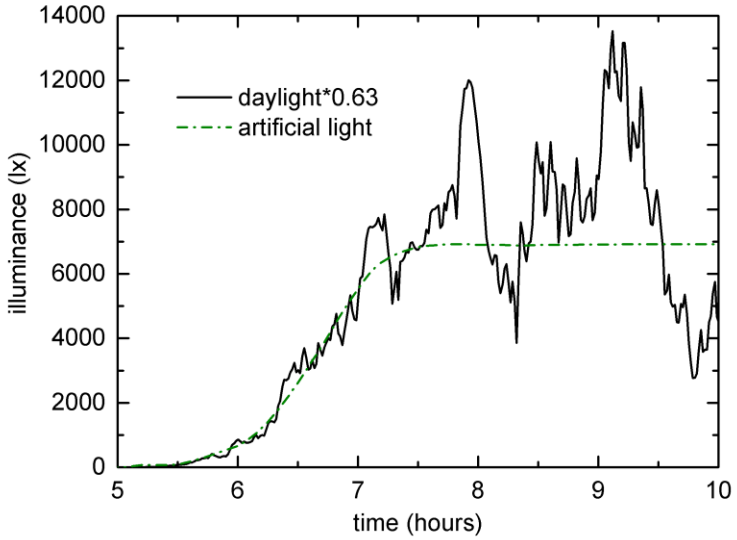

Figure 1.3 Comparison of artificial sunrise/sunset scenario (dash-dotted line) with natural conditions (solid line)

3.4 White nocturnal illumination

Natural nocturnal illumination goes down to values below 1 lx. As fluorescent lamps are dimmable at maximum to 1% of input power, an illuminance level less than 20 lx can only be realized by an additional lamp that is partially covered by an intransparent material or by neutral density filter foil. Therefore all aquaria are equipped with an additional fluorescent lamp for nocturnal illumination, partially covered to adjust the required illuminance. The spectral distribution of nocturnal illumination equals artificial daylight and is not modified to observe the isolated effect of illuminance levels.

3.5 Coloured nocturnal illumination

A straightforward approach for a colour nocturnal illumination is to filter white light, as proposed by Vera et al. (2010). However, filters suffer from a broad bandwidth with a full width at half maximum of 50 to 80 nm. The resulting overlap of spectral transmission may limit the significance of the results and their interpretation. Here, coloured LEDs are advantageous, where bandwidths less than 40 nm are available (Fig. 1.4). In case of LEDs care must be taken to avoid technologies where a blue LED converts light into other spectral regions. In Fig. 1.4 one of the green LEDs shows a significant spectral contribution in the blue spectral range, due to this reason. This kind of LEDs should be excluded for investigations of spectral sensitivities, because depending on the spectral action curve a small amount of radiation in the blue spectral range may have a higher effect in biological action than the radiation in the green spectral range. This is illustrated in Tab. 1.1, where different measures of coloured LED spectra are gathered. The human nocturnal melatonin rating as defined in German standard DIN V 5031-100 (2009) based on the action curve of Gall and Lapuente (2002) decays by orders of magnitudes from the blue to the red LED. The values refer to the spectra given in Figure 5 that are normalized to constant photon flux density of 0.021 $\mu E\ s^{-1}\ m^{-2}$, where the blue and amber Tridonic-LEDs and the green and red Lumitronix-LEDs are selected for aquarium illumination. Photon flux density comes into play when bringing to mind that photometric units are not appropriate for describing physiological action of light on fish. Furthermore from a biological point of view each physiological action is caused by a cascade of biochemical reaction triggered by single photons. Therefore illuminance is listed in Tab. 1.1 only for reference to human light perception.

Tabelle 1.1: Different measures applied to selected coloured LEDs normalized to constant photon flux density as given in Figure 4 (blue and amber: Tridonic LEDs, green and red: Lumitronix-LEDs)

	blue	green	amber	red
full width half maximum (nm)	23	35	18	20
max. spectral irradiance (mW m^{-2} nm^{-1})	0.21	0.13	0.23	0.19
irradiance (mW m^{-2})	5.6	4.8	4.2	3.9
illuminance (lx)	0.15	2.2	2.0	0.62
photon flux density (µE s^{-1} m^{-2})	0.021	0.021	0.021	0.021
human melatonin suppression rating	5.2×10^{-3}	1.8×10^{-3}	1.4×10^{-5}	2.2×10^{-7}

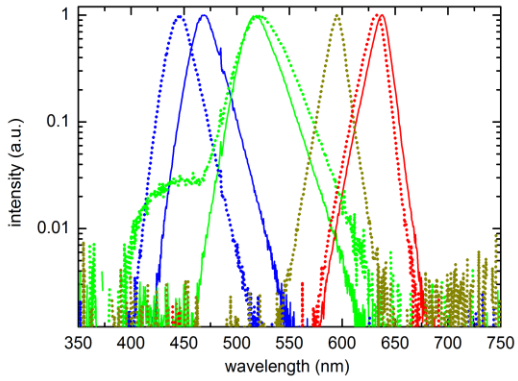

Figure 1.4 Comparison of LED spectra of different manufacturers (solid lines: Lumitronix-LEDs, dotted lines: Tridonic-LEDs)

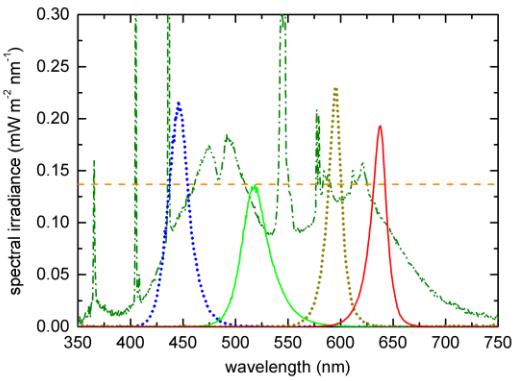

Figure 1.5 Spectra of selected LEDs normalized to constant photon flux density (solid and dotted lines) and compared to an equi-energy spectrum (dashed line) as well as a spectrum of a fluorescence lamp (dash-dotted line) at 10 lx

The following equation describes the conversion of spectral irradiance $\Phi_{\lambda,e}$ to photon flux density Φ_p, where the photon number in mol is commonly given in units of microeinstein (µE):

$$\Phi_p = \int_0^\infty \Phi_{\lambda,e} \frac{\lambda}{119.6\,\mathrm{J\,nm}} \frac{\mu\mathrm{E}}{} \mathrm{d}\lambda \qquad (1)$$

As there is no action curve for nocturnal melatonin suppression of perch, it is appropriate to apply LED spectra of equal photon flux density to fish. The corresponding spectra are given in Fig. 1.5. They are scaled to have a maximum green emission at 0.13 mW m^{-2} nm^{-1}, which is the spectral irradiance of an equi-energy spectrum leading to an illuminance of 10 lx. A spectrum of a fluorescence lamp (OSRAM Biolux) at 10 lx with a total irradiance of about 38 mW m^{-2} and a photon flux density of 0.17 µE s^{-1} m^{-2} is given in the same Figure for reference. Finally it has to be noted that nocturnal illumination of coloured LEDs will still not yield a spectral sensitivity curve, but only indications in which spectral regions the biological action is strongest. Beside blue, green and red LEDs, amber LEDs fill the gap between green and red LEDs and have their peak close to sodium vapor lamps, often used for street illumination in Germany.

3.6 Summary on experimental illumination

It can be summarized that artificial daylight for investigation of melatonin suppression in fish is not designed to perfectly simulate natural daylight conditions but to create an experimental illumination design that resembles natural conditions in key properties, like colour temperature, illuminance level as well as sunrise and sunset time. Fluorescent lamps with best possible continuous spectral emission are chosen, where the colour temperature is around 6,000 K. The illuminance level is chosen to be 6,000 lx throughout the day which is expected to represent a sufficient illuminance level avoiding effects due to a low level photo history. Sunrise and sunset are simulated with constant colour temperature and aim to minimize the onset of light intensity with an appropriate fit to natural conditions. White nocturnal illumination is realized by additional fluorescent lamps of the same colour temperature, whereas coloured nocturnal illumination is realized by coloured LEDs.

4 Biological methods

In fish free melatonin is mainly secreted via the gills (Scott and Ellis 2007). Conjugated hormones might be also secreted with the urine or the faeces (like steroid hormones) but do not represent the physiologically active form. However, free hormones excreted from the gills represent a passive ‘leakage’ expected to match the serum concentrations and by that displaying the status of the physiologically active hormone (Scott and Ellis 2007). In addition Kulczykowska et al. (2006) suggest that melatonin is excreted only in the non-converted form via the gills. For extracting melatonin, the filtered water samples are pumped with a peristaltic pump with Tygon

tubing (Idex) through solid phase extraction cartridges (OASIS HLB Plus, Waters) that have been activated with 5 ml methanol and washed with 5 ml deionized water. Reversed-phase solid phase extraction is a method to concentrate and purify samples for analysis. An extraction cartridge consists of a stationary phase (sorbent) containing hydrocarbon chains. This allows retention of compounds of mid to low polarity out of the mobile phase (water samples) due to the hydrophobic effect. These compounds can be eluted from the sorbent by flushing the cartridge with a non-polar solvent which interrupts the interactions between sorbent and compounds. Additionally the sorbent of the Oasis cartridge contains a component for enhanced retention of polar compounds. The 24-channel peristaltic pump is programmed with a flow rate of approximately 25 ml min^{-1}. After extraction melatonin is eluted from the cartridges with 5 ml ethyl-acetate (Baker). The eluate is evaporated at approximately 50°C with a flow of nitrogen. The extract is reconstituted in 500 µl phosphate buffer and stored frozen until assayed. This procedure has been modified after Ellis et al. (2004) and James et al. (2004). Hormone levels are determined by Enzyme Linked Immunosorbent Assay (ELISA) using commercial Melatonin direct Saliva ELISA-kits (IBL, Hamburg, Germany). ELISA is a method that uses interactions of antigen and antibodies that are linked to an enzyme to cause a colour change. The concentration of the substance to be detected is measured photometrically in comparison to a standard curve. The ELISA used is a test specifically designed for analyzing saliva samples and other aqueous samples with relatively low melatonin contents, compared with blood plasma. In contrast, the saliva ELISA has a detection range of 0.3–50 pg/ml whereas the range of the plasma ELISA is one magnitude higher (1.6–300 pg/ml). The accuracy of the ELISA is 96.8 ±6.8%, i.e. approximately 97% of the substance to be detected is recovered by the test.

5 Mathematical modeling

As described in the previous section, melatonin release rate must be deduced from hormone concentrations in aquarium water. The change in melatonin concentration in aquarium water can be described by a differential equation taking account of melatonin dilution by constant water exchange and melatonin concentration by release of the hormone into the water:

$$\frac{dc}{dt} = -c\frac{1}{V}F + \frac{1}{V}r(t)\,m \qquad (2)$$

The symbols have the following meanings: c - melatonin concentration in water in pg/l, V - aquarium water volume in l, F - flow rate in l/h, r - reduced release rate in pg

h^{-1} kg^{-1}, m - fish biomass in kg. The first question that arises is, if one can estimate the time to achieve a dynamic equilibrium of flow rate and hormone expression rate. To solve the differential equation a volume of 120 l and a fish biomass of 0.5 kg are assumed. The hormone expression rate is simulated by the following ansatz:

$$r = r_0 \left[\frac{\cos\left(2\pi \frac{t + t_0}{24\ \mathrm{h}}\right) + 1}{2} \right]^x + r_1 \qquad (3)$$

Here t is the time in hours and r_0 is the amplitude of the reduced release rate which is set to 100 pg h^{-1} kg^{-1}. The time shift t_0 and offset release rate r_1 are set to zero for the moment. The exponent *x* affects the width of the nocturnal melatonin expression pulse and is set to 4. The temporal course of the reduced release rate is found in Fig. 1.6 with maximum hormone expression at midnight and 24 h cycle.

Let's consider this ansatz as an example to study the behaviour of the system. The evolution of hormone concentration obtained from the solution of the differential equation is given in the same figure for different flow rates. Stationary conditions are achieved after 3 days in case of a flow rate of 10 l/h and for a flow rate of 4 l/h after six days. The relative modulation of hormone concentration is higher with higher flow rate, but absolute modulation is not significantly changing with flow rate. Maximum hormone concentration is delayed with respect to hormone expression by 2.5 h in case of 10 l/h flow rate and by 3 h in case of 4 l/h. It reveals that variation of the maximum hormone expression rate and fish biomass do not affect the establishment of stationary conditions but only the absolute value of hormone concentration. In any case it can be concluded that an acclimation phase of 14 days and a treatment phase of 8 days (as described in section 2) are sufficient to ensure stationary conditions. The second question is how to deduce release rates from measured hormone concentrations. This task is accomplished by rearranging the differential equation, where the temporal derivative of the concentration is approximated by finite differences.

$$r(t_i - \Delta t/2)\, m = V \frac{c(t_i) - c(t_{i-1})}{\Delta t} + \frac{c(t_i) + c(t_{i-1})}{2} F \qquad (4)$$

Here t_i denotes the probing time, t_{i-1} the previous probing time and Δt the time difference between both. Obviously the release rate is obtained in the middle between two probing points. Without loss of generality between two adjacent probing points a constant average release rate can be assumed and the differential equation can be solved analytically. In this case the release rate can be given according to the literature (Kusmic and Gualtieri 2000, Ellis et al. 2004) by:

$$r(t_i - \Delta t/2)\, m = F \frac{c(t_i) - c(t_{i-1}) \exp\left(-\frac{F}{V}\Delta t\right)}{1 - \exp\left(-\frac{F}{V}\Delta t\right)} \qquad (5)$$

Both formulations are equivalent, including the fact that both methods are prone to numerical artifacts in reconstruction of release rates as exemplified in Fig. 1.7. The reconstructed release rate is identical to that one originally given in Fig. 1.6 except at small values. In this case the reconstructed release rate does not go down to zero but takes negative values which are not plausible for the simulated system. These minor numerical artifacts at small release rates observed from numerical experiments indicate that reconstruction might be further limited in evaluation of experimental data superimposed with statistical uncertainties. More details are discussed in the next section.

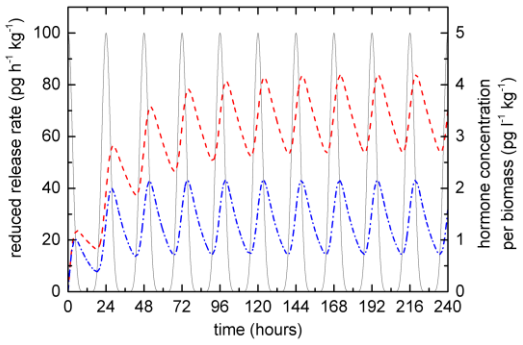

Figure 1.6 Ansatz for temporal course of reduced release rate (solid line) and evolution of hormone concentration at different flow rates (dashed line: 4 l/h, dash-dotted line: 10 l/h)

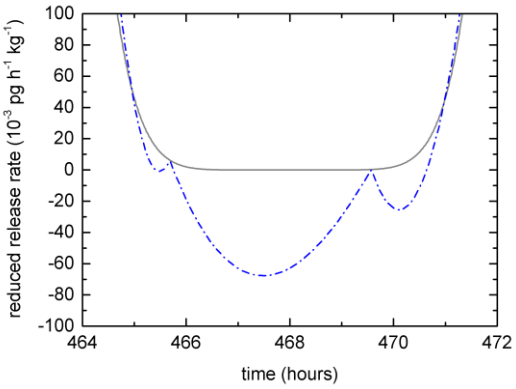

Figure 1.7 Reconstructed release rates from hormone concentrations at a flow rate of 10 l/h (dash-dotted line) compared to given release rate (solid line)

6 Results and discussion

Melatonin concentrations are measured within a 24 h cycle for nocturnal light treatment with minimum illuminance at 0 lx and maximum illuminance at 100 lx as described above. Figure 8 presents melatonin concentration per fish biomass in pg l^{-1} kg^{-1} for 0 lx treatment. The time base is given in hours starting at 0 o'clock of the first day of the probing phase. Water samples are taken from 11 a.m. over 24 h every 3 h including 11 a.m. next day. A periodic continuation of concentrations is assumed as

stationary conditions are expected. This fact is visualized by repeating the 24 h cycle starting with the second day (see Fig. 1.8).

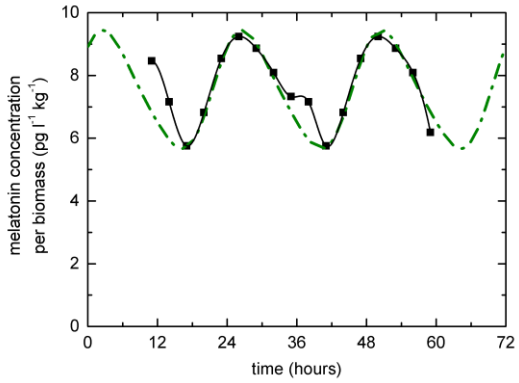

Figure 1.8 Melatonin concentration in aquarium water per biomass at 0 lx (full squares), spline interpolation of experimental values (solid line) and simulated melatonin concentration (dash-dotted line)

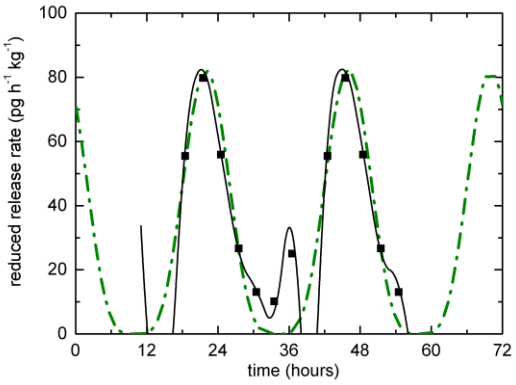

Figure 1.9 Reduced release rate derived from samples at 0 lx (squares), obtained from spline interpolation (solid line) and that one used for simulation (dash-dotted line)

An overlap is given at 11 o'clock of the second day, where the average of concentrations is considered for further evaluation. Furthermore, a spline interpolation of samples is performed with a step size of 1 min to obtain a smooth course of release rates. Release rates are derived applying equation (4) to the samples as well as to the spline interpolant (see Fig. 1.9). On the contrary a simulation of melatonin concentrations is performed assuming a functional dependence of release rates according to Equation (3). Parameters used for simulation are given in Tab. 1.2. The experimental melatonin concentrations are reproduced very well by simulation results for 0 lx treatment (see Fig. 1.8).

Tabelle 1.2: Parameters used for simulation of water melatonin concentration according to equations (2) and (3).

	volume	flow rate	biomass	reduced release rate (pg h^{-1} kg^{-1})			
	(l)	(l/h)	(kg)	amplitude	offset	time shift (h)	exponent (1)
0 lx	84	4.1	0.419	82	0	-2	2
100 lx	84	4.1	0.435	12	0	-2	1.2

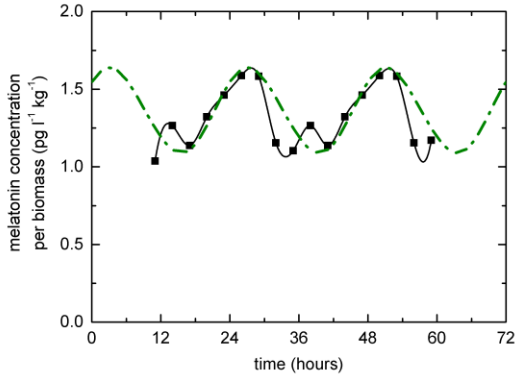

Figure 1.10 Melatonin concentration in aquarium water per biomass at 100 lx (full squares), spline interpolation of experimental values (solid line) and simulated melatonin concentration (dash-dotted line)

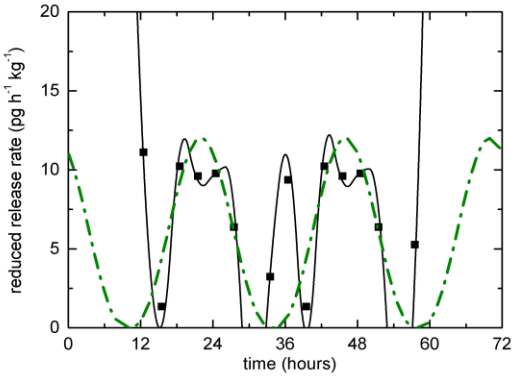

Figure 1.11 Reduced release rate derived from samples at 100 lx (squares), obtained from spline interpolation (solid line) and that one used for simulation (dash-dotted line)

The same procedure is performed for 100 lx treatment. Concentrations and release rates are given in Fig. 1.10 and 1.11, respectively. Several conclusions can be drawn. First of all it has to be noticed that maximum melatonin concentration is more than a factor of 5 less for the 100 lx treatment compared to the 0 lx treatment. Consequently the maximum melatonin release rate is also decreased significantly. That is to say, melatonin expression is efficiently suppressed by nocturnal illumination with white light at 100 lx. But with decreasing melatonin concentration the uncertainty of reconstructed release rates increases and simulation results fit less well to experimental data. In both cases there are negative values of experimental release rates obtained but not displayed in Fig. 1.9 and 1.11, because they are not plausible and caused by numerical effects as discussed in section 5. Even positive release rates around 36 h have to be considered as numerical artifacts due to statistical variations in measured melatonin concentrations. To proof this statement, simulated concentrations are superimposed with normally distributed noise, where the standard deviation is set to 10%.

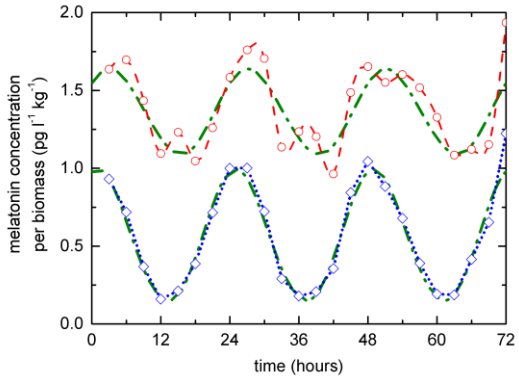

Figure 1.12 Simulated melatonin concentration in aquarium water per biomass at 4.1 l/h and 84 l (dashed line) and at 10 l/h and 60 l (dotted line) disturbed by normally distributed noise

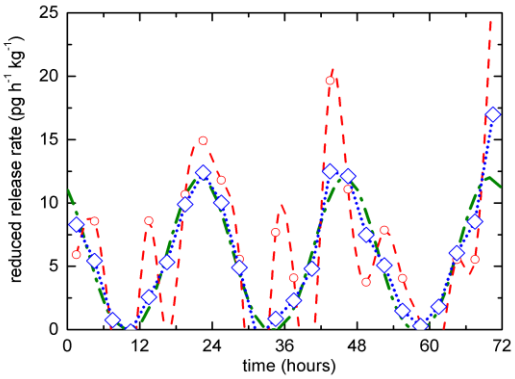

Figure 1.13 Reconstructed reduced release rate from noisy data at 4.1 l/h and 84 l (dashed line) and 10 l/h and 60 l (dotted line**)**

In Figure 12 noisy concentrations are displayed, based on the simulation of the 100 lx treatment, which yields reconstructed release rates given in Fig. 1.13. Variance in experimentally deduced release rate can be reproduced. Moreover it is shown, that numerical artifacts can be reduced by increasing the flow rate at a reduced water volume. Reconstruction of release rates is less affected by numerical uncertainties the higher the relative amplitude of melatonin concentrations is and the less the water melatonin concentration is delayed from melatonin release rates. Although there is no evidence that Equation (3) is the best possible fit to physiological melatonin expression, it reveals that numerical simulation is sensitive to variations in volume, flow rate, biomass and the theoretically assumed release rate. Hence, the principal course of temporal melatonin expression including maximum release rate as well as the width of the nocturnal melatonin expression pulse is fixed within tight limits.

7 Summary

The effect of artificial nocturnal illumination on the melatonin expression of European perch was studied. Measurements of water melatonin concentrations over a period of a nocturnal white light illumination at 100 lx and 0 lx are presented. Based on a mathematical model describing the dilution of melatonin by constant water flux rate and taking into account a time dependent melatonin release rate, the establishment of stationary conditions can be predicted and release rates can be reconstructed from experimental melatonin concentrations. It reveals that at 100 lx nocturnal illumination the melatonin expression is dramatically reduced. Variance of

experimentally obtained release rates can be explained by statistical uncertainties in measured melatonin concentrations, leading to an exclusion of outliers. Furthermore, prospects are given how to increase accuracy in determination of release rates, e.g. by increased flux rates at reduced volumes. The investigations are part of an extended study asking for an onset of nocturnal melatonin suppression and spectral sensitivity of melatonin suppression in fish. The design of the whole study is described in detail focusing on the particular requirements of studies aimed at biological action of light on fish.

Acknowledgements

This work was supported by the German Federal Ministry on Education and Research in the framework of the joint project “Loss of the Night” under grant numbers 033L038A and 033L038G.

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Chapter 2

Spotlight on fish: Light pollution affects circadian rhythms of European perch but does not cause stress

Spotlight on fish: Light pollution affects circadian rhythms of European perch but does not cause stress

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Science of the Total Environment
http://dx.doi.org/10.1016/j.scitotenv.2014.12.094

Highlights

- We studied the impact of light at night on melatonin and cortisol rhythms in perch
- Sampling was non-invasive and hormones were extracted out of the holding water
- Melatonin rhythm was significantly impaired by artificial light at night
- Cortisol was not affected by artificial light at night

Abstract

Flora and fauna evolved under natural day and night cycles. However, natural light is now enhanced by artificial light at night, particularly in urban areas. This alteration of natural light environments during the night is hypothesised to alter biological rhythms in fish, by effecting night-time production of the hormone melatonin. Artificial light at night is also expected to increase the stress level of fish, resulting in higher cortisol production. In laboratory experiments, European perch (Perca fluviatilis) were exposed to four different light intensities during the night, 0 lx (control), 1 lx (potential light level in urban waters), 10 lx (typical street lighting at night) and 100 lx. Melatonin and cortisol concentrations were measured from water samples every 3 h during a 24 hour period. This study revealed that the nocturnal increase in melatonin production was inhibited even at the lowest light level of 1 lx. However, cortisol levels did not differ between control and treatment illumination levels. We conclude that artificial light at night at very low intensities may disturb biological rhythms in fish since nocturnal light levels around 1 lx are already found in urban waters. However, enhanced stress induction could not be demonstrated.

Keywords: Perca fluviatilis; Light pollution; Light intensity; Non-invasive hormone measurement; Fish

1 Introduction

Recent decades have seen a profound transformation of nightscapes, with an increasing proportion of the Earth's surface being illuminated at night. Global light emissions increased at a rate of around 3–6% per year in recent decades (Hölker et al. 2010a). This substantial transformation demonstrates a pressing need to understand the effects of artificial light at night on biological processes. In particular, information regarding the ecological impact of light pollution on animal populations and whole ecosystems is crucial. Possible consequences of artificial light at night on many behavioural and physiological processes in various classes of animals have been reviewed recently (Navara and Nelson 2007, Hölker et al. 2010b, Gaston et al. 2013). Most of these processes are coupled to circadian or seasonal rhythms (Falcón et al. 2003) which may be disrupted by light pollution.

In fish, seasonal rhythms include reproduction, growth and development, and migration, while patterns like locomotor activity, food intake, shoaling or diel vertical migration are mainly driven by circadian rhythms (Lowe 1952, Duston and Bromage 1986, Ryer and Olla 1998, Boeuf and Le Bail 1999, Downing and Litvak 2002, Mehner 2012, Vowles et al. 2014). The most important mechanism of the circadian system in vertebrates is the light-dependent production of melatonin (production at night, suppression during the day) by the pineal organ. The pineal organ of fish is light-sensitive and directly processes photoperiodic information, resulting in a circadian melatonin rhythm that provides periodic information for cells and organs, such as time of the day and season (Underwood and Goldman 1987, Falcón and Collin 1989, Ekström and Meissl 1997, Kulczykowska et al. 2010).

The influence of artificial light at night on fish has been an area of interest for researchers with respect to aquaculture, e.g., how to control growth (Boeuf and Le Bail 1999, Kissil et al. 2001, Biswas et al. 2005), development (Thrush et al. 1994, Porter et al. 1998) and reproduction (Kissil et al. 2001). However, while photoperiod manipulation has proven beneficial for aquaculture, artificial light can have detrimental effects in nature.

Most studies that investigated the influence of artificial light at night on the melatonin rhythm used high light intensities above 100 lx and only a few studies using low light intensities (Bayarri et al. 2002, Migaud et al. 2006a, Takemura et al. 2006), which may occur in light polluted urban areas. However, none of these studies addressed the possible effect of artificial light at night on the physiology of fish and very little is known about dose–response relationships for a range of biological impacts (Gaston et al. 2015).

Cortisol is the most measured indicator for stress in fish. Moreover, two of the major actions of cortisol in fish are hydromineral balance (e.g., seawater adaption in migratory fish) and energy metabolism (carbohydrate, protein, lipid metabolism).

In most fish species, blood levels of cortisol also exhibit a circadian rhythm. However this rhythm is species specific, subjected to seasonal influences and affected by other environmental cues (Wendelaar Bonga 1997). In goldfish (*Carassius auratus*) for instance, the schedule is linked to the photoperiod and peak titres occur around light onset, minimum titres at light offset (Noeske and Spieler 1983). In humans this pattern is called the cortisol awakening response (Kirschbaum et al. 2000) and was also found in Nile tilapia (*Oreochromis mossambicus*) (Binuramesh and Michael 2011). However, in goldfish the feeding schedule can override photoperiod to trigger circadian serum-cortisol variations (Spieler and Noeske 1984). In some salmonids, cortisol peaks were found during the dark phase of the photoperiod but also connected to feeding time (Pickering and Pottinger 1983, Laidley and Leatherland 1988), whereas in sticklebacks (*Gasterosteus aculeatus*) no circadian rhythm could be identified (Audet et al. 1986).

Previous studies regarding the effect of light at night on cortisol levels revealed no obvious impact. But most studies were working with prolonged or continuous photoperiods and relatively high light intensities. Biswas et al. (2006) and Biswas et al. (2008) for example tested a constant illumination of 1500 lx on red sea bream (*Pagrus major*) and striped knifejaw (*Oplegnathus fasciatus*), but cortisol concentrations showed no significant differences to normal photoperiods. Bluefin tuna (*Thunnus orientalis*) showed no changes of circadian cortisol levels when subjected to 5, 15 or 150 lx compared to a 0 lx control (Honryo et al. 2013). However, the invasive sampling procedures (blood or whole body sampling) in the abovementioned examples may have introduced a sampling artefact that obscured potential differences in the stress response between treatments. The dynamics of the stress response to handling stress associated with surgical sampling methods cannot be generalized and seems to be highly species specific ranging from a few minutes (Ramsay et al. 2009) to one or several hours (Wendelaar Bonga 1997). However, with a non-invasive sampling method, existing differences in cortisol rhythms in response to artificial light at night might be uncovered.

Our study presents data on the influence of artificial light at night on circadian rhythm and stress response of European perch (*Perca fluviatilis*). Perch belong to the most dominant fish species in Central European aquatic systems and inhabit a wide range of habitats, including all types of lakes and most streams (Kottelat and Freyhof 2007). Perch are diurnal feeders and become inactive during the night, especially in the presence of nocturnal predators (Hölker et al. 2007).

We measured melatonin concentrations to assess the influence of light on the biological rhythms of perch. Cortisol was measured to evaluate the stress response to light pollution. In contrast to earlier studies, we based our results exclusively on non-invasive measurements, by extracting the hormones from water samples taken without disturbing the experimental animals and determined their concentrations. Ellis et al. (2004) proved a correlation between concentrations of cortisol in water and

serum in rainbow trout (*Oncorhynchus mykiss*) with a phase delay of about 2 h due to excretion route and accumulation in the water. The work of Ellis et al. (2005) indicated a similar correlation for melatonin.
We hypothesised that artificial light at night has a clear effect on the natural circadian rhythm of melatonin production. We expected that with increasing light intensity, the nocturnal melatonin production is suppressed. Furthermore, we hypothesised a stress response caused by the presence of artificial light at night. Using non-invasive methods we expected to identify an increase in cortisol concentrations corresponding to increasing light intensity, especially during the dark phase of the photoperiod.

2 Methods

2.1 Experimental fish

European perch were taken from an existing population at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) in Berlin, Germany. They were originally obtained from the nearby Lake Müggelsee that has a periurban surrounding including forests and housing. Prior to the experiment they were held in 600 L indoor tanks in a flow-through system with ground water, aeration and natural photoperiod and fed daily with frozen red bloodworms. Body mass of the fish at the time of the experiment was 14.2 ± 3.9 g (mean ± SD).

2.2 Experimental setup

Experiments were conducted in a climate chamber in 12 aquaria (80 cm length, 30 cm height, 35 cm width). The temperature of the climate chamber was adjusted to maintain a water temperature of 16 °C. The aquaria were taped with black foil from all sides to make them lightproof.
The lighting system in the cover of the aquaria provided a daytime illumination of up to 7000 lx. Additionally, a three hour dusk and three hour dawn period was programmed to resemble natural twilight conditions. The night-time illumination was adjusted to four different light intensities: 0 lx (control), 1 lx (potential light level in urban waters), 10 lx (nocturnal street illumination) and 100 lx (for details see (Franke et al. 2013)). Each treatment group had three replicates and the experiment was repeated once with a different set of fish to obtain a sufficient replicate number (n = 6) for statistical analysis. Thus, in total 4 × 6 = 24 experimental units were used.

2.3 Experiment

Each aquarium contained 84 L water and was stocked with 30 perch. The flow-through was adjusted to approximately 10 L per hour with aerated tap water. During acclimatisation the photoperiod was 14 hour light/10 hour darkness with complete daylight from 9:30 am to 5:30 pm and dusk and dawn starting at 6:30 pm and 5:30 am, respectively. The night was kept dark in all aquaria and the fish were fed with frozen red bloodworms twice a day. Acclimatisation conditions were applied for 14 days.

After acclimatisation time, the night-time illumination was activated. Water flow was reduced to 4.1 ± 0.2 L/h. When applying the non-invasive method in a flow through system the water exchange rate has to be lowered depending on tank volume and fish biomass to allow the accumulation of the hormones in order to meet the sensitivity range of the applied test.

Fish were not fed during the experiment to maintain water quality. Exposure to experimental night light conditions lasted 10 days.

2.4 Water sampling

After 10 days of experimental exposure, water samples were collected using Tygon tubing (Idex Health and Sciences, Wertheim-Mondfeld, Germany) that extended from every aquarium to a peristaltic pump (24-channel, BVP, Idex Health and Sciences) outside of the climate-chamber. Thus, water samples could be taken without entering the climate chamber and disturbing the fish. Water was pumped via fibre glass syringe filters (1–2 μm; Roth, Karlsruhe, Germany) to remove particular substances. 1 L of water was collected from each tank at each sampling point into 1 L glass bottles. Sampling times were 11 am, 2 pm, 5 pm, 8 pm, 11 pm, 2 am, 5 am, 8 am and 11 am. Water samples were stored at 4 °C in darkness and processed for hormone extractions within 24 h.

2.5 Hormone extraction and assay

The extraction method followed the procedure described in Kidd et al., 2010 but was adjusted to our experimental setup. For extracting melatonin and cortisol, the filtered water samples were pumped through solid phase extraction cartridges (OASIS HLB Plus, Waters, Saint-Quentin En Yvelines Cedex, France) with a peristaltic pump (BVP, Idex Health and Sciences). The cartridges had been activated with 5 mL methanol (Baker®). Before and after processing the tank water, cartridges were

washed with 5 mL deionized water. The 24-channel peristaltic pump was programmed with a flow rate of 25 mL min− 1.
After extraction, cortisol and melatonin were eluted from the cartridges with 5 mL ethyl-acetate (Baker®). The samples were evaporated at 50 °C under a stream of nitrogen. These extracts were reconstituted in 500 µL phosphate buffer saline (PBS + 5%Ethanol + 0.1% bovine serum albumin) and stored at − 20 °C until measured.
Hormone levels were measured by Enzyme Linked Immunosorbent Assay (ELISA) using commercial cortisol-saliva-kits and non-extraction melatonin saliva-kits (IBL, Hamburg, Germany).

2.6 Data handling and statistical analysis

Hormone concentrations in tanks were standardised to 1 kg fish biomass. After confirming that the data met the assumptions of the test (normality of residuals) they were analysed using Linear Mixed Models (LMM, fixed factors: treatment, time; random factor: tank) (Pinheiro and Bates 2000). No significant tank effects could be identified. The analyses were performed with IBM SPSS Statistics (Version 20).
Hormone data was transformed to relative values in relation to the base line ("change vs. control"). The time point with the lowest hormone concentration in the control treatment was defined as baseline and set as 100%. For melatonin the baselines were the 5 pm values, while the 2 am values were set as the cortisol baselines. The relative profiles were tested for rhythmicity, using a nonlinear regression with a cosine function (cosinor-method (Haen 1994)). If the confidence interval of the parameter P (period) contains 0, periodicity is rejected.

3 Results

There were significant differences in melatonin tank water concentrations between the control (0 lx treatment) and all treatments (LMM treatment effect: $F3 = 339.9$; $p = 0.000$, Posthoc test Sidak: df 216; $p \leq 0.05$) with melatonin concentration being up to three times higher in controls compared to light treatments (Fig. 2.1). 1 lx illumination was likewise significantly different to all other treatments and the control (LMM, Sidak).
Control fish produced more melatonin over the 24 h sampling period and exhibited a clear melatonin rhythm with a rise at the beginning of sunset and a decline at sunrise (LMM time effect: $F216 = 3,3$; $p = 0.001$).

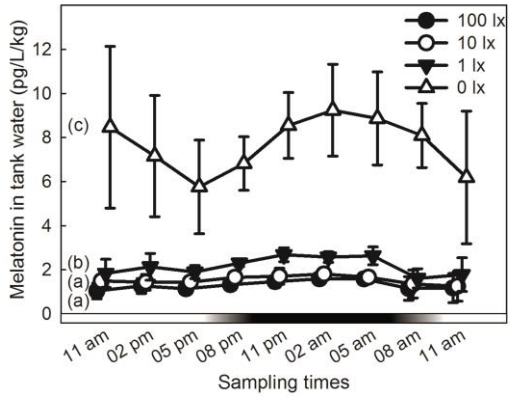

Figure 2.1 Concentration of melatonin per L tank water in pg/L/kg (mean ± SD). Comparison between 4 illumination treatments (n = 6) with 0 lx, 1 lx, 10 lx and 100 lx at night within 24 h. Photoperiod was 14 hour light/10 hour treatment with simulated sunrise and sunset of 3 h (see bar at the bottom of the graph, black indicates night, white indicates daytime). Sunset started at 4 pm and sunrise started at 7 am. Different letters indicate significant differences ($p \leq 0.05$) between treatments.

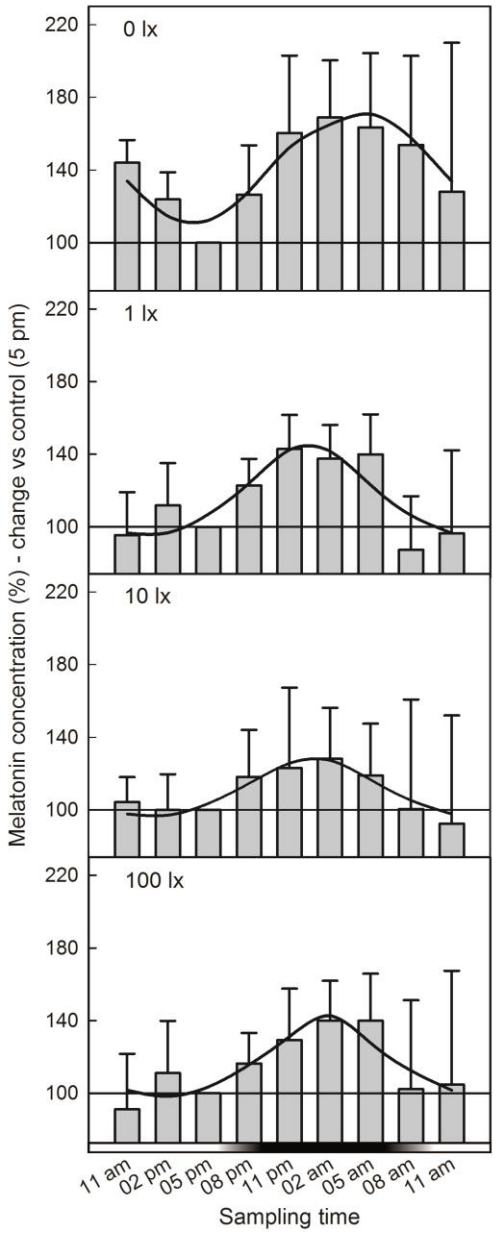

Figure 2.2 Change versus control of relative melatonin concentrations in the tank water of treatments (0, 1, 10, 100 lx). Illustration of the change of melatonin concentration compared to the 05 pm value (baseline); solid line: regression curve of the cosine regression; at the bottom of the columns day and night are represented with white and black bars, respectively.

The cosinor analysis of the relative data found a circadian rhythm in the control and 1 lx treatments (Tab. 2.1, Fig. 2.2), with melatonin tank water concentrations rising in the night and declining during the day. However, the analysis of the data of the 10 and 100 lx treatment indicates an absence of circadian rhythm (confidence interval contains 0, Tab. 2.1). Additionally the R2 value is highest for the control treatment, indicating a better fit of the cosinor model compared to the other treatments.

Table 2.1 Results of the cosinor analysis of relative melatonin and cortisol contents in the tank water over time. R2 > 0.3 indicates sufficient fit of cosinor model; periodicity is rejected if confidence interval of parameter period contains 0.

			Confidence Interval: Period	
	Treatment	R^2	lower	Upper
Melatonin	0 lx	0.3304	17.2698	28.2532
	1 lx	0.0992	10.4561	21.5510
	10 lx	0.0221	-4.3617	52.3617
	100 lx	0.2388	-54.6308	0.2107
Cortisol	0 lx	0.2171	1.9520	46.0480
	1 lx	0.0454	-15.0630	63.0630
	10 lx	0.1388	5.8290	42.1710
	100 lx	0.0427	9.7103	28.7969

Cortisol concentrations in tank water of the 100 lx treatment were significantly lower than 0 lx, 1 lx and 10 lx treatments (LMM treatment effect: $F3 = 5.6$; $p = 0.001$). At the beginning of the sampling period this difference was particularly prominent. Additionally a circadian rhythm of cortisol concentration was identifiable (LMM time effect $F8 = 4.0$; $p < 0.0001$), with a decrease during the night and an increase at the beginning of the day (Fig. 2.3). Fig. 2.4 shows a circadian pattern in treatment underpinned by the results of the cosine regression, which revealed a periodicity for the control treatment and the 10 and 100 lx treatments (Tab. 2.1). However, except for the control treatment, the R2 values are very low suggesting a sufficient fit only for the control treatment.

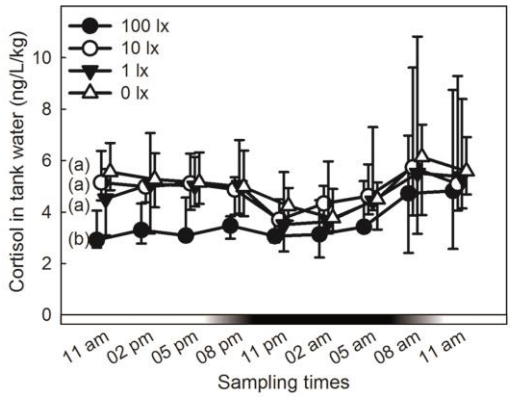

Figure 2.3 Concentration of cortisol per L tank water in ng/L/kg fish (median ± percentiles). Comparison between 4 treatments (n = 6) 0 lx, 1 lx, 10 lx and 100 lx within 24 h. Lighting conditions remain the same during the study period. Different letters indicate significant differences ($p \leq 0.05$) between treatments, day and night are represented with white and black bars, respectively, at the bottom of the graph.

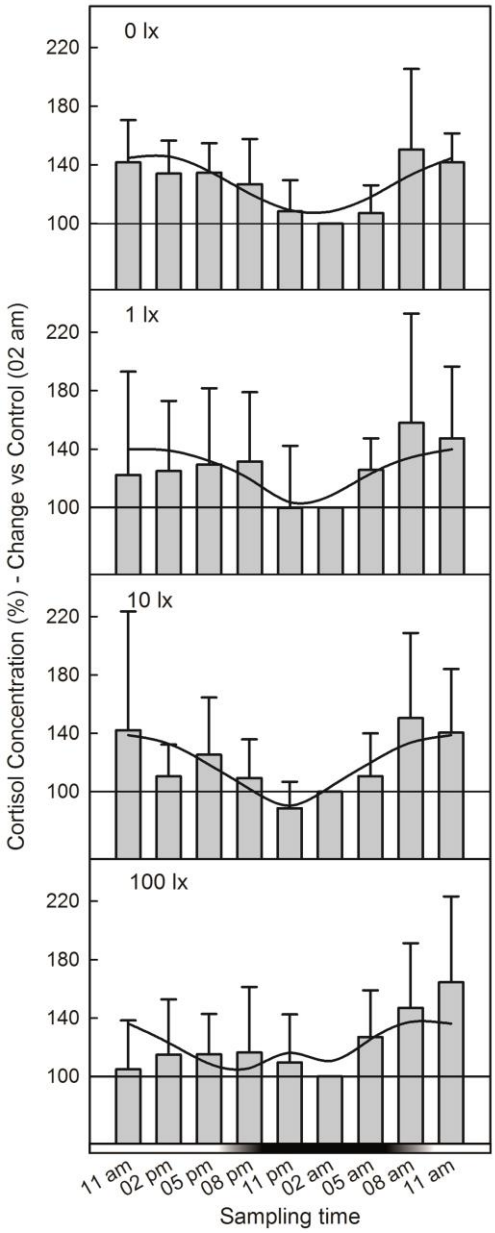

Figure 2.4 Change versus control of relative cortisol concentrations in the tank water. Illustration of the change of cortisol concentration compared to the 02 am value (baseline); solid line: regression curve of the cosine regression; at the bottom of the columns day and night are represented with white and black bars, respectively.

4 Discussion

This study demonstrated that artificial light illuminating the water at night had a strong effect on the rhythm of melatonin production in European perch. Even at low light intensities of 1 lx, which is slightly brighter than the light produced by a full moon, there was already a substantial decrease of melatonin secretion. Fish in treatments with higher light intensities seemed to completely lack any circadian melatonin rhythm. Moreover, melatonin concentration in the water of the control tank was up to 4 times higher compared to experimental tanks (Fig. 2.1). In contrast to the proposed hypothesis, there was no significant difference in cortisol concentrations between control tanks and tanks subjected to varying nocturnal light intensities. Moreover, irrespective of experimental conditions (nocturnal light or darkness), cortisol concentrations exhibited a circadian pattern with a rise in the morning and a decline during the night. Overall, our results strongly suggest that light is the main driver of the circadian melatonin rhythm in perch. In contrast, there was no significant difference in tank water cortisol, suggesting that artificial light at night is not an additional stress factor for perch.

Since the investigation of ecological light pollution remains an emerging area of focus for researchers (Hölker et al. 2010a), not much is known about its potential effects on aquatic ecosystems (Perkin et al. 2011). How artificial light at night in urban waters may affect physiological patterns, notably the melatonin rhythm in wild fish populations, has currently only been hypothesised and very little is known about a threshold, above which these patterns are impaired or the overall shape of the dose–response function is altered.

There are several studies that investigated the effects of light at night on behaviour or physiology of different fish species. However, most of these studies used higher light intensities usually > 10 lx (Imsland et al. 1995, Koskela et al. 1997, López-Olmeda et al. 2009, Brüning et al. 2011, Riley et al. 2013). With a few exceptions none of these studies attempted to identify the threshold, above which, nocturnal light intensities begin to effect fish behaviour or physiology. For example, light levels < 1 lx have been shown to be an important cue for both predator avoidance and feeding in some salmoniformes (Metcalfe et al. 1997, Takemura et al. 2006, Ohlberger et al. 2008). Takemura et al. (2006) studied cultured pineal glands of rabbitfish (*Siganus guttatus*), another perciformes, which have lunar spawning rhythms (Ikegami et al. 2014). Accordingly, the nocturnal fluctuations of serum melatonin concentrations at different moon phases were examined. At 0.1 lx and 0.5 lx, melatonin was significantly lowered. Even though there are major physiological differences between rabbitfish and European perch and a display of a full circadian rhythm in rabbitfish is missing, the low light threshold of rabbitfish pineal is consistent with findings in the current study that artificial light, at low intensities, can disturb nocturnal melatonin production. Porter et al. (2001) found that Juvenile Atlantic salmon (*Salmo salar*)

subjected to brighter nocturnal light levels (20, 200 and 400 lx) produced less melatonin compared to salmon exposed to lower light intensity levels (0 and 1 lx). In contrast to our results from European perch, the melatonin rhythm in salmon was not completely diminished but was gradually decreased with increasing light intensity. However, due to differences in circadian systems between species (Falcón et al. 2010, Kulczykowska et al. 2010) the response to artificial light at night may be species-specific.

As shown by our experiment, artificial light at night resulted in significantly decreased secretion of melatonin in European perch. In preliminary experiments we found that under daylight conditions of the present experimental setup melatonin is relatively stable concerning degradation processes (see Supplementary). These results suggest that differences in melatonin concentrations originate from the impaired melatonin production rather than any degradation of melatonin due to artificial illumination. Extremely low energetic illumination of 1 lx caused a significant drop in melatonin levels indicating that European perch are very sensitive to nocturnal light emissions. We provided an experimental light environment that effectively resembles that of natural conditions at daytime, including dusk and dawn. Excluding other modifying abiotic factors like temperature and based on the non-invasive approach that excludes disturbances from sampling, the results from fully dark reference conditions can be considered the baseline against which the results of the light treatments are evaluated. A circadian melatonin rhythm with a strong increase at night and a comparable decrease during the day was only seen in the control treatment. The overall higher amount of melatonin in the control treatment (Fig. 2.1) is a result of the non-invasive sampling method with lowered water flow rate. The low flow rate guaranteed an accumulation of hormones in the water to a baseline in order to meet the sensitivity range of the assay. The relatively high accumulation of melatonin in the 0 lx treatment, i.e., a higher baseline value leads to the assumption that the additional night light inhibited the production of melatonin secretion in the other treatments from the first night of the experimental phase resulting in a lower baseline.

The consequences of an impaired melatonin rhythm can be varied, since the melatonin rhythms drive the biological clock and influence many other physiological processes. The influence of light or in particular long day photoperiods, in fish has predominantly been a focus for researchers with an interest in aquaculture, since the manipulation of photoperiod is an efficient tool to induce reproductive events (Randall et al. 1995). For example, both percid and salmonid farmers utilise photoperiod manipulation to induce reproductive maturation and egg ovulation outside of natural spawning periods (Macquarrie et al. 1979, Kolkovski and Dabrowski 1998). However, in nature, artificial photoperiods might cause problems, such as disturbance of synchronous hatching and swim bladder inflation of fish larvae, which may reduce survival chances (Brüning et al. 2011). The reproductive

process in temperate fish species is characterized by a distinct seasonal pattern since they mostly reproduce only once a year. Potential effects of a disturbed melatonin rhythm on reproductive processes are summarized by Falcón et al. (2010). Although studied in different fish species, it is clear that a missing circadian rhythm and subsequent decrease in melatonin production can interrupt reproduction. This hypothesis is supported by numerous studies in aquaculture, proving an inhibition of sexual maturation at constant light conditions in several species e.g., haddock (*Melanogrammus aeglefinus*) (Davie et al. 2007), Senegalese sole (*Solea senegalensis*) (García-López et al. 2006), Sea bass (*Dicentrarchus labrax*) (Rodríguez et al. 2005) and Atlantic salmon (*S. salar*) (Porter et al. 1999). Migaud et al. (2006b) demonstrated a complete interruption of gonadogenesis in *P. fluviatilis* by 500 lx of continuous light. Combined with our result that night light intensities at a very low level can diminish the melatonin rhythm, it is most likely that light pollution has the potential to interrupt the reproductive process of European perch and may therefore reduce the fitness of this species.

The threshold light intensity for this interruption could only be estimated but our results suggest that it is lower than 1 lx. Although the melatonin concentration at 1 lx was substantially reduced compared to the control, it was higher compared to 10 and 100 lx and a rhythm was still identifiable. The challenge for future studies would be to examine, whether concentration or rhythm alone is driving light dependent behaviours and physiological processes.

However, since light levels around 1 lx were already found in urban waters at night, e.g., in Berlin (Perkin et al. 2014), it is most likely, that circadian rhythms in fish can be impaired, especially when considering, that the problem of light pollution is continuously increasing (Hölker et al. 2010a).

None of the different light treatments resulted in a significant elevation of cortisol levels. Moreover, in the 100 lx treatment, cortisol was significantly reduced at the beginning of the sampling period. We had assumed that the non-invasive and non-disturbed measurement of cortisol would reveal differences in the cortisol response to different light intensities, whereas these differences are blurred by the handling stress when taking plasma samples by invasive methods (Biswas et al. 2006, Biswas et al. 2008, Bani et al. 2009). This assumption could not be verified, since our results correspond to existing knowledge about the stress response to night light in other species, e.g., goldfish (*C. auratus*) (Spieler and Noeske 1984), sea bream & striped knifejaw (*P. major, O. fasciatus*) (Biswas et al. 2006, Biswas et al. 2008); great sturgeon (*Huso huso*) (Bani et al. 2009) or bluefin tuna (*T. orientalis*) (Honryo et al. 2013). However, we cannot assess the impact of long term exposure to artificial light at night on the fitness of European perch. Light pollution may have an effect on reproduction and has also been shown to modulate the immune system of fish (Bowden 2008). Therefore, long term effects of light pollution on physiology and behaviour need to be assessed in future studies.

Cortisol rhythm in general seems to be influenced by more than one factor, e.g., feeding and onset of light (dawn) (Cerdá-Reverter et al. 1998). The so called cortisol awakening response known for humans doesn't seem to occur in all fish species. In salmonids for instance, a peak in plasma cortisol concentrations occurs during the night, as reported for brown trout (*Salmo trutta*) (Pickering and Pottinger 1983) and Atlantic salmon (*S. salar*) (Nichols and Weisbart 1984). In three spined sticklebacks (*G. aculeatus*) no peak at all was found, suggesting that this species has no identifiable circadian rhythm of cortisol (Audet et al. 1986). Oliveira et al. (2013) exposed Senegalese sole (*S. senegalensis*) to 11 days of continuous low light (0.5–1 lx). As well as under natural conditions, the cortisol concentrations followed a circadian rhythm although the peak was delayed. However, under continuous low light, the concentrations were lower than under natural photoperiod. In our experiment the morning rise in water cortisol was visible in all treatments (Fig. 2.3) although periodicity was significant for all treatments except 1 lx. The relative concentrations in relation to the basic value at 02 am (Fig. 2.4) reveal an increase of about 50% in the morning, suggesting that cortisol in perch is following a circadian rhythm, associated with the onset of light and is not disturbed by artificial light at night.

Our results suggest that artificial light at night (ranging from 1 to 100 lx) is not inducing a stress response in perch and that these results are consistent with the abovementioned previous studies.

However, the study took place in a controlled laboratory context, lacking environmental influences, such as predators, prey or refuges that are normally present under natural conditions. In a natural context with multiple stress factors, light pollution as an additional stressor may have additive, antagonistic or even synergistic effects (Perkin et al. 2011).

5 Conclusion

We found a strong effect of artificial light at night on the melatonin rhythm which leads us to conclude that light pollution in urban waters at realistic light levels has significant potential to inhibit circadian melatonin patterns. Considering the main task of melatonin - mediating between the current photoperiod and the different circadian and seasonal rhythms in a fish's life - it is likely that these rhythms will be altered too. Although light pollution is not yet measured consistently in urban waters, the trend of lighting up cities including lake and river promenades is rising. Even though a clear stress response towards night lighting was not found, our results are a step towards finding the threshold light intensity above which, at least in perch, an impairment of biological rhythms occur.

Acknowledgements

The authors thank T. Mehner, Alex Lumsdon and D. Murray for the helpful comments on the manuscript and M. Kunow and the students at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries for the technical support. Funding was provided by the Verlust der Nacht project (Federal Ministry of Education and Research, Germany, BMBF-033L038A).

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Chapter 3

Impact of different colours of artificial light at night on melatonin rhythm and gene expression of gonadotropins in European perch

Impact of different colours of artificial light at night on melatonin rhythm and gene expression of gonadotropins in European perch

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Science of the Total Environment
http://dx.doi.org/10.1016/j.scitotenv.2015.11.023

Highlights

- We studied the impact of different wavelengths of artificial light at night on melatonin rhythm
- We examined the effect of different light colours and intensities of white light on gene expression of gonadotropins in perch
- Melatonin rhythm was significantly impaired by blue, green and red light at night, blue light was less suppressive
- Gene expression of gonadotropins was suppressed by white light of 1 lx and higher but not by blue, green and red light

Abstract

The distribution and intensity of artificial light at night, commonly referred to as light pollution, is consequently rising and progressively also ecological implications come to light. Low intensity light is known to suppress nocturnal melatonin production in several fish species.

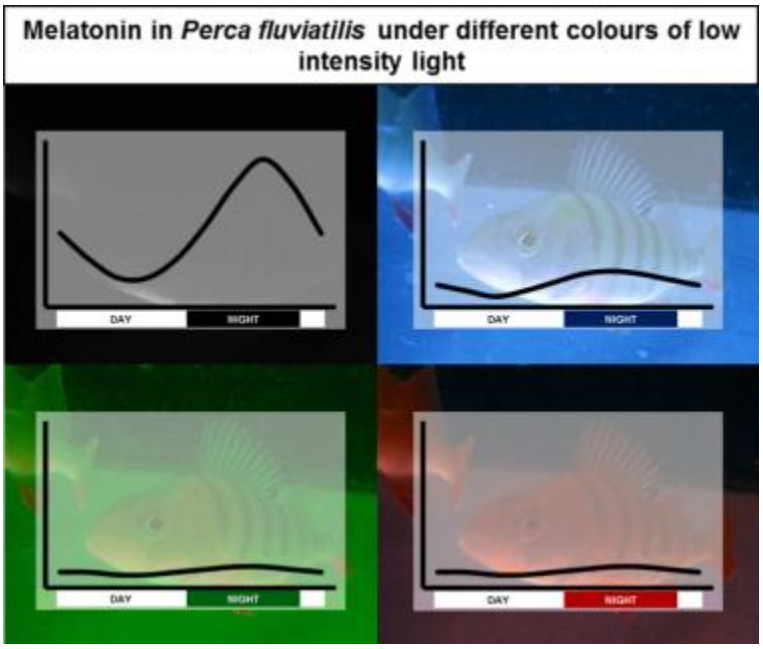
This study aims to examine the least suppressive light colour for melatonin excreted into the holding water and the influence of different light qualities and quantities in the night on gene expression of gonadotropins in fish. European perch (*Perca fluviatilis*) were exposed to light of different wavelengths during the night (blue, green, and red). Melatonin concentrations were measured from water samples every 3 h during a 24 h period. Gene expression of gonadotropins was measured in perch exposed to different light colours and was additionally examined for perch subjected to different intensities of white light (0 lx, 1 lx, 10 lx, 100 lx) during the night.

All different light colours caused a significant drop of melatonin concentration; however, blue light was least suppressive. Gene expression of gonadotropins was not influenced by nocturnal light of different light colours, but in female perch gonadotropin expression was significantly reduced by white light already at the lowest level (1 lx).

We conclude that artificial light with shorter wavelengths at night is less effective in disturbing biological rhythms of perch than longer wavelengths, coinciding with the light situation in freshwater habitats inhabited by perch. Different light colours in the night showed no significant effect on gonadotropin expression, but white light in the night can disturb reproductive traits already at very low light intensities. These findings indicate that light pollution has not only the potential to disturb the melatonin cycle but also the reproductive rhythm and may therefore have implications on whole species communities.

Keywords: *Perca fluviatilis*; Light pollution; Melatonin; Spectral sensitivity; Reproduction; Gonadotropins; Fish

Graphical abstract



1 Introduction

In December 2013 the UN declared the year 2015 as the international year of light. The initiative focuses on light sciences and its application. But it also raises awareness about ‘light pollution’. The distribution and intensity of artificial light at night (ALAN) is consequently rising since the invention of the electric light and progressively also ecological implications come to light (Longcore and Rich 2004, Navara and Nelson 2007, Hölker et al. 2010a, Hölker et al. 2010b, Gaston et al. 2013) pointing out the influence of ALAN on flora and fauna and even on whole ecosystems. Historically, research on this topic focused on humans (Griefahn et al.

2006, Kantermann and Roenneberg 2009) and terrestrial organisms (Redlin 2001, Stone et al. 2009, Kempenaers et al. 2010, van Langevelde et al. 2011, Davies et al. 2012, Nordt and Klenke 2013, Lewanzik and Voigt 2014). The impact of ALAN on aquatic systems has attracted special attention only in recent years (Meyer and Sullivan 2013, Perkin et al. 2014b, Brüning and Hölker 2015, Hölker et al. 2015).

The influence of ALAN on fish was studied mainly with regards to aquacultural interests like improvement of growth, decreasing stressful conditions or control of reproductive processes (Boeuf and Le Bail 1999, Kissil et al. 2001, Biswas et al. 2006, Davie et al. 2007, Honryo et al. 2013). However, the beneficial effects of ALAN in aquaculture might be detrimental for biological rhythms of fish in natural environments, even though ALAN in nature has mostly low light intensities of up to 4 lx (Meyer and Sullivan 2013, Perkin et al. 2014a).

In contrast to mammals that possess only ocular photoreceptors (Peirson et al. 2009) the circadian rhythm of fish may be controlled by multiple tissues and receptors beside rods and cones. The most important are:

- Deep brain photoreceptors – photoresponsive molecule in the brain that may also respond to photic stimuli (Kojima et al. 2000, Philp et al. 2000, Fernandes et al. 2012)
- The saccus vasculosus may be a sensor for seasonal information and modulates the thyroid system in some fish species (Tsuneki 1992, Nakane et al. 2013)
- Non-image forming photoreceptors such as melanopsin in the retinal ganglion cells have often peak sensitivity for blue light and horizontal and amacrine cells can possess vertebrate ancient opsin with maximum sensitivity for green. Both may also signal environmental irradiance (Kojima et al. 2000, Philp et al. 2000, Peirson et al. 2009).
- The pineal complex, a part of the brain that is located under a translucent window of the scull, consists of cone-like photoreceptor cells and is therefore light sensitive. I.e. the fish pineal is able to directly transduce light signals into hormonal signals with circulating melatonin as main output (Falcón and Meissl 1981, Falcón et al. 1992).

However, the circadian control of melatonin production differs in some teleosts (Migaud et al. 2007b). In salmonids it seems to be the pineal alone that controls melatonin production, which is also the case in goldfish, *Carassius auratus* (Kezuka et al. 1992). In this species, melatonin produced by the eyes would not contribute to plasma and CBS (cerebrospinal fluid) melatonin. In contrast, in sea bass (*Dicentrarchus labrax*) and cod (*Gadus morhua*) light perceived by the eyes may regulate melatonin synthesis by the pineal gland through neural projections into the brain (Migaud et al. 2007b). The same study suggested that in Nile tilapia and catfish

it is ocular light input alone, that modulates melatonin production, although in Tilapia melatonin production by the pineal gland could be observed ex vivo.

In any case the production of melatonin is suppressed by light, thus melatonin levels are high at night and low during the day. The oscillations and shape of this curve change throughout the year and thereby the melatonin rhythm provides information about day and season. ALAN can impair these rhythms, as reported in several fish species (Porter et al. 1999, Bayarri et al. 2002, Vera et al. 2005, Ziv et al. 2007, Brüning et al. 2015). Most of the studies used higher light intensities as assumed for light polluted freshwater habitats. Perkin et al. (Perkin et al. 2014a) found light levels of up to 1.4 lx in an urban river (River Spree, Berlin, Germany). Intensities of up to 4 lx were found in river Scotio in Columbus, Ohio (Meyer and Sullivan 2013). In experimental field in Westhavelland, equipped with actual streetlamps, intensities of up to 17 lx where measured in a drainage channel 3 m away from the light sources (Hölker et al. 2015). In the very few studies that investigated the effect of very low ALAN intensities at 1 lx and lower (Bayarri et al. 2002, Migaud et al. 2006a, Takemura et al. 2006, Brüning et al. 2015) a similar impairment of the melatonin rhythm was found.

The photoperiod is one of the most important triggers for the timing of reproduction and consequently reproductive processes are affected by light pollution as well. Reproduction in temperate freshwater fish species is normally initiated by changing the zeitgeber temperature and photoperiod (via melatonin rhythm or other physiological indicators) in the fall. Under natural conditions, the gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH), stimulate gonadogenesis/gametogenesis and thereby the production of sex steroids. Inhibition of melatonin rhythm can implicate a suppression of gonadotropin production. Melatonin is known to be able to alter several components of the hypothalamus–pituitary–gonadal (HPG) axis such as gonadotropins, sex steroids or gonadal maturation (Khan and Thomas 1996, Amano et al. 2000, Chattoraj et al. 2005, Bhattacharya et al. 2007, Sébert et al. 2008, Carnevali et al. 2011). This is underpinned by numerous studies that used continuous light or altered photoperiods to prevent or delay maturation (Porter et al. 1998, Porter et al. 1999, Rodríguez et al. 2005, García-López et al. 2006, Davie et al. 2007) or to shift spawning incidences (out of season spawning) (Macquarrie et al. 1979, Thrush et al. 1994, Kolkovski and Dabrowski 1998). However, information about the influence of low intensity ALAN, as it occurs in light polluted aquatic environments on reproductive processes is missing.

When talking about light, the light spectrum is another factor that has to be taken into account. In aqueous environments light propagation differs from terrestrial habitats. In water, each wavelength is attenuated differently. The attenuation also depends on the composition of the water. In general you can say that in sea water short

wavelengths are dominant (Clarke 1956) whereas in most lakes yellow light penetrates the water deepest (Lythgoe 1988, Wetzel 2001).
As a result the pineal or other non-visual photoreceptors of marine fish have different sensitivities from freshwater fish, resulting from different pigments. Indeed a variety of pigments has been identified so far in fish pineal and other non-visual photoreceptors, including rod-like and cone-like opsins, VA- and VAL-opsin (vertebrate ancient and vertebrate ancient long opsin), extra-retinal rod-like opsin and parapinopsin (Blackshaw and Snyder 1997, Soni and Foster 1997, Kojima et al. 2000, Forsell et al. 2001, Bellingham et al. 2003). The degree of expression and combination of the pigments in extraretinal photoreceptors seems to be highly species specific and depending on aquatic environment and information available only in selected species. A lot of research has been done concerning the spectral sensitivity of retinal photoreceptors and the influence of different light colours on promoting growth or reducing stress (Volpato and Barreto 2001, Ruchin 2004, Owen et al. 2010, Heydarnejad et al. 2013). However, most of the studies dealing with the effect on melatonin rhythm revealed that marine fish are most susceptible to blue light (Migaud et al. 2007a, Oliveira et al. 2007, Vera et al. 2010). In contrast, the melatonin system in freshwater fish seems to be more receptive to green light and longer wavelengths (Falcón and Tanabe 1983, Forsell et al. 2001).
This study focuses on the influence of artificial light on circadian and seasonal rhythms in European perch (*Perca fluviatilis*). European perch is one of the most dominant fish species in Central Europe and in habit various habitats including all kinds of lakes and streams (Kottelat and Freyhof 2007). In a previous study we investigated the impact of different intensities of white light, 0 lx, 1 lx, 10 lx and 100 lx, on the melatonion rhythm of European perch. We found a disturbance of the rhythm already at the lowest light intensity tested (1 lx) (Brüning et al. 2015). In this study we want to follow up on these results and test if these intensities have an influence on reproduction. We hypothesise that these lighting conditions affect reproduction by impairing gene expression of the gonadotropins LH and FSH.
In a second experiment we examined the influence of different light qualities (blue, green, red) on the melatonin rhythm and gene expression of LH and FSH. We expect that longer wavelengths (green and red) are more effective in suppressing the night time production of melatonin as well as the gene expression of the gonadotropins.

2 Methods

2.1 Experimental fish

European perch (*P. fluviatilis*) were obtained from the stock at the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB) in Berlin, Germany, and originated

from the nearby Lake Müggelsee. Prior to the experiment, fish were held in indoor tanks in a flow-through system with tap water and aeration under natural photoperiod and fed daily with frozen red bloodworms. Fish body mass at the time of the experiment was 31.8 ± 10.1 g (mean ± standard deviation (SD)). Temperature was 16 °C and water parameters were checked regularly to maintain optimum conditions.

2.2 Experimental setup

The experimental setup followed the methods described by Brüning et al. (2015).
Briefly, the experimental tanks were stocked with 30 perch each; each treatment consisted of 6 replicates (6 tanks).
In Brüning et al. (2015) we already showed the effect of different light intensities at night on the melatonin rhythm of perch. To complement these results, we present here the effect of these conditions on the gene expression of gonadotropins.
For the light intensity experiment (LI), night-time illumination was adjusted to four different white light intensities: 0 lx (control), 1 lx (0.017 μmol s^{-1} m^{-2}), 10 lx (0.17 μmol s^{-1} m^{-2}) and 100 lx (1.7 μmol s^{-1} m^{-2}). Light measurements were taken with an ILT1700 Research Radiometer (range: 0.00167–1,670,000 lx, International Light Technologies, Peabody, MA) equipped with an SUD033 Underwater Broadband Silicon Detector (200–1100 nm, 950 nm peak), thus the light intensity of the control treatment would be < 0.00167, referred to as 0 lx. During acclimatization, complete daylight was from 9:30 to 17:30 h and dusk and dawn of three hours was provided, starting at 6:30 and ending at 20:30 h, respectively (October conditions).
For the colour experiment (LC), the night-time illumination was adjusted to three different spectral qualities: red, green and blue LED light and a dark control. The intensity of the LED lights was equalized to a photon flux density of 0.021 μmol s^{-1} m^{-2} (for details see Franke et al. (2013) and Tab. 3.1). Photon flux is a more accurate way to quantify different spectra in biological experiments because biochemical reactions are normally triggered by single photons and the number of photons differs for spectra of different wavelengths at the same irradiance level.
During acclimatization in the experimental tanks the photoperiod was adjusted to autumn (September) conditions. Complete daylight was from 10:00 to 16:00 h, complete darkness or experimental night lighting was from 19:00 to 07:00 h and dusk and dawn (3 h each) started at 07:00 and ended at 19:00 h respectively.
In both experiments the night was dark in all aquaria during acclimatization and the fish were fed with frozen red bloodworms twice a day. Simulated daylight of approximately 7000 lx was realized by three fluorescent bulbs (white light, 6000 K, Osram Biolux T8/18 W see Franke et al., 2013).Water temperature was approximately 16 °C and flow rate was 10 L/h. Acclimatization conditions were applied for 14 days.

Table 3.1 Different measures applied to selected coloured LEDs normalized to constant photon flux density (blue: Tridonic LEDs, green and red: Lumitronix-LEDs). modified after Franke et al. (2013).

	blue	**green**	**red**
peak wavelength (nm)	446	518	638
full width half maximum (nm)	23	35	20
max. spectral irradiance (mW m^{-2} nm^{-1})	0.21	0.13	0.19
irradiance (mW m^{-2})	5.6	4.8	3.9
illuminance (lx)	0.15	2.2	0.62
photon flux density (µmol s^{-1} m^{-2})	0.021	0.021	0.021

After acclimatization time, the night-time illumination was activated. Water flow was reduced to 4.1 ± 0.2 L/h to obtain higher concentrations of melatonin in order to meet the sensitivity range of the applied test. Experimental night light conditions lasted for 10 days.

2.3 Water and tissue sampling

For water sampling in the LC experiment we followed the procedure as described in Brüning et al. (2015). 1 L water samples were collected non-invasively without disturbing the fish. Water was pumped via fibre glass syringe filters (1–2 µm; Roth, Karlsruhe, Germany) to remove particular substances. Sampling times were 11:00, 14:00, 17:00, 20:00, 23:00, 02:00, 05:00, 08:00 and 11:00 h. Water samples were stored at 4 °C in darkness and processed for hormone extractions within 24 h.
The night after the last water sampling of the LC and LI experiment, we excised the pituitaries from 30 fish per treatment (randomly taken from 3 tanks). Fish were killed by cutting the neck, the top of the skull was removed and the brain was exposed. Pituitaries were removed with sharp forceps. Pituitary tissue was stored in RNAlater® (Sigma-Aldrich) for 24 h at 4 °C before removing the RNAlater and freezing the samples at − 20 °C.

2.4 Melatonin extraction and assay

For melatonin extraction, the filtered water samples were pumped through solid phase extraction cartridges (25 mL min^{-1}) (OASIS HLB Plus, Waters, Saint-Quentin En Yvelines Cedex, France). The cartridges have been activated with 5 ml methanol (Baker®). Before and after processing the tank water, cartridges were washed with

5 ml deionized water. After extraction, melatonin was eluted with 5 ml ethyl-acetate (Baker®). The samples were evaporated at 50 °C under a stream of nitrogen and reconstituted in 500 µl phosphate buffer saline (Sørensen's PBS + 5% ethanol + 0.1% bovine serum albumin, pH 7.2).
Melatonin levels were measured by enzyme linked immunosorbent assay (ELISA) with non-extraction melatonin saliva-kits (IBL, Hamburg, Germany).

2.5 RNA extraction and reverse transcription

Total RNA from pituitaries was extracted using an RNeasy extraction kit (Qiagen) following manufacturers protocol. Concentration of total RNA was measured by UV absorption spectrometry using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).
The RNA samples were diluted to a concentration of 10 ng RNA/µl RNAse-free water.
For a subset of RNA samples, RNA integrity number (RIN) was determined with an Agilent 2100 bioanalyser (Agilent Technologies). RIN ranged between 7.6 and 8.3, thus confirming a good integrity of RNA (Fleige and Pfaffl 2006).
Reverse transcription of RNA was carried out using Affinity Script Multiple Temperature Reverse Transcriptase (Stratagene). Reverse transcription was carried out using 1.5 µl poly-dT-Primer, 1:5 dilution (CCTgAATTCTAgAgCTCA(T)17, Biometra, Göttingen), 2 µl 10 × AffinityScript RT buffer (Stratagene), 1 µl 10 nmol of each dNTP, 2 µl DTT 100 mM (Stratagene), 1 µl Affinity Script Multiple Temperature Reverse Transcriptase (Stratagene) in a reaction volume of 10 µl.

2.6 Gene expression analysis by RT-qPCR

Since there were no sequences of the target genes available for perch, we tested primers of a closely related species, the pikeperch, *Sander lucioperca* (Hermelink et al. 2011). Pikeperch-primers for FSHβ-subunit and ribosomal protein L8 (rpL8) as housekeeping gene fitted also for perch and were validated. For LHβ-subunit, primers had to be designed based on the sequence in the above mentioned study and were validated and established for perch (Tab. 3.2). The identity of the products was confirmed after direct sequencing (SEQULAB, Göttingen) and analysis with the database homology search tool BLAST and multiple alignment sequence comparison. Identities were 97% and 100% for *S. lucioperca* LH and FSH respectively (Accession numbers: HQ259049.1 and HQ259048.1).

Table 3.2 Overview of primer specific PCR conditions.

Target gene	Forward primer	Reverse primer	T_A (°C)	Primer-konz.	Product size (bp)	PCR-efficiency
L8	GTTATCGCCTCTGCC AAC	ACCGAAGGGATGCTC AAC	62	375 nM	167	2.02
FSHß	CCTACTGGCAGGGA AGAAC	CCTACTGGCAGGGAA GAAC	64	375 nM	85	1.92
LHß	GGCTGTCCAAAGTGT CACCT	GGGAGAACAGTCAGG GAGCTTAA	62	188 nM	158	1.9

T_A – annealing temperature

Real-time PCR was carried out with a Mx3005 qPCR Cycler (Stratagene) using hot start Taq polymerase (Platinum, Invitrogen) and SYBR Green in a 20 µL reaction volume (2 µL diluted (1:30) cDNA, 0.17 µL dNTP-solution (10 mM per dNTP), 1 × Taq buffer, 2 mM $MgCl_2$, 0.1 µL of 200-fold diluted SYBR-Green I solution, 0.15 µL Primer sense, 0.15 µL Primer antisense (Tib MolBiol), 1 U polymerase) under the following conditions: 95 °C initial denaturation for 7 min 40 s, followed by 40 cycles of 95 °C denaturation for 17 s, primer annealing for 25 s, and 72 °C extension for 25 s.

2.7 Relative mRNA quantification

PCR efficiencies were determined in triplicate with dilution series of pooled pituitary cDNA and ranged between 1.9 and 2.02 (Tab. 2.1). All PCRs were run in duplicate. Expression was determined by the comparative C_T method ($\Delta\Delta C_T$) according to Pfaffl (2001) considering a calibrator sample (pooled pituitary cDNA) and correction for the specific PCR efficiency. Melting curve analyses were carried out to assure assay specificity. Samples were normalized to rpL8 mRNA which was not differentially expressed between all investigated fish.

2.8 Data handling and statistical analysis

Melatonin concentrations in tanks were standardized to 1 kg fish biomass. After confirming that the data met the assumptions of the test (normality of residuals) they were analysed using Linear Mixed Models (LMM, fixed factor: treatment; random factor: tank) (Pinheiro and Bates 2000). No significant tank effects could be identified. The gene expression data was analysed likewise.

Melatonin data was transformed to relative values in relation to the baseline ("change vs. basal value", the time point with lowest hormone concentration in control treatment ➔ 20:00 was defined as baseline and set as 100%). The relative profiles

were tested using a nonlinear regression with a cosine function $f(x) = M + A * \cos(6.283185307/P * (h - K))$ (cosinor-method; (Baganz et al. 2005)),where x is the given time data in hours, M is the MESOR (mean of the data series), A is the amplitude, P is the period length in hours and K is the acrophase in hours (the phase angle of the peak of a cosine wave fitted to the raw data of the time series). The calculated overall solutions for single treatments and control were compared by an F-Test and differences of regression parameters between control and single treatments were evaluated by Student's t test and level of significance for all tests was set at 95%. False discovery rate (FDR) was applied to control for type I errors from multiple testing (Nichols and Hayasaka 2003).
For evaluating the influence of different light colours with the same energy level on nocturnal production, the night-time melatonin concentrations (20:00–08:00 h) of the colour treatments (blue, green, red) were summed up for every replicate individually. After log-transformation to meet the assumptions of normality and equal variance, the difference between the light colour treatments was analysed with ANOVA (post-hoc-test: Sidak).
Additionally, we compared the gene expression data of the control treatments of both experiments and both sexes against each other with a one-way-ANOVA.
The analyses were performed using IBM SPSS Statistics (Version 20).

3 Results

3.1 Melatonin

We found significant differences in melatonin tank water concentrations between the control (dark treatment) and all treatments (LMM treatment effect: $F_3 = 119.13$; $p = 0.000$, posthoc test Sidak: df 216; $p = 0.00$) with melatonin concentrations being up to three times higher in controls compared to light colour treatments (Fig. 3.1). Control fish produced more melatonin over the 24 h sampling period and exhibited an apparent melatonin rhythm with a rise at beginning of sunset and a decline at sunrise.

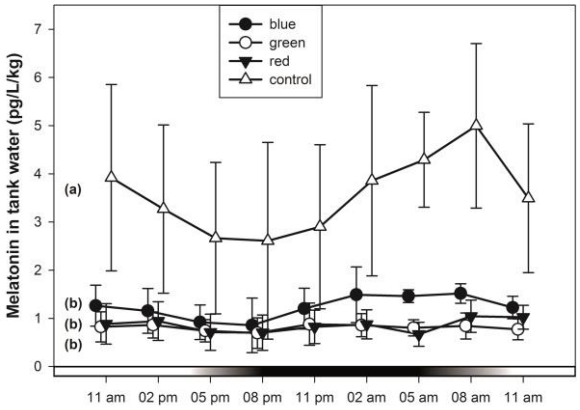


Figure 3.1 Concentration of melatonin per L tank water in pg/L/kg perch biomass (mean ± standard deviation (SD)). Comparison between 4 illumination treatments (n = 6) with blue, green, red and no light (control) at night within 24 h. Photoperiod was 14 h light/10 h treatment with simulated sunrise and sunset of 3 h (see bar at the bottom of the graph, black indicates night, white indicates daytime). Different letters indicate significant differences ($p \leq 0.05$) between treatments.

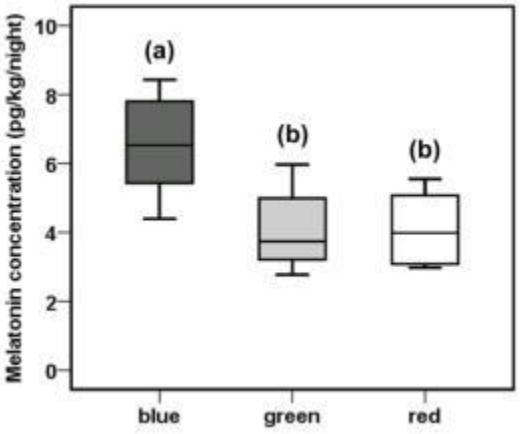


Figure 3. 2 Comparison of nocturnal melatonin concentrations (20:00–08:00 h) in the tank water with different colour treatments. Data are shown as box-and-whisker plots. Different letters indicate significant differences ($p \leq 0.05$) between treatments.

The ANOVA of the total nocturnal melatonin concentrations demonstrated significantly lowered nocturnal melatonin concentration in the green and red treatment (ANOVA treatment effect: $F_2 = 6.026$, $p < 0.05$, post-hoc test: Sidak) compared to the blue treatment (Fig. 3.2). This is confirmed by the cosinor regression results. The exposure to green and red light altered the rhythm significantly (F-test, $p < 0.05$; FDR-controlled), whereas there is no significant differences between the control and the blue treatment. The R^2 value is highest for the control treatment followed by the blue treatment, indicating the best fit of the cosinor model compared to the other treatments (Fig. 3.3). The rhythmical parameters of the cosinor analysis MESOR (M), amplitude (A), acrophase (K) and Period (P) are shown in Tab. 3.3. The rhythmical parameters of the blue treatments were not significant different from control, whereas MESOR and amplitude of the green and red treatment where significantly lower compared to the control treatment. The acrophase as well was lower in the green and red treatment although only significant for green. The period length was the same in all treatments.

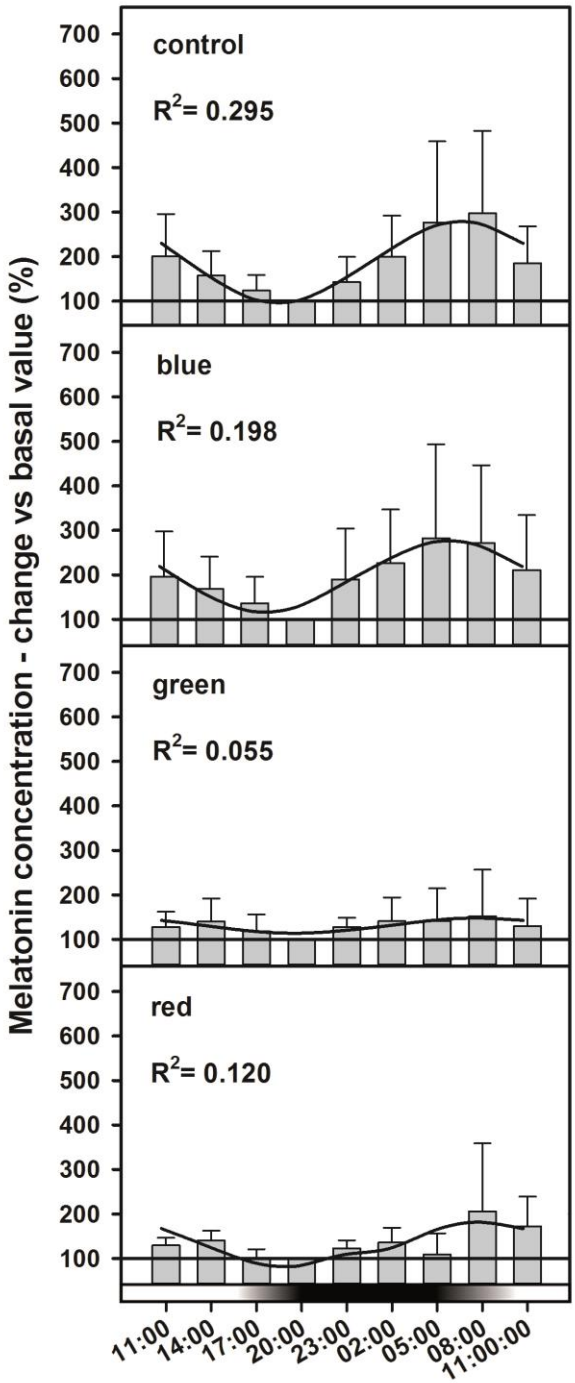


Figure 3.3 Change versus basal value at 20:00 h of relative melatonin concentrations (mean ± SD) in the tank water of treatments (control, red, green, blue). Illustration of the change of melatonin concentration compared to the 20:00 h value (baseline); solid line: regression curve of the cosine regression; at the bottom of the columns day, dawn and dusk and night is represented by white, grey and black respectively.

Table 3.3 Results of Student's t-test (p-values). Rhythmical parameters of cosinor analysis: MESOR (M), amplitude (A), acrophase (K) and period length (P) of light colour treatments compared to control (dark treatment). Significant differences are shown by *> (significantly higher than control, FDR-controlled), *< (significantly lower than control, FDR-controlled), ns (no significant difference).

	treatment					
parameter	blue		green		red	
M (%)	0.695	ns	0.001	*<	0.002	*<
A (%)	0.744	ns	0.000	*<	0.026	*<
K (hours)	0.698	ns	0.005	*>	0.311	ns
P (hours)	0.989	ns	0.871	ns	0.441	ns

3.2 Gene expression of LHß- and FSHß-subunits

There were no significant differences in the mRNA expression of LHβ and FSHβ in male and female perch respectively, when subjected to different light colours at 0.021 µmol s^{-1} m^{-2} (0.15–2.2 lx depending on colour treatment) or no light in the night with a photoperiod simulating September conditions (Fig. 3.4). In contrast, gene expression analyses of LHβ and FSHβ from female perch subjected to different light intensities in the night with a photoperiod simulating October conditions revealed significant differences in both, LHß and FSHß gene expression (Fig. 3.5) that was highest in the control treatment and significantly lowered in light treatments. On the contrary, different light intensities did not affect mRNA expression of LHβ and FSHβ in male perch. Direct comparison between male and female controls did not reveal significant differences of mRNA expression levels of LHβ and FSHβ (Fig. 3.6). A comparison between the controls of the different treatments resulted in significantly higher mRNA expression levels of FSHß in males of the LI experiment (October light conditions) relative to the LC experiment (September light conditions) (Fig. 3.7). Similar tendencies are visible for LHß in male and both gonadotropins in female but differences are not significant.

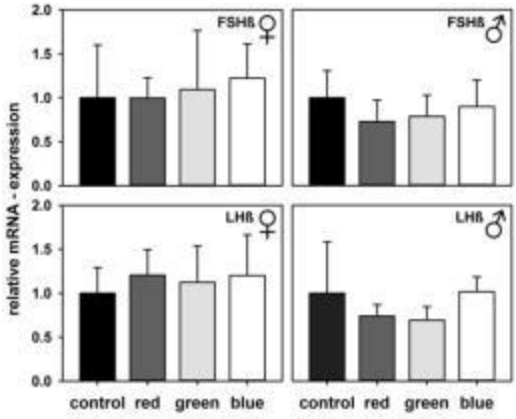


Figure 3.4 Relative mRNA expression of luteinizing hormone (β-subunit; LHβ) and follicle-stimulating hormone (β-subunit; FSHβ) in brain-pituitary tissue of *P. fluviatilis* subjected to different qualities of night-time illumination in September: control (male/female: N = 8), red (male: N = 8, female: N = 7), green (male/female: N = 8) and blue (male/female: N = 9). Data is shown as mean ± SD. Photon flux density was 0.021 µmol s^{-1} m^{-2} (0.15–2.2 lx depending on colour treatment). ΔΔCT values are shown relative to the control treatment.

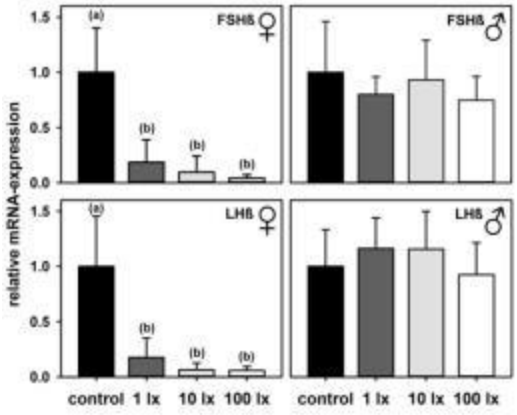


Figure 3.5 Relative mRNA expression of luteinizing hormone (β-subunit; LHβ) and follicle-stimulating hormone (β-subunit; FSHβ) in brain-pituitary tissue of *P. fluviatilis* subjected to different quantities of white night-time illumination in October: control (male: N = 8, female: N = 7), 1 lx (male/female: N = 3), 10 lx (male/female: N = 8) and 100 lx (male/female: N = 9). Data is shown as mean ± SD. ΔΔCT values are shown relative to the control treatment. Different letters indicate significant differences ($p \leq 0.05$) between treatments.

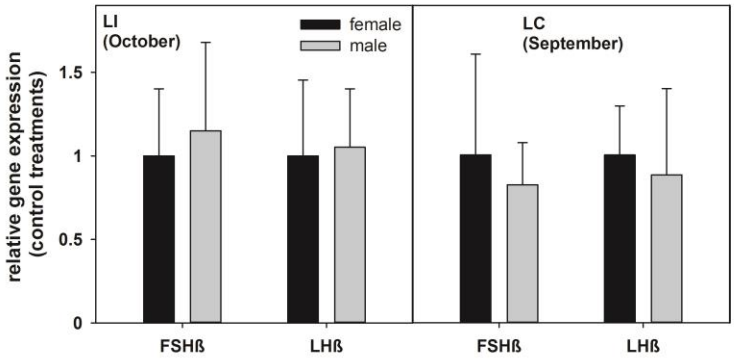


Figure 3.6 Relative mRNA expression of LHß and FSHß subunits in brain-pituitary tissue of *P. fluviatilis*. Comparison between control treatments of male and female for both experiments, light intensity (LI) and light colour (LC). Data is presented as mean ± SD. Male ΔΔCT values are shown relative to the female values.

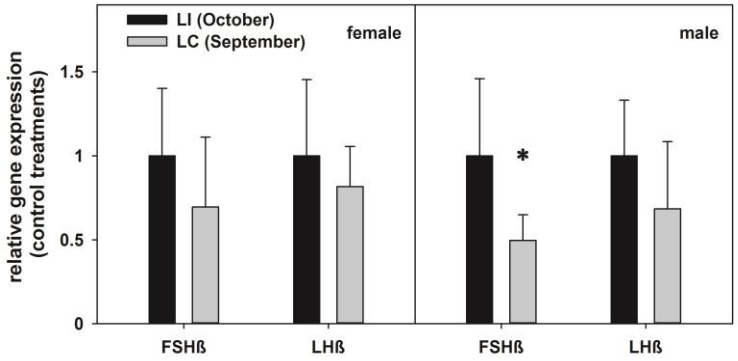


Figure 3.7 Relative mRNA expression of LHß and FSHß subunits in brain-pituitary tissue of *P. fluviatilis*. Comparison between control treatments of the different experiments (LI and LC) for male and female. Data is presented as mean ± SD. Data is presented as mean ± SD. LC ΔΔCT values are shown relative to the LI treatment. Significant difference (Anova, $p \leq 0.05$) is marked by asterisk.

4 Discussion

This study demonstrated that blue, green and red artificial light at night of same photon flux density (0.021 μmol s^{-1} m^{-2}) had a strong effect on melatonin production in European perch. We found a substantial decrease of melatonin concentration in the tank water at all tested wavelengths compared to a dark control. Melatonin concentration in the water of the control tanks was up to 4 times higher compared to all tanks illuminated at night (Fig. 3.1). We investigated, which wavelength has the least effect on melatonin suppression. Fish treated with longer wavelengths seemed to lack a circadian melatonin rhythm, whereas in the blue treatment there was still a rise in melatonin concentration. Statistical comparison of the nocturnal melatonin concentrations of the colour treatments confirmed the differences in the effects of different wavelengths. Blue light had a weaker effect on melatonin suppression with melatonin concentrations during the night being significantly higher (Fig. 3.2), thus the perception of the pineal or other non-visual photoreceptors of European perch are less sensitive to blue light compared to red or green light.

The cosinor analysis of the relative melatonin concentrations revealed differences in the R^2 values. The control treatment had the best fit of the cosine model, followed by the blue treatment ($R^2 \geq 0.2$), suggesting a circadian rhythmicity in both groups which seems to get lost in treatments with green or red light (Fig. 3.3). The comparison of the single treatments with the control showed significant differences between control and green treatment (F-Test, $p < 0.05$; FDR-controlled). In contrast, there were no significant differences between control and the blue treatment. This is confirmed by the comparison of the rhythmical parameters, with significantly smaller means and amplitudes in the red and green treatment compared to the control and no differences

between blue and control (Tab. 3.3). Additionally the value for the acrophase was significantly higher in the green treatment compared to the control, indicating a displacement of the peak of the nocturnal rise of melatonin towards the end of the night. In brief, every light colour lowered the melatonin concentration at night but in contrast to green and red light, blue light had no inhibitory effect on rhythmicity (Fig. 3.1- 3.3).

In preliminary experiments we found that under daylight conditions of the present experimental setup melatonin is stable with respect to degradation (Brüning et al. 2015). These results suggest that differences in melatonin concentrations originate from the impaired melatonin production rather than any degradation of melatonin due to artificial illumination.

Up to now, there is not much information about the effect of artificial light colours at night on biological rhythms of fish. Findings in marine or diadromous fishes of the upper 'photic' zone indicate great sensitivity of the pineal or other non-visual photoreceptors to blue light. For example, 1 h pulses of blue light in the mid of the dark phase were proved to be more efficient in suppressing melatonin than green or red light in sea bass (*D. labrax*) (Bayarri et al. 2002). Salmon pineal ex and in vivo exhibited a greater melatonin suppression under blue and green light during the night (12 h) when compared to red light and control (Migaud et al. 2007a, Vera et al. 2010). Also in Senegalese Sole (*Solea senegalensis*) short wavelengths (far violet; 365 nm) were most effective when compared with red light (600 nm), that failed to reduce plasma melatonin (Oliveira et al. 2007).

In general, the spectral sensitivity of pineal photoreceptors and perhaps other melatonin modulating structures seem to differ depending on the environment. In terrestrial mammals (including humans) melatonin is most suppressed by blue light (Brainard et al. 1984, Miyamoto and Sancar 1998, Lockley et al. 2003). In aquatic ecosystems the water acts as a light filter. Thus in different aquatic ecosystems, different photic environments exist. Non turbid marine environments appear to be blue, whereas most lakes and streams are dominated by yellow light (Wetzel 2001). Unfortunately there is no information about spectral sensitivities of other non-visual photoreceptors. However, Forsell et al. (2001) found evidence for pigments with sensitivity in the green and ultraviolet part of the spectrum in the pineals of several fish species including freshwater and marine species, but the sensitivity of pineal photopigments still seems to be species specific (Ekström and Meissl 2010). Falcón and Tanabe (1983) for example found maximum photoresponses of the pike pineal at mid wavelengths (green and yellow). That corresponds to our findings in perch where blue light is less affective, compared to green and red light. These similarities make sense since pike and perch use the same habitats.

It is commonly known that the melatonin rhythm provides information about time of the day and the season (Underwood 1989, Iigo and Aida 1995, García-Allegue et al. 2001). In middle Europe fish reproduction is characterized by a distinct seasonal

pattern and along with temperature, photoperiod is the most important factor. The timing of reproduction in fish strongly depends on light and the light dependent production of melatonin is one of the decisive factors. This is where ALAN may intervene. Falcón et al. (2010) reviewed the interaction of the photoperiod or rather melatonin with the reproductive process in fishes and pointed out the effects of ALAN on reproduction, indicating that continuous photoperiod during a crucial period for reproduction in temperate fish species (fall) leads to a hormonal cascade in the hypothalamic–pituitary–gonad axis. In this scenario continuous light suppresses the melatonin rhythm, and also leads to a suppression of gonadotropin production, production of sex steroids and maturation of gonads. In a previous study we investigated the influence of artificial white light at night of different intensities on the melatonin rhythm of European perch (Brüning et al. 2015). We found that white light at intensities of 1 lx suppressed production of melatonin under the experimental conditions. With this work, we present evidence, that this light level also has the potential to disrupt reproductive processes. The expression of LHß and FSHß in female perch was significantly lowered by light at approximately 1 lx and above (Fig. 3.3). This is supported by numerous studies that investigated continuous light or prolonged photoperiods in order to control reproductive processes of fish in aquaculture (Macquarrie et al. 1979, Thrush et al. 1994, Kolkovski and Dabrowski 1998, Kissil et al. 2001, Rodríguez et al. 2005, García-López et al. 2006, Davie et al. 2007). We couldn't find differences in gonadotropin expression in male perch. That may be due to the fact, that male reproductive cycle starts earlier and the maturation of the gonads ends earlier than in females (Migaud et al. 2006b). It is most likely, that an important task of the gonadotropins, initiating gonadal recrudescence, was already fulfilled at this time. Another possibility is that male perch are not that sensitive to ALAN or already reached a "point of no return", where such disturbances have no influence anymore. This is supported by the comparison of the gene expression levels between male and female (Fig. 3.6), where no significant differences could be found in the control treatments. As a result, gonadotropin gene expression of male perch in the light treatments has to be considered as similarly high.

In contrast to the LI-experiment, we did not find any differences in LHß and FSHß gene expression of perch subjected to light of different wavelengths in the night, although these colours had a strong effect on the melatonin rhythm. One assumption is, that the perch in our experiment where not yet susceptible to light disturbances regarding reproductive processes. This experiment was carried out in September, under simulated natural photoperiodic conditions. Another study in male and female perch (Sulistyo et al. 1998, Sulistyo et al. 2000) examined the reproductive cycle by measuring the sexual steroids 17β-estradiol, 11-ketotestosterone and testosterone. All these hormones started to rise around October/November. Probably, in our light colour experiment, the reproductive cycle had not yet begun and thus FSHß and LHß expression was too low to get any differences between dark and colour treatments.

This is supported by the comparison between the control treatments of both experiments. Both, male and female showed, though only significant in male for FSH, higher gonadotropin gene expression levels in the light intensity treatment with the October light conditions.
In conclusion, we found differences in the impact of different light colours in the night on melatonin concentration. Compared to a dark night, all colours suppressed melatonin production. Comparison of the different wavelengths on nocturnal melatonin proved that blue light is less effective for melatonin suppression in perch, corresponding to the light conditions in their habitats. We couldn't find proofs that longer wavelengths have a greater impact on reproduction. However, we found a great impact of low intensity white light (1 lx and higher) at night, significantly suppressing gonadotropin production of female perch. Thus, ALAN of different colours is able to affect biological rhythms by disturbing the melatonin rhythm of perch and at least white light is proved to interfere with reproductive traits. In 2001 it was estimated that around 19% of the global land surface is above a light threshold set for a polluted status. Until 2010, continental trend is towards increasing light pollution (Baganz et al. 2005, Bennie et al. 2014). Taken together, these results suggest that the increasing illumination of the night can be a serious threat to fish communities and, taken into account the various examples of implications on other species, on whole ecosystems.

Acknowledgements

The Authors like to thank M. Kunow and the students at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries for the technical support. Funding was provided by the Verlust der Nacht project (Federal Ministry of Education and Research, Germany, BMBF-033L038A).

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Chapter 4

Influence of light intensity and spectral composition of artificial light at night on melatonin rhythm and expression of gonadotropins in roach *Rutilus rutilus*

Influence of light intensity and spectral composition of artificial light at night on melatonin rhythm and expression of gonadotropins in roach *Rutilus rutilus*

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Abstract

In this study we investigated the influence of artificial light at night (ALAN) of different intensities (0, 1, 10, 100 lx) and different colours (blue, green, red) on the daily melatonin rhythm and reproductive traits of roach *Rutilus rutilus*.
Melatonin concentrations were significantly lowered under nocturnal white light already at 1 lx. Low intensity blue, green and red ALAN decreased the melatonin concentration significantly in comparison to a dark control. We conclude that ALAN can disturb melatonin rhythms in roach already at very low intensities and at different wavelengths and thus light pollution in urban waters has the potential to impact biological rhythms in fish. Gene expression of gonadotropins was not affected by ALAN during the period of the experiments. We assume that roach possess a "photo-labile" period, a small time frame during which an impact of ALAN on reproduction is possible.

Keywords: biological rhythms; fish; light colour; light pollution; reproduction

1 Introduction

Light pollution research is not anymore restricted to astronomy (Riegel 1973) or to technical enhancements to save costs and energy use (Isobe and Hamamura 2000, Kyba et al. 2014). In the past 20 years it expanded more and more towards investigation of ecological implications (Hölker et al. 2010b, Gaston et al. 2012, Gaston et al. 2015). A wide range of species was investigated with regard to the effect of ALAN on physiology and behaviour. These studies include research on humans (Pauley 2004, Anisimov 2006, Chepesiuk 2009), other mammals (Redlin 2001, Bird et al. 2004), birds (Miller 2006, Titulaer et al. 2012, de Jong et al. 2016), reptiles (Witherington and Bjorndal 1991, Tuxbury and Salmon 2005), amphibians (Buchanan 1993, Baker and Richardson 2006), and insects (van Langevelde et al. 2011, Perkin et al. 2014b, Jones et al. 2015). Only recently also publications with a

more general approach emerged, dealing with the effects of ALAN on species communities, ecosystems and social aspects (Hölker et al. 2010a, Hölker et al. 2010b, Perkin et al. 2011, Davies et al. 2012, Gaston 2013, Gaston et al. 2013, Kurvers and Hölker 2014, Hölker et al. 2015).

Many studies have focused on light-induced effects in fish. The majority of this research, however, relates to aquaculture, i.e. control of reproduction by the use of artificial light (Porter et al. 1999, Zakes and Szczepkowski 2004, Mylonas et al. 2010) and improvement of growth and survival (Oppedal et al. 1997, Taranger et al. 2006, Villamizar et al. 2009). Studies focusing on biological rhythms in fish related to ALAN are less frequent (Brüning et al. 2011, Becker et al. 2013, Perkin et al. 2014c, Brüning et al. 2015, Newman et al. 2015, Riley et al. 2015, Brüning et al. 2016, Brüning et al. submitted).

The natural circadian rhythm of fish is mainly controlled by the pineal organ, a light sensitive part of the brain. It transduces light signals into hormonal signals, i.e. melatonin. The pineal complex is considered to be the major circadian pacemaker (Underwood 1989, Falcón et al. 2009). However, other tissues as well are known to be light sensitive and may play a role in modulating the biological rhythm, e.g. deep brain photoreceptors (Kojima et al. 2000, Philp et al. 2000, Fernandes et al. 2012), the saccus vasculosus that may be a sensor for seasonal information (Tsuneki 1992, Nakane et al. 2013) or non-image forming (or even visual) photoreceptors in the retina (Kojima et al. 2000, Philp et al. 2000, Peirson et al. 2009).

Nevertheless, melatonin is considered to be the key driver of biological rhythm in fish (Ekström and Meissl 1997, Falcón et al. 2006, Falcón et al. 2011). Melatonin levels are high at night and low during the day. The oscillations and shape of this melatonin rhythm provides information about day and season that can be used to synchronize physiological and behavioural processes with the photoperiod. ALAN has been proven to impair this rhythm by suppressing melatonin in several fish species (Porter et al. 1999, Bayarri et al. 2002, Vera et al. 2005, Ziv et al. 2007). Most of these studies used relatively high light intensities but light pollution is regarded to be of considerable low intensity. It is characterized as direct glare from streetlamps, lighted buildings or advertisements or indirect as sky glow, artificial light that is repulsed from the cloud coverage (Longcore and Rich 2004). Especially in the vicinity of urban areas these lights can be bright enough to penetrate water, causing e.g. light levels of 1.4 lx at 50 cm depth in a turbid stream in Berlin (Perkin et al. 2014a). Street lights (70W high-pressure sodium lamps, 4.75 m height) 3 m from the water's edge have been found to produce light level of 6.8–8.5 lx at the sediment surface of a ditch in 50 cm depth (Hölker et al. 2015). Few research has been done to assess the impacts of such low light intensities on biological rhythms of fish and mostly in commercial marine fish species; only few attempts had been made to find the lowest effective light intensity to impact e.g. the melatonin rhythm (Bayarri et al. 2002, Migaud et al.

2006a, Takemura et al. 2006, Oliveira et al. 2007, Vera et al. 2010, Brüning et al. 2015).

In previous studies (Brüning et al. 2015, Brüning et al. 2016) we investigated the effect of low light intensity ALAN on melatonin rhythm in diurnal European perch *Perca fluviatilis* L. 1758, and subsequent effects on reproductive traits. Already at 1 lx nocturnal melatonin concentration was substantially lowered and reproductive traits were affected. Also in a field study, impacts of ALAN on reproduction of *P. fluviatilis* and roach *Rutilus rutilus* (L. 1758) were found, although differences in melatonin could not be discriminated (Brüning et al. submitted). However, in Falcón et al. (2010) and Migaud et al. (2010) the probable link between the daily and seasonal melatonin rhythm and maturation and reproduction in fish is reviewed comprehensively.

The globally changing nocturnal light regime includes also spectral alterations, especially in view of the constantly developing lighting technologies (i.e. LED) that are increasingly used in public lighting (Hölker et al. 2010a). Only recently this problem and the effect on different species, excluding fish, has been addressed (Davies et al. 2013).

The influence of different light colours on fish was mostly investigated in terms of stress physiology, growth and behaviour (Volpato and Barreto 2001, Marchesan et al. 2005, Villamizar et al. 2009) whereas the spectral sensitivity of fish was first and foremost studied in terms of retinal physiology (Cameron 1982, Douglas 1986, Cowing et al. 2002). Differential effects of different wavelengths on the circadian axis, particularly melatonin suppression, received attention mainly in recent years. The current results suggest that the spectral sensitivity of the circadian system is species specific and obviously an expression of habitat use. Marine environments are dominated by short wavelengths and consequently melatonin in marine species is most suppressed by light in this range (Bayarri et al. 2002, Migaud et al. 2007, Oliveira et al. 2007, Vera et al. 2010). European freshwater habitats are often dominated by yellow light and longer wavelengths, depending on suspended material like algae or humic substances (Wetzel 2001). Accordingly visual and non-visual photoreceptors of e.g. zebrafish *Danio rerio* (Hamilton, 1822) and pike *Esox lucius* L. 1758 (Falcón and Meissl 1981, Falcón and Tanabe 1983, Enright et al. 2015) and presumably *P. fluviatilis* (Brüning et al. 2016) are more susceptible to green and red light. For cyprinids no such information is available.

This study focuses on the light sensitivity of the melatonin rhythm and reproductive components in *R. rutilus*, a common omnivore in Eurasian fresh waters. *R. rutilus* are usually diurnal and crepuscular (Hölker and Breckling 2005). They show a strong antipredator behaviour to nocturnal predators by reduced activity and habitat shifts (Hölker et al. 2007). Nocturnal feeding has also been reported, e.g. in a periurban lake, which is experiencing ALAN (Okun and Mehner 2005, Perkin et al. 2014a).

We hypothesize that white ALAN has a clear effect on the natural circadian rhythm of melatonin production, already at low light intensities. Furthermore we hypothesize that blue light has a weaker effect on melatonin suppression compared to green or red light, as anticipated for European freshwater fishes that use similar habitats to *E. lucius* and *P. fluviatilis*.
Finally we examine the influence of different light intensities and colours on the gene expression of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as important components of the reproductive process.

2 Methods

Experiments were conducted with the legal review of animal testing and approval of the Berlin State Office of Health and Social Affairs (LAGeSo, reference number G 0113/12).

2.1 Experimental Fish

R. rutilus were obtained from a local fish farmer (Martin Keil, Teichwirtschaft Hammermühle, Lindena, Germany) where they were kept in outdoor ponds. After transportation to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, IGB, in Berlin, they were held in indoor tanks in a flow-through system with freshwater, aeration and natural photoperiod and fed daily with dry food (Aller Silver 2mm, Emsland-Aller Aqua). Fish body mass at the time of the experiment was 28.3 ± 9.9 g (mean ± SD). Temperature was 15 °C and water parameters were checked regularly.

2.2 Experiment

The experimental setup was previously described by Brüning et al. (2015) and Brüning et al. (2016). Briefly, each treatment was performed with 6 replicates (6 experimental tanks, 84 L). Stocking density was 30 fish per tank. In the light intensity experiment (LI), nocturnal white illumination was 0 lx (control), 1 lx, 10 lx and 100 lx. White light was provided by fluorescent bulbs with best available natural spectrum. In the light colour experiment (LC), night-time illumination was a dark control, blue, green and red light, provided by narrow bandwidths LED lights (for lighting details see Franke et al. (2013)). LEDs were normalized to a photon flux density of 0.021 µmol s^{-1} m^{-2}; equivalent to 0.15 lx (blue), 2.2 lx (green) and 0.62 lx (red).

During acclimatisation, complete daylight was from 9:00 to 15:00 h and dusk and dawn of three hours was provided, starting at 6:00 and 15:00 h, respectively (December conditions). Daylight intensity was about 7000 lx, provided by 3 fluorescent bulbs per tank.
During a 14-days acclimatization period, the night was dark in all aquaria. Fish were fed with dry food (0.5% of body mass) twice a day. Water temperature was 12 °C and flow rate was 10 L/h. After acclimatization, the nocturnal illumination was activated. Water flow was reduced to 4.1 ± 0.2 L/h to obtain necessary concentrations of hormones to meet the sensitivity range of the applied test-kits. Experimental night light conditions lasted 12 days.

2.3 Water and tissue sampling

Water and tissue sampling was performed as described by Brüning et al. (2015) and Brüning et al. (2016).
1 L water samples were collected non-invasively and pumped through a fibre glass Whatman filter paper to a glass bottle. Sampling times were 08:00 h, 11:00 h, 14:00 h, 17:00 h, 20:00 h, 23:00 h, 02:00 h, 05:00 h, 08:00 h. All water samples were taken simultaneously by a multichannel peristaltic pump. Water samples were stored at 4 °C in darkness and processed for melatonin extraction within 24 h.
The night after the last water sampling, pituitaries were excised from 30 fish per treatment (randomly taken from 3 tanks). Pituitary tissue was stored in RNAlater® (Sigma-Aldrich) for 24 h in the fridge before removing the RNAlater and freezing the samples at -20 °C.

2.4 Melatonin extraction and assay

Filtered water samples were pumped through solid phase extraction cartridges (25 mL min^{-1}) (OASIS HLB Plus, Waters, Saint-Quentin En Yvelines Cedex, France) that were activated with 5 ml methanol (Baker®) and washed with destilled water before and after processing tank water. Melatonin was eluted from the cartridges with 5 ml ethyl-acetate (Baker®). Samples were evaporated at 50 °C under a stream of nitrogen and reconstituted in phosphate buffer saline (*Sørensen's* PBS + 5 % Ethanol + 0.1 % bovine serum albumin, pH 7.2).
Hormone levels were measured by enzyme linked immunosorbent assay (ELISA) using commercial non-extraction melatonin saliva-kits (IBL, Hamburg, Germany).

2.5 RNA extraction and reverse transcription

RNA extraction and reverse transcription was carried out as previously described (Brüning et al. 2016). Total RNA from pituitaries was extracted using RNeasy extraction kit (Qiagen) and concentration of total RNA was measured by UV absorption spectrometry (Nanodrop ND-1000 spectrophotometer; Thermo Fisher Scientific).
RNA integrity number (RIN) was determined with an Agilent 2100 bioanalyser (Agilent Technologies) and ranged between 7.6 and 8.3, confirming a good integrity (Fleige and Pfaffl 2006).
Total RNA was reversely transcribed with AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene).

2.6 Gene expression analysis by RT-qPCR

For qPCR of LHß, FSHβ and ribosomal protein L8 (rpL8) as housekeeping gene, the primers designed by Trubiroha et al. (2012) were used (Accession-numbers: FJ769369 (FSHß); AY770579 (LHß); FJ769335 (rpl8)). The identity of the products was confirmed by direct sequencing (SEQULAB, Göttingen) and analysis with the database homology search tool BLAST.
PCR was carried out with a Mx3005 qPCR cycler (Stratagene) using hot start Taq polymerase (Platinum, Invitrogen) and SYBR Green in a 20 µL reaction volume as described previously by Brüning et al. (2016).
PCR efficiencies were determined in triplicate with a dilution series of pooled pituitary cDNA and ranged between 1.93 and 1.98. All PCRs were run in duplicate. Expression was determined according to Pfaffl (2001) by comparative C_T method ($\Delta\Delta C_T$) with respect to a calibrator sample (pooled pituitary cDNA) and correction for the specific PCR efficiency. A melting curve analysis was carried out to assure assay specificity. Samples were normalized to rpL8 mRNA (reference gene) which was not differentially expressed between all investigated fish.

2.7 Data handling and statistical analysis

Melatonin concentrations in tanks were normalised to 1 kg fish biomass. After confirming that the data met the assumptions of the test (normality of residuals) they were analysed using Linear Mixed Models (LMM, fixed factor: treatment, time; random factor: tank) (Pinheiro and Bates 2000). No significant tank effects could be identified. The gene expression data was analysed likewise.

In addition, we compared the gene expression data of the control treatments of both sexes for both experiments using One-Way-Anova.
For evaluating the influence of different light colours with the same energy level on nocturnal melatonin production, night-time melatonin concentrations (20:00–08:00 h) of the colour treatments (blue, green, red) were summed up for every replicate individually. After log-transformation to meet the assumptions of normality and equal variance, the difference between the light colour treatments was analysed with ANOVA (post-hoc-test: Sidak).
The level of significance for all tests was set at p= 0.05.
The analyses were performed with IBM SPSS Statistics (Version 20).

3 Results

We found significant differences of melatonin concentrations in the tank water between control and the LI treatments (LMM; treatment effect: $F_{3,216}$ = 249.8; $p < 0.05$).
The melatonin level in the control treatments was up to 20 times higher in comparison to the other treatments. However, a distinct raise of melatonin concentration is visible in the 1 lx treatment towards the end of the night (05:00 h). This is supported by the LMM with a significant time effect for the control treatment ($F_{8, 51} = 2.9$; $p < 0.05$) and 1 lx treatment ($F_{8, 54} = 19.2$; $p < 0.05$) (Fig. 4.1).

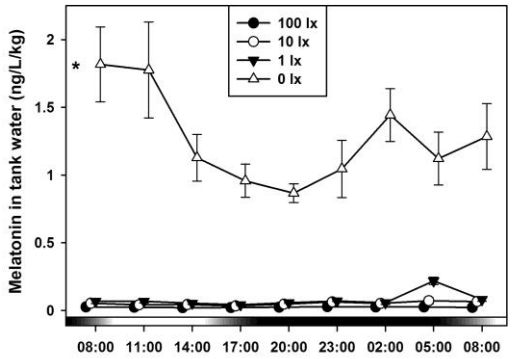

Figure 4.1 Concentration of melatonin in tank water in ng/L/kg roach biomass (mean ± SD). Comparison between 4 illumination treatments (n=6) with 0 lx, 1 lx, 10 lx and 100 lx at night within 24 h. At the bottom of the graph day, dawn, dusk and night are shown as white, grey and black bars. Significant difference ($p \leq 0.05$) between 0 lx and the other treatments is represented by asterisk.

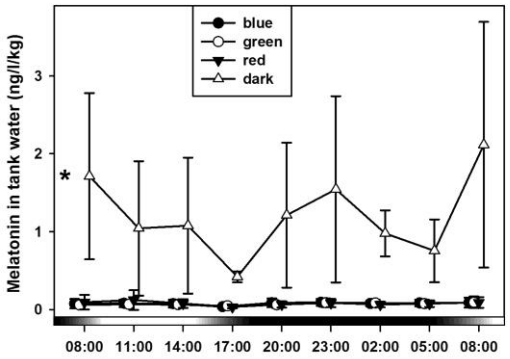

Figure 4.2 Concentration of melatonin per L tank water in ng/L/kg roach biomass (mean ± SD). Comparison between 4 illumination treatments (n=6) with blue, green, red and no (”dark”) light at night within 24 h. At the bottom of the graph day, dawn, dusk and night are shown as white, grey and black bars. Significant difference ($p \leq 0.05$) between “dark” and the other treatments is represented by asterisk.

Melatonin concentrations in the tank water of the blue, green and red treatments were significantly lowered compared to the control (LMM, treatment effect: $F_{3, 216} = 77.7$; $p < 0.05$). A significant time effect (LMM, time effect: $F_{8, 54} = 2.2$; $p < 0.05$) was only detected in the control treatment (Fig. 4.2).
There were no differences in nocturnal (20:00- 08:00) melatonin production between the three light colour treatments (Fig. 4.3). No significant differences were found in the expression of pituitary gonadotropins LH and FSH, neither in the LI experiment nor in the LC experiment (Fig. 4.4).

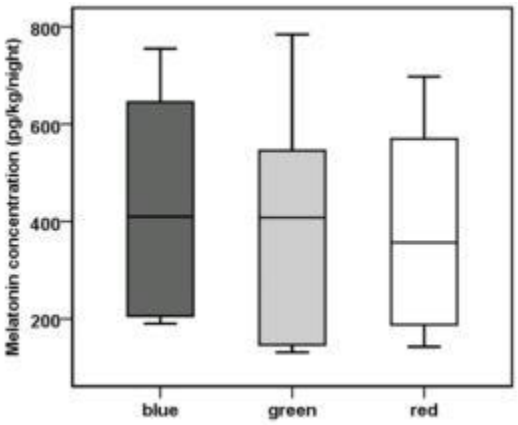

Figure 4.3 Nocturnal melatonin concentrations (20:00 – 08:00 h) in the tank water. Comparison of different colour treatments. Data is shown as box-and-whisker plots. There was no significant difference between the blue, green and red treatment

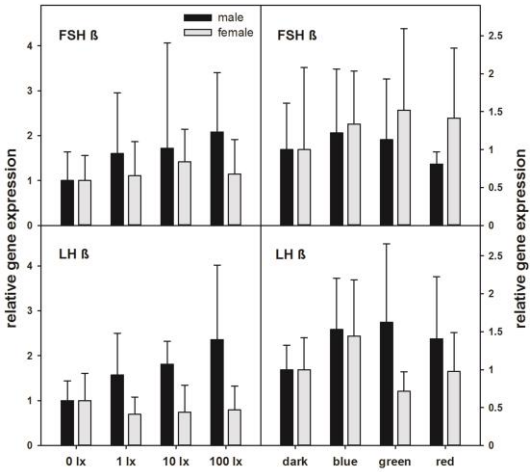

Figure 4.4 Relative mRNA expression of luteinizing hormone (β-subunit; LHβ) and follicle-stimulating hormone (β-subunit; FSHβ) in brain-pituitary tissue of *R. rutilus* subjected to different quantities of white night-time illumination: 0 lx (control; male: N= 3, female: N= 15), 1 lx (male: N= 5, female: N= 12), 10 lx (male: N= 4, female: N= 12) and 100 lx (male: N= 7, female: N= 9) and different qualities of night-time illumination: dark (control; male: N= 7, female: N= 8), red (male: N= 4, female: N= 10), green (male: N= 3, female: N= 11) and blue (male: N= 3, female: N= 13). Data is shown as mean ± SD.

We tested the control treatments of male and female *R. rutilus* against each other for both experiments in order to detect gender-specific differences in gonadotropin gene expression. Gene expression levels of LH and FSH in the LI experiment was significantly higher in females (ANOVA; LH: $F_{1, 16} = 9.6$; FSH: $F_{1, 16} = 5.9$; $p < 0.05$), whereas in the LC treatment gene expression levels of FSH were higher in males and

LH expression levels were higher in females (ANOVA; LH: $F_{1, 12} = 8.2$; FSH: $F_{1, 12} = 14.8$; $p < 0.05$) (Fig. 4.5).

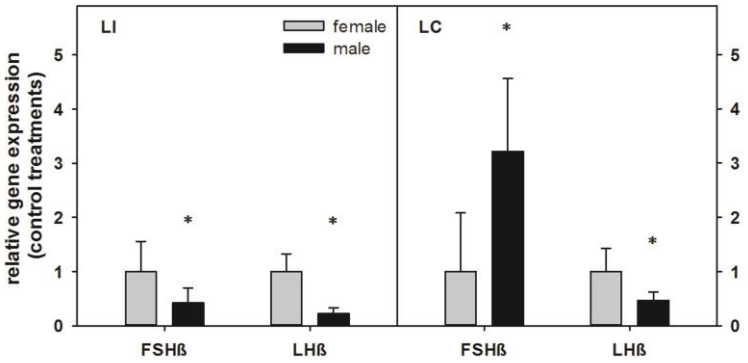

Figure 4.5 Relative mRNA expression of LHß and FSHß subunits in brain-pituitary tissue of *R.rutilus*. Comparison between control treatments of male and female for both experiments, light intensity (LI) and light colour (LC). Data is presented as mean ± SD. Significant difference ($p \leq 0.05$) is represented by asterisk.

4 Discussion

With this study we demonstrate that low intensity ALAN has a strong effect on the natural melatonin rhythm of *R. rutilus*. We found a significant decrease of melatonin concentration in the tank water already at 1 lx of white ALAN. Furthermore, we found that light at all tested colours substantially decreased the melatonin concentration in the tank water. Consequently, it can be stated that low intensity white ALAN as well as low intensity narrow bandwidths (blue, green and red) interfere with the natural rhythm of melatonin production of fish, i.e. roach.

However, although the melatonin concentration in the 1 lx treatment was up to 20 times lower than the control, there was still a noticeable increase towards the end of the night (Fig. 4.1). Therefore, the threshold light intensity for melatonin suppression must be even lower than 1 lx. These results demonstrate that light is a main driver of melatonin rhythm in *R. rutilus* and ALAN is able to modify these rhythms. It is already known for several fish species, that light or photoperiod modification can also modify concomitant traits or behaviours, particularly when they are based on diurnal or seasonal rhythms. In aquaculture, this has been used e.g. to improve growth with prolonged photoperiods and thus prolonged feeding possibilities (Duray and Kohno 1988, Imsland et al. 1995, Oppedal et al. 1997, Biswas et al. 2005, Veras et al. 2013, Imsland et al. 2014) or to control swimming depth and shoaling to prevent crowding (Juell and Fosseidengen 2004). In nature, however, ALAN can be also disadvantageous, since growth improvement is often a consequence of delayed

maturation and impaired reproduction. Furthermore, brighter light conditions in the water may extend the feeding window of visual predators into the night and thereby lead to a unnatural top-down regulation in fish communities (Becker et al. 2013). For fry of Atlantic salmon *Salmo salar* L. 1758, the period between dispersal and the establishment of defended feeding territories is a critical period (Armstrong et al. 2003). A dose–response study for both, a delay in dispersal timing and disruption of diurnal pattern, was not linear, with a strong effect apparent at 1 lx, and little or no additional impact seen when the light intensity was increased further (Riley et al. 2015), which corresponds to our findings concerning melatonin suppression. Altogether this may lead to a change in species composition and thus even affect whole ecosystems.

The influence of different light colours on biological rhythm of fish seems to be species specific and a reflection of the habitat the fish lives in. Seabass *Dicentrarchus labrax* (L. 1758) for example is a marine fish species whose habitat is mostly dominated by short wavelengths. Bayarri et al. (2002) found that in this species circulating melatonin is most effectively suppressed by blue light. In contrast, in *P. fluviatilis* blue light is less effective in suppressing nocturnal melatonin, compared to red and green light (Brüning et al. 2016) and in *E. lucius* pineal photoreceptor pigments are likewise maximum sensitive to red and green (Ekström and Meissl 2010). Unlike *D. labrax*, *P. fluviatilis* inhabit mainly freshwater lakes and streams that are dominated by green-yellow light or longer wavelengths resulting from suspended particles like algae or humins (Wetzel 2001). Since *R. rutilus* evolved under similar environmental conditions, we expected melatonin suppression to be likewise less sensitive to blue light. Contrary to our expectations, there were no statistical differences in tank water melatonin concentrations between the three LC treatments as in the above mentioned study with *P. fluviatilis*; moreover, the melatonin concentration was significantly lower in all colour treatments, compared to the dark control (Fig. 4.2). In contrast to *P. fluviatilis*, where blue light had the least influence on nocturnal melatonin suppression (Brüning et al 2016), a similar comparison of the nocturnal melatonin concentrations (20:00 – 08:00) in *R. rutilus* yielded no differences between the three light colours (Fig. 4.3). With these results we conclude, that low intensity white, blue, red and green light in the night are similarly capable of reducing the nocturnal melatonin concentration in *R. rutilus*. Obviously the sensitivity of *R. rutilus* towards light, especially low intensity narrow bandwidths is higher compared to *P. fluviatilis*. Studies with even lower light intensities should reveal threshold light intensities and effects of different light colours on the circadian axis.

When considering that light pollution in some urban areas like Berlin can cause underwater brightness of 1.4 lx at 50 cm depth (Perkin et al. 2014a), even in a turbid river like the river Spree, it is reasonable to conclude that light pollution has the potential to impact the natural melatonin rhythm and subsequently rhythmical

physiological and behavioural processes in fish, especially *R. rutilus* as species that is apparently more light sensitive.

The greatest advantage of the use of light in aquaculture is the possibility to control reproduction by photoperiod manipulation. It is a common practice to prevent or delay maturation in order to improve somatic growth or induce out of season spawning. In several farmed finfish these effects are well studied (Macquarrie et al. 1979, Thrush et al. 1994, Oppedal et al. 1997, Kolkovski and Dabrowski 1998, Kissil et al. 2001, García-López et al. 2006, Migaud et al. 2006b, Davie et al. 2007). Since most of these processes are related to the melatonin rhythm, it is most likely that ALAN can modify them already at a very low level. The disturbance of the melatonin rhythm by low light levels at night and concomitant interference with certain components of the reproductive cycle has been lately proven for *P. fluviatilis* (Brüning et al. 2015, Brüning et al. 2016). Also in other species the link between melatonin and reproduction has been demonstrated (Amano et al. 2000, Bayarri et al. 2004, Oliveira et al. 2010, Renuka and Joshi 2010). In the present experiments we did not find a negative impact of white or coloured ALAN and concomitant depletion of melatonin on the gene expression of gonadotropins (Fig. 4.3) that play a key role in vertebrate reproduction (Planas and Swanson 2008). Falcón et al. (2010) reviewed the role of melatonin in fish behaviour and physiology. Concerning reproduction, they suggested a photo-labile phase, a period during the reproductive cycle where fish are highly susceptible for manipulation. In some fish, this period coincides with the naturally de- or increasing photoperiods in fall and spring. In male *D. labrax*, for example, a period between August and November, coinciding with pre-gametogenesis and gametogenesis was found to be most likely effective in manipulating maturation with continuous light (Felip et al. 2008, Carrillo et al. 2009). Since most fish from temperate European waters, i.e. also *R. rutilus* are seasonal spawners, it is most likely that they possess some kind of photo-labile period, presumably around the onset of the reproductive phase in autumn. If this is the case, the discrepancy between the melatonin suppression by ALAN (Fig. 4.1 and 4.2) and the lacking impact on gonadotropin expression (Fig. 4.4) can be explained by the timing of the experiments. *P. fluviatilis* were found to have a photo-labile period around October (Brüning et al. 2016) in a laboratory experiment with artificial illumination and static temperature. In a field experiment (Brüning et al. submitted) ALAN in August caused impacts on reproduction in both *P. fluviatilis* and *R. rutilus*. The onset of gonadogenesis in *P. fluviatilis* under natural conditions is around August and September and spawning takes place around April (Treasurer 1988, Wang and Eckmann 1994, Sulistyo et al. 1998, Sulistyo et al. 2000). Thus, it is reasonable to assume a photo-labile period during this time. The reproductive cycle *R. rutilus* has more or less the same time frame, with onset of gonadogenesis between August and September and spawning in early May (Jamet and Desmolles 1994, Geraudie et al. 2010, Trubiroha et al. 2012). Presumably a photo-labile period would occur around

August as well. This is confirmed for roach in field experiments of Brüning et al. (submitted), where ALAN during August caused significant impacts on gonadotropin gene expression and serum sex steroid concentrations. Both of the experiments in the present study took place in winter with a photoperiod resembling December light conditions. A photo-labile period around August may be accountable for the ineffectiveness of ALAN in disrupting gonadotropin gene expression in the present study.

Significant differences were found in gene expression levels of LHß and FSHß between male and female *R. rutilus* (Fig. 4.5), which were, except for FSHß in the LC treatment, always higher in female roach. These gender specific differences can be explained by the fact, that gonads mature faster in female than in male *R. rutilus* and also the gonadosomatic index is usually higher (Jamet and Desmolles 1994).

In conclusion, our experiments demonstrated a strong ability of low intensity white light, and low intensity, narrow bandwidths blue, green and red light, to suppress nocturnal melatonin production in *R. rutilus*. ALAN was not effective in disturbing gene expression of gonadotropins in roach during the investigated seasonal period. We conclude that roach have a photo-labile phase around the onset of the reproductive cycle, where ALAN is able to interfere.

Acknowledgements

The authors like to thank Kunow and students at Leibniz-Institute of Freshwater Ecology and Inland Fisheries for technical support. Funding was provided by the "Verlust der Nacht" project (Federal Ministry of Education and Research, Germany, BMBF-033L038A).

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Chapter 5

Influence of artificially induced light pollution on the hormone system of perch and roach in a rural habitat

Influence of artificially induced light pollution on the hormone system of perch and roach in a rural habitat

Anika Brüning, Franz Hölker, Torsten Preuer, Werner Kloas

Abstract

Almost all life on earth has adapted to live in cycles of light and dark by evolving biological rhythms at different temporal scales (e.g., circadian and circannual rhythms) for behavioural and physiological synchrony. Artificial light at night (ALAN) might most likely interfere with these rhythms. In this study we examined the influence of ALAN on nocturnal melatonin and sex steroid concentration in the blood and gene expression of gonadotropins in the pituitary of European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in a rural experimental setting. Fish were held in net cages in drainage channels experiencing either additional ALAN of approximately 15 lx at the water surface or natural light conditions with sampling around half moon. No differences in melatonin concentrations between ALAN and natural conditions could be detected. However, blood concentration of sex steroids (17β-estradiol; 11-ketotestosterone) as well as gene expression of gonadotropins (luteinizing hormone, follicle stimulating hormone) was reduced in both fish species. We conclude that ALAN can disturb biological rhythms in fish in urban waters. However, impacts on melatonin rhythm might have been blurred by individual differences and natural nocturnal light. The most sensitive effect of ALAN was found on biomarkers of reproduction, suggesting a photo-labile period around the onset of gonadogenesis, including the experimental period (August). Light pollution therefore has a great potential to influence crucial life history traits with unpredictable outcome for aquatic ecosystems.

Keywords: fish; light pollution; melatonin; reproduction; gonadotropins; sex steroids

1 Introduction

Light is fundamental for the existence of flora and fauna on earth. It serves not only as a source of energy but also as a source of information to organisms that drives daily and seasonal cycles of behaviour, phenology and physiological changes. The natural alteration and length of day and night sets the internal clock of organisms and ensures that behaviours and physical processes are synchronized with time of day and season (Gaston et al. 2013). This is particularly important in reproduction of many

animals, e.g. for timing of courtship and mating, which ensures that rearing of offspring can be coordinated with availability of natural resources and optimizes its survival chances.

However, especially in urban areas, nightscapes are increasingly polluted by ALAN (Hölker et al. 2010). Streetlights, illuminated advertising and other kinds of public lighting are not only causing direct glare. Mostly unshielded against the sky, their irradiance is reflected by airborne particles, aerosols and other molecules. The result is that the nocturnal urban sky can be brighter than a full moon night (Kyba et al. 2011, Kyba et al. 2015). Ecological consequences are known for almost all classes of organisms (Rich and Longcore 2006, Schroer and Hölker in press). Impacts on humans are also discussed, e.g. an increased cancer risk (Haim and Portnov 2013). The impact on migrating birds is well studied. Often they migrate at night and can be greatly attracted to artificial lights, get trapped there and lose important energy reserves (Gauthreaux Jr. and Belser 2006). Similar behaviour is known from insects that perish on streetlights (Eisenbeis 2006). Also in aquatic environments impacts of ALAN on organisms are known. Daphnia exhibit changes in their daily vertical migration pattern in response to ALAN at very low light levels as produced by skyglow (Moore et al. 2006). This may lead to an increase in algae biomass (reduced grazing) and general changes of food web interactions. Drift patterns of aquatic insect larvae are likewise modified by ALAN of about 1 lx (Perkin et al. 2014b).

In fish, many behaviours and physiological functions underlie either daily or seasonal rhythms. The European eel performs a spawning migration from freshwater to the ocean in autumn and spring. Eels migrate during dark nights and rest during bright nights around full moon (Stein et al. 2015). ALAN is likewise able to disrupt this migration (Lowe 1952, Vøllestad et al. 1986), which is also known for other migratory fish species like salmon (*Salmo salar*) (Hansen and Jonsson 1985, Greenstreet 1992). Thus, it is reasonable to assume that illuminated bridges or lighting along the embankment can interfere with this reproductive trait. In fish, several reproductive traits are clock-controlled and depend, apart from temperature, on light as an important cue. Apart from other photoreceptor systems and endogenous oscillators the photosensitive pineal complex is considered to be highly important in regulating rhythmicity (Ekström and Meissl 1997, Falcón et al. 2010). The daily fluctuating levels of the main output hormone, melatonin, always resembles the photoperiod. Since it is suppressed by light, levels are high at night and low during daytime. Thus the pineal complex transduces photoperiodic information into hormonal signals (i.e. melatonin) that can be used by other organs to synchronize physiological and behavioural processes with daytime and season.

It is commonly accepted that light plays a key role in mediating reproductive processes in temperate freshwater fish. Therefore it is a common practice in science and aquaculture to control these processes by manipulating the photoperiod with artificial light. The aquacultural interest led to research focusing on the interaction

between light and reproduction (Kolkovski and Dabrowski 1998, Porter et al. 1998, Porter et al. 1999, Zakes and Szczepkowski 2004, Maitra et al. 2013).

Gametogenesis and final maturation are regulated by a hormonal cascade from the hypothalamic–pituitary–gonad (HPG) axis. In a simplified scheme, the HPG axis is modulated by several environmental and social factors like photoperiod, temperature, growth and social interactions. This information is integrated and gonadotropin releasing hormone (GnRH) stimulates the secretion of pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are transported to the gonads via the bloodstream and stimulate in the gonads the synthesis of sex steroids that play a role in cell proliferation and differentiation and final gonadal maturation. In a negative feedback loop they also act on the pituitary and regulate gonadotropin secretion.

Light is known to manipulate these axis components. In pejerrej (*Odontesthes bonariensis*) for example short photoperiods are known to suppress gene expression of GnRH (Miranda et al. 2009). Artificial light or photoperiod manipulation can also have an impact on gonadotropins. Gene-expression or plasma concentrations of gonadotropins are significantly suppressed by continuous high intensity lighting (Bayarri et al. 2004, Rodríguez et al. 2005) and also sex steroid concentrations are affected. In general, maturation, onset of gonadogenesis or spawning events can be controlled by photoperiod manipulation. Fish farmers and scientist are using this to induce e.g. off season spawning in fish that normally reproduce only once per year or to prevent precocity in commercial aquaculture species (Macquarrie et al. 1979, Kolkovski and Dabrowski 1998, Rodríguez et al. 2005, Carrillo et al. 2009).

However, this kind of research predominantly took place in the lab or in artificial environments and mostly with high intensity night lighting. The potential impacts of ALAN on fish in natural habitats at typical light levels (as found in light polluted areas) are not well studied. In previous lab experiments we assessed the impact of low light intensity ALAN on the circadian rhythm of melatonin and gene expression of gonadotropins of European perch (Brüning et al. 2015, Brüning et al. 2016) and roach (unpublished) under laboratory conditions. We found that the melatonin rhythm was substantially diminished already at light intensities of 1 lx and above. Furthermore, in perch also gene expression of LH and FSH was significantly impaired.

Falcón et al. (2010) reviewed the possible interactions between melatonin and the HPG axis and suggested that fishes have a photo-labile period, the decreasing day lengths in fall (in fish species of temperate waters). In this period of about two month, fish would be susceptible to photoperiod manipulation, and continuous light would cause decreased synthesis and secretion of gonadotropins and sex steroids and gonadal maturation would fail.

With the present field study we attempt to investigate the impact of ALAN, represented by streetlights along an embankment, in a naturally dark environment.

The experimental fish species are perch and roach, two of the most abundant fish species in European freshwaters with diverging life history traits.
European perch are known to feed on zooplankton when young, but undergo an ontogenetic niche shift towards a diet of benthic organisms and fish when older (Persson 1986). They are diurnal and crepuscular consumers. Onset of gonadogenesis occurs in late summer, around end of August and September (Sulistyo et al. 1998, Sulistyo et al. 2000) and spawning takes place during April and May (Treasurer 1988, Wang and Eckmann 1994).
Roach are also diurnal and crepuscular (Hölker and Breckling 2005), but nocturnal feeding has been reported for roach in periurban lakes experiencing ALAN (Okun and Mehner 2005). They feed on benthic invertebrates, zooplankton or plant material. Onset of gonadogenesis appears to be around August and spawning takes place between April and May (Trubiroha et al. 2012).
We expect the photo-labile period in perch and roach to be in August/September. We hypothesize, that ALAN in a natural environment during this period is able to cause significantly decreased levels of melatonin and the sex steroids, 11-ketotestosterone (11-KT) and 17β-estradiol (E2), in the blood and significantly reduced gene expression of gonadotropins (LH, FSH).

2 Methods

2.1 Field site

The field site is situated about 70 km north-west of Berlin, Germany, in the Westhavelland Nature Park (52’69° N, 12’46° E). The Nature Park is one of the least-illuminated areas of Germany and has recently been designated an “International Dark-Sky Reserve” by the International Dark-Sky Association (IDA) (www.darksky.org).
The experimental field sites consisted of two light-naïve grassland fields along a drainage ditch that were under agricultural use (mown twice per year, not fertilized). The ditch itself is approximately 5 m wide and 50- 80 cm deep, depending on precipitation. Each of the field was equipped with 12 streetlights (20m apart) in 3 rows parallel to the ditch. The first row (4 streetlights) was installed 3 meters away from the edge of the water. On one field (lit field) the streetlights were equipped with high-pressure sodium lamps in 4.75 m height (70 W, 2000 K, 96 lmW21; Osram Vialox NAV-T Super 4Y, Munich, Germany).
Nocturnal light levels in the lit field ranged from 13.3 to 16.5 lx at the water surface and 6.8–8.5 lx at 50 cm depth. The experimental sites are separated by 600 m (Euclidian distance) and a row of trees ensures there is no influence of the

experimental lights on the control site (Hölker et al. 2015). The streetlights in the other field (dark field) were not equipped with lamps.
In front of each streetlight at the first row along the ditch, two net cages (1x1x1 m, aluminium frame with nylon net, 1 mm mesh size) were installed in the ditch, approximately 1 m away from the water edge, thus in total there were 8 net cages per field. Water depth during the experimental period was approximately 80 cm, so that the cages covered the water column from bottom to surface. The two sites were similar in terms of morphology, catchment characteristics and abiotic parameters (Holzhauer et al. 2015).
On August 7th 2012 each net cage was equipped with 6 perch and 6 roach and left under the respective conditions for approximately one month.

2.2 Experimental fish

European perch, *Perca fluviatilis*, and roach, *Rutilus rutilus*, from the stock of the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB) in Berlin, Germany, originated from the nearby Lake Müggelsee, a periurban lake surrounded by forests and housing and thus partly experiencing ALAN (Perkin et al. 2014a). Before transportation to the field site fish were kept in indoor tanks in a flow-through system with tap water and aeration under natural photoperiod. They were fed daily with frozen chironomid larvae (perch) and dry food (roach). Temperature was 15.5° C and water parameters were regularly controlled to maintain optimum conditions. The mean fish biomass was 32.1 ± 13.2 g (mean ± standard deviation) for perch and 29.6 ± 11.9 g for roach.

2.3 Blood and tissue sampling

Sampling took place in 4 consecutive nights around half moon from September 6 – 9 2012. Due to the half-moon, natural nocturnal light levels of up to 0.02 lx were measured at the water surface of the control site. Four cages per night were sampled, two from each field in randomized order.
The fish were taken out with a landing net and transferred to a bucket with ice water. Fish were immediately sampled in a transporter equipped with a mobile laboratory.
Blood samples were taken with heparinized syringes from the caudal vein and centrifuged at 7,000 rpm for two minutes. The serum was stored in a polystyrene box containing a freezing mixture (crushed ice + NaCl, -20° C) for the duration of the sampling and transferred to a freezer thereafter.

The pituitary was dissected and transferred to RNAlater (Sigma-Aldrich) to preserve the mRNA. The pituitary tissue was left in RNAlater for approximately 24 h at 4° C and frozen thereafter.

2.4 Hormone extraction and assay

Blood serum samples (70-100 µL) were transferred to 5ml glass vials and 500 µL ethyl acetate (J.T.Baker) was added to each vial. Each vial was vortexed for 30 seconds and then left for 5 minutes to allow phase separation. Afterwards the vials were frozen at -80° C for 15 min and the liquid phase was transferred to a new vial. The procedure was repeated once and the supernatant of both extractions was pooled for each sample.
The supernatant was then evaporated under a stream of nitrogen at 45° C. The residue was reconstituted with 0.5 mL EIA-buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA and 0.1% sodium azide, Cayman Chemicals).
Melatonin, E2 and 11-KT levels were measured by enzyme linked immunosorbent assay (ELISA) using commercial kits (RE54021, RE52041, CM582751 (Cayman Chemicals), IBL, Hamburg, Germany).

2.5 RNA extraction and reverse transcription

Total pituitary RNA was extracted with RNeasy extraction kit (Qiagen) following the manufacturers protocol. Concentration of total RNA was measured by UV absorption spectrometry with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).
Reverse transcription of total RNA was performed using Affinity Script Multiple Temperature Reverse Transcriptase (Stratagene).

2.6 Gene expression analysis by RT-qPCR and relative mRNA quantification

For LHß, FSHβ and ribosomal protein L8 (rpL8) as housekeeping gene, we used primers established by Trubiroha et al. (2009) for roach and for perch we used primers established by Brüning et al. (2016) (Tab. 5.1). The identity of the products was confirmed by direct sequencing (SEQULAB, Göttingen) and comparison with the database homology search tool BLAST.
PCR was carried as previously described by Brüning et al. (2016). PCR efficiencies were determined with pooled pituitary cDNA and ranged between 1.90 and 2.02;

gene expression was determined by comparative CT method (ΔΔCT) with using a calibrator sample (pooled pituitary cDNA) and correction for the PCR efficiency according to Pfaffl (2001).

Table 5.1 Overview of primer specific PCR conditions (primer sequences L8, FSHß, LHß)

	Target gene	Forward primer	Reverse primer	T_A (°C)	Primer-conc.	Product size (bp)	PCR-efficiency
P. fluviatilis	L8	GTTATCGCCTCTGCCAAC	ACCGAAGGGATGCTCAAC	62	375 nM	167	2.02
	FSH	CCTACTGGCAGGGAAGAAC	CCTACTGGCAGGGAAGAAC	64	375 nM	85	1.92
	LH	GGCTGTCCAAAGTGTCACCT	GGGAGAACAGTCAGGGAGCTTAA	62	188 nM	158	1.9
R. rutilus	L8	ATCCCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAACAG	62	375 nM	94	1.98
	FSH	CTGTCGGCTTTCCAATATC	GGCTACGGTAAACTCTTTC	62	375 nM	119	1.93
	LH	TAGGTGATGTGCGGGTCCAC	AAGAGCTGTCCGAAATGC	62	375 nM	187	1.94

T_A – annealing temperature

2.7 Data handling and statistical analysis

Both fish species were randomly distributed into the net cages and later on data for each species and treatment was pooled, since no significant differences have been found for all parameters (Kruskal-Wallis-test; $p > 0.05$). Hormone data was standardised to pg/ml and tested for normal distribution (Kolmogorov-Smirnov test). Gene expression data was likewise tested for normality. In case of normality, data was compared with student's t test. Non-parametric data was compared with Mann-Whitney-U test. Mortalities were compared by Binomial test. The level of significance for all tests was set at $p = 0.05$.

3 Results

Mortality for the ALAN field was 25% and 12.5% and for the control field 10.4 % and 14.6 % for perch and roach respectively. There were no significant differences in mortality between the treatments (binomial test, $p > 0.05$).

3.1 Melatonin

There were no significant differences in the concentration of melatonin in blood serum of perch subjected to either ALAN or no additional ALAN (natural half-moon conditions). Also for roach no significant differences in serum concentrations of melatonin were found (Fig. 5.1).

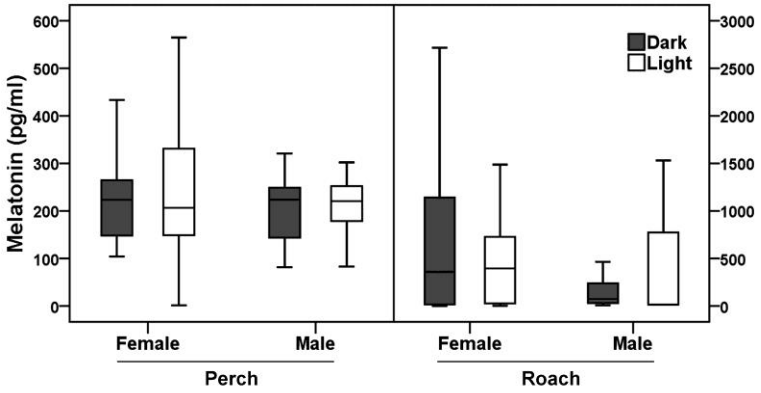

Figure 5.1 Concentrations of melatonin in blood serum. Comparison between effects of ALAN ("Light") and natural half-moon conditions ("Dark") on female (light: N=22; dark: N=23) and male (light: N=5; dark: N=19) perch and female (light: N=33; dark: N=35) and male (light: N=3; dark: N=5) roach. No significant differences were found.

3.2 Gene expression of gonadotropins

Gene expression analyses of LHβ and FSHβ from female and male perch subjected to ALAN or no artificial light in the night revealed significant differences in both, FSHß and LHß mRNA expression (FSH: Students t-test, $p \leq 0.001$ (female) and $p \leq 0.01$ (male); LH: Mann-Whitney U test, $p \leq 0.001$) being highest in the control (dark) treatment and significantly lowered in the ALAN treatment (Fig. 5.2). Also in roach significant differences in mRNA expression of gonadotropins were found. FSHß-mRNA expression was significantly lowered in the ALAN treatment (female: Students t-test, $p \leq 0.001$; male: Mann-Whitney U test, $p \leq 0.05$; Fig. 5.3). However,

while LHß expression is significantly lowered by ALAN in female roach (Students t-test, $p \leq 0.001$), the similar tendency in male roach turned out not to be statistically different.

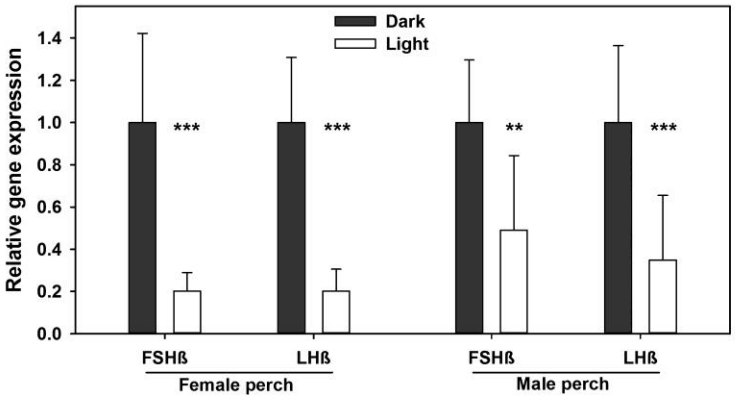

Figure 5.2 Relative mRNA expression of follicle-stimulating hormone (β-subunit; FSHβ) and luteinizing hormone (β-subunit; LHβ) in brain-pituitary tissue of *P. fluviatilis*, subjected ALAN ("Light") or natural half-moon conditions ("Dark"): female, (dark: N= 13, light: N= 15) and male (dark: N= 13, light: N= 8). Data is shown as mean ± SD. Significant differences are represented by asterisks (***: $p \leq 0.001$; **: $p \leq 0.01$). Gene expression of FSHβ and LHβ was significantly reduced in the ALAN treatment.

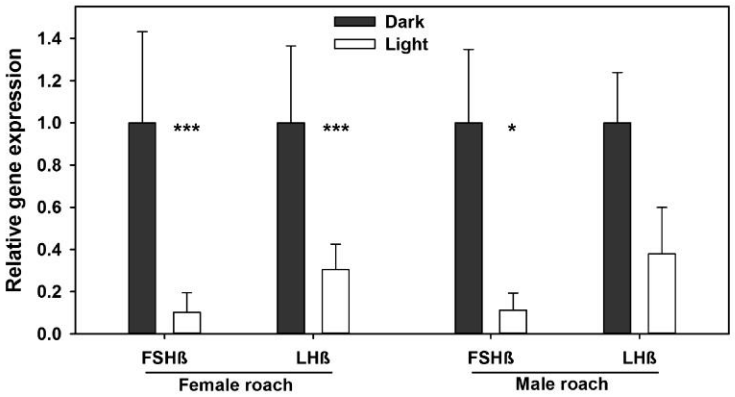

Figure 5.3 Relative mRNA expression of follicle-stimulating hormone (β-subunit; FSHβ) and luteinizing hormone (β-subunit; LHβ) in brain-pituitary tissue of *R. rutilus* subjected ALAN ("Light") or natural half-moon conditions ("Dark"): female (dark: N= 25, light: N= 28) and male (dark: N= 5, light: N= 3) Data is shown as mean ± SD. Significant differences are represented by asterisks (***: $p \leq 0.001$; *: $p \leq 0.05$). Except for LHβ in male roach, gene expression of gonadotropins was significantly reduced in the light treatment.

3.3 11-Ketotestosterone

The analyses of the blood serum of perch revealed significant differences in 11-KT concentrations between ALAN and control treatment in female (median (interquartile range, IQR) in pg/ml: 3.7 (2.6 – 5.4) versus 20.8 (7.7 – 27.3)) and male (53.5 (44.3 – 234.4) versus 455.1 (270.4 – 786.2)) (Mann-Whitney-U test: $p \leq 0.001$). The serum of perch subjected to natural half-moon conditions (control) contained up to 10 times more 11-KT compared to the ALAN treatment. Similar results were obtained for roach (Fig. 5.4). 11-KT concentrations in blood serum were significantly lower in the ALAN treatment compared to the control treatment in female (5.2 (2.9 – 8.0) versus 8.0 (5.7– 170.3)) and male roach (8.4 (4.4 – 12.6) versus 408.2 (275.8–850.6)) (Mann-Whitney-U test: $p \leq 0.001$).

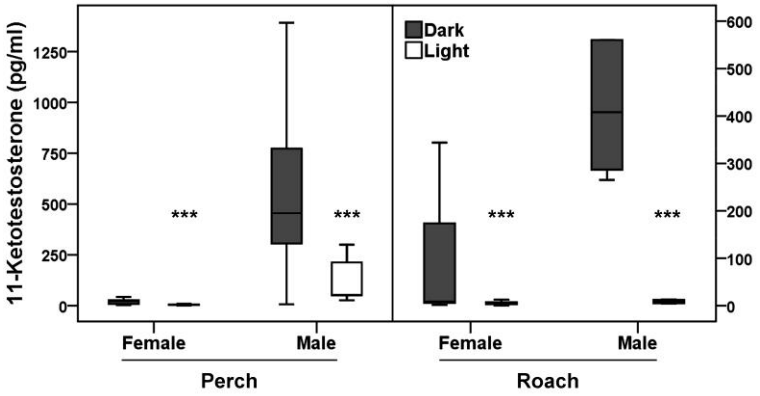

Figure 5.4 Concentrations of 11-KT in blood serum of perch and roach. Comparison between effects of ALAN ("Light") and natural half-moon conditions ("Dark") on female (light: N=22; dark: N=23) and male (light: N=5; dark: N=19) perch and female (light: N=33; dark: N=35) and male (light: N=3; dark: N=5) roach. Significant differences are represented by asterisks (***: $p \leq 0.001$). 11KT concentrations of female and male of both species are significantly reduced in the ALAN-treatment.

3.4 17ß-Estradiol

The concentrations (pg/ml) of E2 were significantly lowered by ALAN compared to the control treatment in female of both, perch (72.4 (63.6 – 122.3) versus 162.6 (80.2 – 269.2)) and roach (55.7 (37.4 – 71.9) versus 79.7 (38.7 – 126.7)) (Mann-Whitney-U test, t-test: $p \leq 0.001$). However E2 concentrations in male perch and roach were not affected by ALAN (Fig. 5.5).

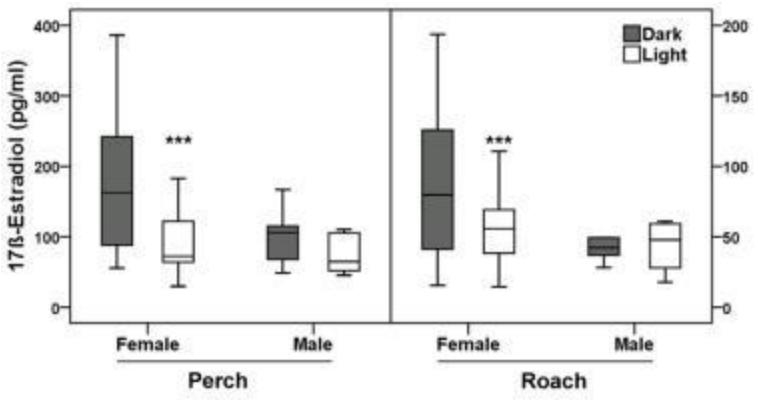

Figure 5.5 Concentrations of E2 in blood serum of perch and roach. Comparison between effects of ALAN ("Light") and natural half-moon conditions ("Dark") on female (light: N=22; dark: N=23) and male (light: N=5; dark: N=19) perch and female (light: N=33; dark: N=35) and male (light: N=3; dark: N=5) roach. Significant differences are represented by asterisks (***: $p \leq 0.001$). E2 concentrations are significantly reduced by ALAN in female but not in male perch and roach.

4 Discussion

This study demonstrated that ALAN had a strong effect on reproductive traits in European perch and roach under *in situ* conditions. Mortality rates were similar in the two experimental sites, substantiating comparability of the results. Occurrence of mortality was attributed to handling stress, since it occurred only at the beginning of the experiments, directly after transferring the fish into the net cages. Gene expression of LH and FSH and the production of sex steroids were profoundly affected by ALAN in both species (Figs. 5.2 - 5.5). There was no effect on serum melatonin detectable (Fig. 5.1), which seem to contradict our previous results in the lab (Brüning et al. 2015), where 1 lx white light already caused a substantial drop in melatonin. However, no NOEC (no observed effect concentration) was found in the above mentioned study, implying that the threshold is lower. The sampling for the present experiment took place around half moon, which was producing light levels of up to 0.02 lx at the water surface. Although this might initially sound very low, several fish species are known to be affected by very low light intensities. The European eel, *Anguilla anguilla*, in its glass eel stage for example avoids light levels of about 0.07 lx and less (Bardonnet et al. 2005) and the common bream, *Abramis brama*, is able to detect prey at light levels of 0.005 lx (Townsend and Risebrow 1982). In tench, *Tinca tinca*, melatonin levels dropped to daytime levels after a 1h light pulse of 0.3 lx in the middle of the night (Vera et al. 2005). Special cases are lunar spawners, e.g. rabbitfish, which synchronize their reproduction with the moon. The pineal organs of the golden rabbitfish, *Siganus guttatus*, are able to perceive light intensities as low as 0.1 lx (Takemura et al. 2006). However, in the latter 2 studies, no NOEC was found, suggesting that the threshold light intensities are even lower. It is

likely that perch and roach are as well able to perceive the natural half-moon intensities via the pineal complex or other melatonin influencing photoreceptors. Thus, melatonin levels in perch and roach might be influenced by natural half-moon light conditions during sampling. The differences in melatonin levels between ALAN and natural half-moon conditions are therefore smaller compared to the lab experiments in Brüning et al. (2015). Together with the high individual variabilities (Fig. 5.1) even the natural light during half-moon could have blurred possible differences between the treatments.

However, the effects of ALAN on biological rhythms are clearly pronounced in the reproduction results (Fig. 5.2 – 5.5). Gonadotropins and thereby controlled sex steroids are sensitive endpoints when studying reproductive rhythms. Thus, the impact of ALAN on sex steroids and gonadotropins corresponds to our expectations that light pollution in a realistic scenario has a great potential to disrupt crucial rhythms like reproduction in fish.

Reproduction in fish species of temperate freshwaters is subjected to a seasonal rhythm and entrained by the cyclic environmental changes during a year, among which photoperiod and temperature are regarded as most important zeitgeber for the majority of species (de Vlaming 1972, Migaud et al. 2010, Hermelink et al. 2011). In contrast to mammals, fish possess other tissues and receptors, besides ocular photoreceptors, to entrain endogenous rhythms by light. The pineal gland is commonly regarded to be of most importance. Its cyclical output, melatonin, is a signal that translates light information about day and season into endogenous signals. Generally, the period of nocturnal melatonin secretion is equal to night length and transducable into seasonal information. This is where light may intervene with the HPG axis. In European perch, for example, the decreasing photoperiod in fall is necessary to induce gametogenesis (Migaud et al. 2003) and an artificially increasing photoperiod or continuous lighting can severely disturb the schedule of reproduction (Fontaine et al. 2006). Studies in salmonids and a species of Neotropical silversides have shown that light can act directly on the upper part of the axis, the gonadotropin-releasing-hormone (GnRH) by manipulating its timing (Amano et al. 1994, Miranda et al. 2009). The main task of GnRH is stimulation of the expression and secretion of gonadotropins, FSH and LH. Consequently gonadotropins can be a target for photo-induced disruption too. In the present study the gene expression of gonadotropins of European perch and roach was substantially reduced (Fig. 5.2 and 5.3). However, in male roach the differences were not significant, likely due to the low sample size. In an earlier lab study we already proved the suppression of gonadotropin expression by low light intensities in perch (Brüning et al. 2016). As reported in different fish species, gonadotropins play a critical role in early stages of gonadal maturation, in synthesis of sex steroids and consequently in spermatogenesis and vitellogenesis (Patiño and Sullivan 2002, Schulz and Miura 2002, Mateos et al. 2003, Yaron et al. 2003). There is little data available on the influence of low intensity ALAN, as

referred to as light pollution, on gonadotropin expression in fish. However, gonadotropin gene expression was inhibited by continuous light of 650 lx (Rodríguez et al. 2005), and daily plasma concentrations of LH and its storage where significantly altered by artificial light in European sea bass (*Dicentrarchus labrax*) (Bayarri et al. 2004). In Brüning et al. (2016) we verified a significant reduction in gene expression of both gonadotropins by low intensity white (1 lx) and coloured light (0.15 - 2.2 lx) in perch (*P. fluviatilis*) under artificial conditions. There is also strong evidence, that photoperiod manipulation, e.g. mimicking short or long day photoperiods, can change the timing of gonadotropin production and subsequent spawning events (Breton et al. 1977, Bromage et al. 1982, Felip et al. 2008, Miranda et al. 2009).

However, all of these studies were carried out under artificial conditions. The present study is the first evidence, that light pollution in a realistic natural context can suppress gonadotropin gene expression in the preparatory phase of reproduction and confirms our previous results of lab experiments (Brüning et al. 2016).

One of the major roles of gonadotropins is triggering the synthesis of sex steroids from the gonads. Sex steroids in turn exhibit positive or negative feedback on gonadotropins and brain. They are involved in vitellogenesis and final gamete maturation and are also important for the control of sexual behaviour and spawning (Zohar et al. 2010). In our study the plasma concentrations of 11-KT were significantly reduced by ALAN in both sexes of perch and roach (Fig. 5.4). Plasma levels of E2 were likewise reduced in the light treatment in female perch and roach (Fig. 5.5). However, in males E2 levels where already low in the control treatment, thus no effect of ALAN was detectable. These findings are in accordance with studies in roach (Trubiroha et al. 2010) where E2 levels in males were significantly lower than in females. The suppression of sex steroids by ALAN was also reported for other fish species. As shown in male sea bass, continuous light was very effective in inhibiting the increase of 11-KT in plasma during the reproductive cycle (Rodríguez et al. 2005, Felip et al. 2008). Also in Senegalese sole plasma E2 levels in female and 11-KT levels in male fish were significantly lower compared to conspecifics under a natural photoperiod (García-López et al. 2006). A concomitant failure in gonad maturation was reported for several fish species, i.e. sea bass (Begtashi et al. 2004), Atlantic cod (Taranger et al. 2006), turbot (Imsland et al. 2003) or Nile tilapia (Rad et al. 2006). Also Migaud et al. (2004) found a significant suppression of E2 by high intensity continuous light (500 lx) in male and female perch. These results were obtained in the lab in September (experimental period September 1st – October 6th); in July/August (experimental period July 17th - September 1st), no differences were found. This is in contrast to our results in the field, where an exposition in August/September (August 7th - September 6th - 9th) elicited a significant suppression. However, they found no differences in plasma 11-KT in both experimental periods. This raises a question about a photo-labile period where fish are particularly

susceptible for additional light at night or photoperiod manipulations. Indeed recently, evidence for such a period could be found in some fish species. Falcón et al. (2010) suggested a photo-labile period for fish in general, coinciding with the onset of gonadogenesis at de- or increasing photoperiods in spring or fall, depending on species. Indeed, in male sea bass this period is a short time frame of about two month, including September (Rodríguez et al. 2012). In Atlantic cod it is the decreasing photoperiod in fall, where photoperiod manipulations were effective (Davie et al. 2007). The clear inhibition of parts of the HPG-axis in perch and roach suggest for both species that the photo-labile period must be the period around the onset of gonadogenesis, including August, when our experiments took place.
Taken all these information together, it becomes clear, that ALAN can interfere with various components of the HPG-axis. It obvious, that photoperiod manipulation can be a powerful aquacultural tool for controlling reproductive events. But in nature ALAN might be an unpredictable risk for light sensitive species, species communities and subsequently biodiversity.

Acknowledgements

The Authors like to thank M. Kunow and L. Lehmann at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries for technical support. Funding was provided by the Verlust der Nacht project (Federal Ministry of Education and Research, Germany, BMBF-033L038A).

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General discussion

Plants and animals have evolved over millions of years under a natural cycle of day and night, resulting in behavioural patterns and physiological processes being controlled by a circadian clock. However, until now little is known about ecological and evolutionary consequences of interrupting these circadian patterns in nature. The influence of ALAN on fish was mostly studied to enhance rearing conditions, growth and survival in aquaculture. Continuous lighting or prolonged photoperiods are used to extend the period of food intake and prevent maturation and thereby improve the growth of fish, e.g. in sea bass *Dicentrarchus labrax* (Rodríguez et al. 2005, Felip et al. 2008, Villamizar et al. 2009), Atlantic salmon *Salmo salar* (Oppedal et al. 1997, Porter et al. 1999), turbot *Scophthalmus maximus* (Imsland et al. 1995, Imsland et al. 2003) or cod *Gadus morhua* (Taranger et al. 2006, Davie et al. 2007). However, although ALAN can be advantageous for fish production in aquaculture, it might create significant bottlenecks for natural populations if it affects fundamental elements in the life cycle of a species (Brüning et al. 2011).
The aim of this study was to evaluate the effect of low intensity ALAN on the melatonin rhythm, stress response and reproductive parameters of two highly abundant European freshwater fish species, European perch *Perca fluviatilis* and roach *Rutilus rutilus*. Since the two species differ in life history, an additional objective of this work was to compare the effects of ALAN on perch and roach and assess possible species specific differences.

Is it possible to mimic natural light conditions in the laboratory?

The first objective of this thesis was to establish a laboratory setup with lighting conditions that resembles natural conditions in key properties, like colour temperature, illuminance level as well as sunrise and sunset time. A light setup was established with simulated daylight that was realized by fluorescent lamps with the best available continuous spectrum at a colour temperature around 6,000 K that is considered to be an appropriate fit to the natural daylight spectrum (Fig. 1.2). A light intensity of 6000 lx could be achieved, resembling a cloudy day in the northern latitudes. Simulated sunrise and sunset were realized by a programmable time switch, where the illuminance level is gradually increased or decreased within 3 hours as found with natural conditions (Fig. 1.3). Nocturnal white light of 1 lx, 10 lx and 100 lx was realized with similar fluorescent bulbs that were dimmed and shaded. Nocturnal coloured light was provided by light emitting diodes (LEDs) because here bandwidths less than 40 nm are available (Fig. 1.4). Different LEDs were normalized to photon flux density, since biochemical reaction to light are triggered by single

photons (Fig. 1.5). Melatonin concentrations of perch were measured from water samples within a 24 h cycle for nocturnal white light illumination at 100 lx and 0 lx and release rates were calculated. The melatonin rhythm in the control treatment reflected the natural wavelike shape (Fig. 1.8). Maximum melatonin concentration was more than a factor of 5 less for the 100 lx treatment compared to the 0 lx treatment and consequently also the maximum melatonin release rate is significantly decreased (Figs 1.8-1.10). Therefore, the experimental setup and the biological methods (measuring of waterborne melatonin as a non-invasive method) could be validated, since a distinct rhythm of melatonin was found for perch and concentrations and release rates were reduced in the positive control (100 lx) (Franke et al. 2013).
Hence, this experimental setup effectively resembles light conditions in nature. By keeping constant other abiotic factors this setup is very suitable to study the impact of ALAN on fish.

How do different intensities of white ALAN influence daily rhythms of melatonin and cortisol in perch?

In the first laboratory study, the effects of different intensities of white ALAN (0 lx, 1 lx, 10 lx, 100 lx) on melatonin and cortisol rhythms of European perch were investigated. It was expected, that nocturnal light decreases melatonin and elevates cortisol in a dose dependent manner. Concentrations of melatonin, as marker for the circadian rhythm and cortisol as indicator for stress were measured from water samples, providing a non-invasive method to minimise handling influences. To yield a daily profile, samples were taken every three hours during a 24 hour period as described in Chapter 1. As water samples were taken with a multichannel peristaltic pump, all experimental tanks could be sampled simultaneously, enhancing comparability of the treatments.
Cortisol exhibited a distinct rhythm in all light treatments with an increase of concentrations in the morning (Fig. 2.3). Also in other species light at night had no effect on stress response (Spieler and Noeske 1984, Biswas et al. 2006, Biswas et al. 2008, Bani et al. 2009, Honryo et al. 2013). However, if ALAN is not perceived as stressor, fish have no need to avoid artificial light. Moreover, since it enhances feeding possibilities, fish may be attracted to it and thereby increase the probability of a disturbance of biological rhythms.
Since no effect of broad spectrum white light on cortisol concentration was found, this parameter was not examined further in the following experiments.
In the control treatment the expected melatonin rhythm of high levels during the night and low levels during the day could be observed. However, at all tested light intensities the melatonin concentrations were significantly lowered (Fig. 2.1). In

Atlantic salmon parr, melatonin suppression gradually increased with increasing light intensity (Porter et al. 2001). In perch, no such dose dependent reaction could be found at the tested light intensities above 1 lx. However, melatonin levels of perch in the 1 lx treatment exhibited a noticeable increase during the night. Also rhythmical parameters of the melatonin profile were most pronounced in the control and 1 lx treatment as shown by the cosinor analysis (Fig. 2.2). These results indicate that in perch the threshold light intensity for melatonin suppression must be close to 1 lx. Since light levels around 1 lx were already found in urban waters at night, e.g. in Berlin (Perkin et al. 2014), it is most likely, that light pollution in urban waters at realistic light levels has a great potential to inhibit circadian melatonin patterns (Brüning et al. 2015). The consequences of this disturbance can be very broad, since melatonin rhythms drive the biological clock and influence many physiological aspects including reproduction.

What is the spectral sensitivity of the circadian system of perch and does ALAN influence reproduction?

In a second experiment, the influence of different light colours during the night on the melatonin rhythm of perch was examined. Intensities of the light colours ranged from 0.15 – 2.2 lx, depending on colour. Additionally, the impact of both, white and coloured ALAN on gene expression profiles of the gonadotropins LH and FSH was investigated to reveal possible consequences on reproduction. Melatonin profiles were measured as described above (Chapter 1+2, (Franke et al. 2013, Brüning et al. 2015)) and gene expression of gonadotropins was determined with qPCR from pituitary tissue.

Results of the melatonin measurements resembled those of the light intensity experiment. While melatonin concentrations exhibited the expected rhythm with low concentrations during the day and higher concentrations at night in the control group, all three light colours suppressed nocturnal melatonin levels (Fig. 3.1). However, blue light was found to be less effective in supressing melatonin, with significantly higher nocturnal melatonin concentrations compared to the treatments with red or green light (Fig.3.2) Only in the control and blue light treatments the melatonin profile exhibited a clear rhythmicity (Fig. 3.3). In humans and other mammals it is a given fact that melatonin suppression is highest under short wavelengths, i.e. blue light (Brainard et al. 1984, Brainard et al. 2001, Cajochen et al. 2005). In fish, spectral sensitivities may differ given the light absorption properties of different waterbodies (Wetzel 2001). Falcón and Tanabe (1983) for example found maximum photoresponses of the pike pineal at mid wavelengths (green and yellow). Also for goldfish, a domesticated form of Prussian carp (*Carassius gibelio*), a cyprinid, pineal photosensitivity peaked in the green range of the visible spectrum (Meissl et al. 1986). Cyprinids and pike inhabit

rather turbid waters like lakes and streams that are dominated by green and yellow light. Perch use similar habitats and have therefore similar spectral sensitivities. Conclusively, it is possible that the circadian axis of fish from more or less turbid freshwater bodies is generally more sensitive to longer wavelengths at night.
The timing of reproduction in fish strongly depends on light and the light dependent production of melatonin is one of the decisive factors. Since ALAN of all tested intensities and wavelengths diminished the melatonin rhythm, reproduction is likely to be affected too. Therefore it was expected that ALAN influences reproduction of perch by impairing the secretion of LH and FSH. For technical reasons, the light colour experiment and the light intensity experiment were conducted at different times. Experimental fish ought to have experienced natural light conditions. Therefore, different photoperiods resembling the actual photoperiods were used for every experiment. The light intensity experiment was conducted with a simulated photoperiod resembling October light conditions and the light colour experiment was conducted with September light conditions. Different colours of ALAN during September had no effect on the gonadotropin expression of the experimental fish (Fig. 3.4). Gene expression of gonadotropins was only effected in female perch in the light intensity treatment by white light at approximately 1 lx and above (Fig. 3.5). Influences of ALAN on gonadotropin expression of male perch were not observed. That may be due to the fact, that the reproductive cycle of males starts earlier and the maturation of the gonads ends earlier than in females (Migaud et al. 2006). However a comparison of the control treatments of the two experiment indicated that gene expression of gonadotropins in October was generally higher (Fig. 3.7). These results suggest the existence of a photo-labile phase in perch, where reproduction of fish may be particularly sensitive to ALAN. Under the present experimental conditions, this period seems to be around October.

Are the findings in perch applicable to roach, a fish species with a different biology?

The third experiment was performed to evaluate, whether the impacts of ALAN on perch can be also found in other species. Therefore, the above mentioned experiments were conducted with roach, a similar abundant species as perch. Roach use the same habitats but life history and biology differ in some aspects. Roach were exposed to four different light intensities and colours during the night, respectively (0 lx, 1 lx, 10 lx and 100 lx; dark control, blue, green and red). Melatonin concentrations were measured from the holding water and gene expression profiles of LH and FSH were examined from pituitary tissue. As in perch, it was expected that ALAN supresses the nocturnal increase of melatonin concentrations and reduces the gene expression of gonadotropins in comparison to the control treatments. A significant decrease of

melatonin concentration was found already at 1 lx of white ALAN (Fig. 4.1) and all tested wavelengths also substantially decreased the melatonin concentration in the tank water (Fig. 4.2). Consequently, it can be stated that low intensity white ALAN as well as low intensity narrow bandwidths (blue, green and red) interfere with the natural rhythm of melatonin production. The melatonin rhythm in the control treatments showed the typical increase at night and decrease during the day; whereas all light intensity treatments diminished this rhythm. As in perch, nocturnal melatonin concentration increased slightly the 1 lx treatment. However, whereas in perch a gradual in- and decrease over the whole nocturnal period was found (Fig. 3.1), melatonin increased only at one time point during the night in roach (05:00, Fig. 4.1). Dose-dependent effects could not be found. These results indicate that the threshold light intensity for melatonin suppression in roach is lower than 1 lx as well. Moreover, this species might be even more sensitive to light compared to perch. In the light colour experiment all three light colours with intensities ranging from 0.15 to 2.2 lx, depending on colour, suppressed nocturnal melatonin concentrations in comparison to the control treatment. In roach all tested light colours during the night elicited an almost complete suppression of the melatonin rhythm with no differences between night and day levels (Fig. 4.2). Since perch and roach inhabit similar habitats, it would be expected that melatonin suppression in roach is more sensitive to longer wavelengths as well, but our results do not confirm this. Although intensities were considerably low (Tab. 3.1), melatonin suppression did not differ between the tested colours (Fig. 4.3). The results, however, support the assumption, that the circadian axis of roach is generally more sensitive to ALAN than that of perch.

ALAN during simulated October light conditions caused a suppression of gonadotropin gene expression in perch. In roach, experiments were carried out under December conditions corresponding to the actual photoperiod prevailing during the experimental phase. Although melatonin is known to be able to alter several components of the HPG-axis such as gonadotropins, sex steroids or gonadal maturation (Khan and Thomas 1996, Amano et al. 2000, Chattoraj et al. 2005, Bhattacharya et al. 2007, Sébert et al. 2008, Carnevali et al. 2011), neither white ALAN of different intensities nor coloured ALAN and concomitant melatonin depletion had an effect on gene expression profiles of gonadotropins in roach (Fig. 4.4). The assumption that perch possess a photo-labile phase might therefore also hold true for roach. Falcón et al. (2010) suggested thath this photo-labile phase coincides with the naturally de- or increasing photoperiods in fall and spring. It has been stipulated that this period occurs during the onset of the reproductive phase, but only in sea bass it has been proven so far (Felip et al. 2008, Rodríguez et al. 2012). Nevertheless, roach, as well most fish from temperate waters are seasonal spawners and rely on seasonal information for the timing of reproduction. Thus, the existence of a photo-labile phase is most likely. Under natural conditions, the onset of reproduction in roach is between August and September (Jamet and Desmolles 1994,

Geraudie et al. 2010, Trubiroha et al. 2012). A photo-labile phase during this time would explain the lack of impact of ALAN on reproduction in these experiments, although melatonin is strongly affected.

Are implications of ALAN on perch and roach under laboratory conditions also found in a natural environment?

Laboratory experiments cannot fully display natural environments. To attribute above mentioned impacts to the presence of ALAN, temperature was kept constant in the laboratory experiments of this study. However, temperature is also an important cue for the timing of reproductive events. However, the potential impacts of ALAN on fish in natural habitats at typical light levels (as found in light polluted areas) are not well studied. Therefore an additional experiment was conducted under natural conditions in the field. Perch and roach were held in net cages in drainage channels and were exposed to street light or natural conditions for four weeks in August/September. Concentrations of melatonin and sex steroids and gene expression levels of gonadotropins were determined.

Surprisingly, no effect of ALAN on serum melatonin was detectable (Fig. 5.1) which seems contradictory to previous results in the lab (Brüning et al. 2015, Brüning et al. 2016, submitted-a), where 1 lx white light already caused a substantial drop in melatonin. Since no NOEC (no observed effect concentration) was found in the lab experiments, it must be assumed that the light threshold is even lower. Sampling took place in four consecutive nights around half moon. Natural half-moon conditions are accompanied with light levels of up to 0.02 lx at the water surface. Several fish species are known to be affected by and use very low light intensities (Townsend and Risebrow 1982, Bardonnet et al. 2005, Vera et al. 2005, Takemura et al. 2006). Perch and roach might as well able to perceive the natural half-moon intensities via the pineal complex or other melatonin influencing photoreceptors. In golden rabbitfish for example midnight melatonin concentrations in the blood during full moon are reduced by half as compared to new moon (Ikegami et al. 2014). Thus, melatonin levels in perch and roach in this study might have been influenced by natural half-moon light conditions during sampling. Differences in melatonin levels between ALAN and natural half-moon might therefore be smaller compared to laboratory experiments in Brüning et al. (2015), Brüning et al. (2016) and Brüning et al. (submitted-a). High individual variabilities in blood concentrations of melatonin (Fig. 5.1), sample processing and the natural light during half-moon most likely blurred possible differences between the treatments.

However, the impact of ALAN is clearly pronounced in the HPG-axis. In addition to gene expression of gonadotropins that are sensitive endpoints when studying reproductive rhythms, we also measured sex steroid concentrations in the blood.

Concentrations of E2 and 11-KT, as well as gene expression levels of gonadotropins were significantly reduced in the ALAN treatments in both fish species (Fig. 5.2 - 5.5). The distinct inhibition of key enzymes of the HPG-axis in perch and roach around August/September suggests for both species that the photo-labile period must be around this time of the year, when our experiments took place and the reproductive cycle of perch and roach commences. Thus it is most likely, that discrepancies in the effects of ALAN on reproduction between laboratory experiment with static temperature (photo-labile phase in October, Brüning et al. (2016)) and field experiment with natural temperature conditions (photo-labile phase in August, (Brüning et al. submitted-b)) are related to temperature. However, although the influence of ALAN on melatonin could not be demonstrated in the field experiments, the impact of ALAN on sex steroids levels and gonadotropin expression corresponds to our expectations that light pollution in a realistic scenario has a great potential to disrupt crucial rhythms like reproduction in fish.

Major findings, conclusions and future directions

The aim of this thesis was to study the impact of ALAN on fish. Light intensity thresholds as well as spectral sensitivities of biological rhythms ought to be investigated in two widespread fish species with diverging life histories.

The major findings are:

- White ALAN supresses the melatonin rhythm of perch already at an intensity of 1 lx but has no effect on cortisol (Chapter 2).
- Blue, green and red ALAN suppress the melatonin rhythm of perch, with blue light having a weaker effect. ALAN with a simulated photoperiod resembling October impacts reproduction, but not ALAN with a simulated September photoperiod (Chapter 3).
- White and coloured ALAN suppress the melatonin rhythm of roach but reproduction is not affected under simulated December light conditions (Chapter 4).
- In a natural setting comparing ALAN and half-moon conditions in August, an effect of ALAN on melatonin could not be demonstrated in perch and roach. However, ALAN significantly suppressed plasma sex steroid concentrations and gene expression of gonadotropins, key enzymes of the HPG-axis of fish.

In conclusion, light pollution has a great potential to impact biological rhythms of fish. The impact on the circadian melatonin rhythm is evident for perch in roach in both laboratory experiments (Brüning et al. 2015, Brüning et al. 2016, submitted-a)

with a very low threshold (> 1lx). Results of the melatonin measurements from field experiments confirm a very low light threshold in both species, since concentrations are obviously influenced already at moonlight intensities, which is likely the main reason for the discrepancy between laboratory (where the response of 30 fish was integrated) and field (individual) results. To discover dose-dependent effects of light intensity, experiments have to be conducted, that resolves the range of 0-1 lx in a fine scale.

Spectral sensitivities could only be obtained for perch, implying, that the circadian axis in this species is more sensitive to longer wavelengths, i.e. green and red, as compared to blue light. Indeed, the circadian system of fish from clear waters, such as oceans, is more susceptible to blue light or shorter wavelengths, e.g. in European sea bass (Bayarri et al. 2002) or Senegalese Sole *Solea senegalensis* (far violet; 365 nm) (Oliveira et al. 2007). Consistently, in zebrafish *Danio rerio* and rainbow trout, preferring mostly clear streams, melatonin suppression is highest in blue-green to green light (Morita 1966, Max and Menaker 1992, Kusmic et al. 1993, Ziv et al. 2007). The spectral sensitivity of roach circadian system is not studied so far. However, roach and perch prefer similar habitats, thus similar spectral sensitivities can be assumed. Roach, however, seem to react more sensitive to ALAN, as discussed in Chapter 4. This might be attributed to its life history. Roach are usually diurnal and crepuscular (Hölker and Breckling, 2005) and known to be able to use very low light intensities and also feed during the night, a strategy to avoid visual predators like perch. A higher sensitivity to very low light levels might therefore be beneficial. Conclusively, light thresholds and spectral sensitivities are species specific, depending not only on habitat but also on the varying life histories.

When evaluating impacts of ALAN on a fish species it is highly important to consider reproduction as it is closely linked to the fitness of a species. However, in both studied species, reproduction was affected only at a specific time of the year, referred to as photo-labile phase. As mentioned before, the onset of gonadogenesis around August is the particular phase where the HPG-axis perch and roach is most susceptible to photomanipulation (Brüning et al. submitted-b). However, conclusive results still have to be obtained from the majority of fish species to evaluate potential implications of ALAN e.g. on species composition. Impacts, especially when they are species specific, may cause shifts in population sizes and thereby alter also the structure of fish communities with unpredictable outcome for aquatic ecosystems.

Important for future research is to increase the knowledge about the implications, which ALAN can have on different physiological and behavioural aspects of fish, as well as other aquatic organisms to derive to what extant light pollution can damage aquatic ecosystems.

One of the aims of the research project “Verlust der Nacht” (http://www.verlustdernacht.de/), in which the present work was embedded, was to give recommendations about how to deal with public lighting in the future.

However, it is very difficult to make general recommendations, even if only aquatic ecosystems are considered. European freshwaters alone comprise 546 native fish species, including lampreys (Kottelat and Freyhof 2007) with individual life histories, inhabiting all kinds of habitats and niches. Consequently also sensitivities to light might be different. Furthermore, reproduction happens almost all year round in European freshwaters (Kottelat and Freyhof 2007).

Therefore it is almost impossible to propose the optimum lighting concept, especially for aquatic environments. The "Loss of the Night" most likely has impacts on biodiversity and ecology of aquatic ecosystems that are only fragmentarily investigated until now. There is still a substantial need for research to provide realistic solutions about how to deal with light pollution. The suppression of the melatonin rhythm of fish might yield several adverse effects that are not sufficiently studied so far. Impacts on the immune system, growth and development as well as behavioural traits can influence not only individuals. Impacts on reproduction and species specific sensitivities may cause unnatural niche shifts, affect predator prey relationships and influence species communities and whole ecosystems.

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Acknowledgements

I would like to express my gratitude to my supervisors Werner Kloas and Franz Hölker. Particularly, I thank Prof. Dr. Werner Kloas for giving me the opportunity to do my PhD at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), for being a supportive advisor, for providing the necessary equipment and for many helpful ideas and suggestions.
I also want to especially thank PD Dr. Franz Hölker, head of the interdisclipinary research project "Verlust der Nacht", for giving me the opportunity to work at this exciting topic, which allowed me to present and discuss my research at several international conferences and meetings.
Steffen Franke from the INP Greifswald supported this work by providing the lighting set-up. I have to thank him many times for his efforts. It was a pleasure to work with him and expand my horizon.
A very special thanks goes to Torsten Preuer who supported me in the development of the experimental set-ups and methods. His many inspiring and fancy ideas contributed substantially to the success of this thesis. Lilli, my student, and Tine: Thank you for helping me so much, it was great to work with you both because you had so much affection for this topic! I am also very grateful to Wibke Joseph for performing the gene expression analyses for me, to Matthias Kunow and Ingo Cuppock for endless supply of fishes and technical support for my experiments, to the IT-team of the IGB and to Klaus Kopf for the opportunity to occupy his lab space.
I would like to express my gratitude to Kirsten Pohlmann, who helped me not only with statistical problems but also always encouraged me and finally helped me to organize the final phase of my PhD. Your advices prevented me from freaking out.
I owe great thanks to everyone who supported me with my sampling during many many long nights. Although it was exhausting, it was great fun with all of you. And sorry for disturbing your biological rhythm!
I am extremely grateful to my colleagues and friends from the IGB who made my work so enjoyable and entertaining. Frauke Hoffmann and Nike Sommerwerk were the best office mates in the world; it was always funny with you. Thank you so much! Thanks to the whole aquarium hall crew and especially Björn, Christian and Hendrik and of course my girls, Bine and Karin. Each one of you considerably enriched my life.
Finally, I am greatful to my friends Kete, Conrad, Rip, Mirko, Uta and Konne. I especially have to thank Lars, my best friend ever, who is part of my life since nearly 20 years, for his unconditional friendship.

And last but not least: The work on this PhD-project would not have been possible without the unlimited support and encouragement of my family: mum, dad, my sister Saskia and my brother in law Basty, and of course little Vince and Hoschy.
Mama und Papa: Ihr habt mich immer in allem unterstützt, habt mir immer versichert, dass ihr für mich da seid, egal was passiert. Ich bin euch so dankbar für eure Liebe und das Gefühl von Geborgenheit, das ihr mir gebt!

Statement of academic integrity

I hereby certify that the submitted thesis “Spotlight on fish: The biological impacts of artificial light at night” is my own work, and that all published or other sources of material consulted in its preparation have been indicated. Where any collaboration has taken place with other researchers, I have clearly stated my own personal share in the investigation. I confirm that this work, in the same or a similar form, has not been submitted to any other university or examining body for a comparable academic award.

Berlin, 22 March 2016

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Anika Brüning