Effects of skyglow on the physiology of Eurasian perch, *Perca fluviatilis*

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“...as far as we are capable of knowledge,
we sin in neglecting to acquire it...”

— Gottfried Wilhelm Leibniz
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“Wissen und Erkennen sind die Freude
und die Berechtigung der Menschheit;“

— Alexander von Humboldt
Kosmos: Entwurf einer physischen Weltbeschreibung

Contents

Summary

Artificial light at night (ALAN) is emitted from centers of human activities and increasingly brightens up nights and can disturb biological rhythms of humans and wildlife. Skyglow is a diffuse brightening of the night sky due to reflection and scattering of ALAN, which indirectly illuminates large areas of urban and suburban ecosystems. As centers of human activities are usually located close to rivers and lakes, skyglow may disproportionally affect wildlife of freshwater.

In this thesis, I present research on the effects of ALAN and particularly the dim light intensities of skyglow on the physiology of Eurasian perch *Perca fluviatilis* – a common freshwater fish species in temperate Eurasia. Results of three experiments are presented: One experiment under controlled conditions in a climate chamber at low illuminances mimicking skyglow exposure (nocturnal illumination of 0.01, 0.1 and 1 lx at the water surface, all chapters). A second experiment was also carried out under controlled conditions in a climate chamber, but with higher light intensities (1, 10, 100 lx) to investigate effects for which little information on ALAN effects was available from previous literature (Chapter 3 and 4). In the third experiment, skyglow was mimicked with an illuminance of 0.06 lx at the water surface of large lake enclosures in a field experiment (Chapter 2).

In Chapter 1, significant suppression of nocturnal melatonin under very dim ALAN (0.01, 0.1, 1 lx) in controlled conditions clearly show the potential of skyglow to disrupt biological rhythms. Compared to the existing literature, the reduction in melatonin at only 0.01 lx is among the lowest light intensities at which ALAN effects have been demonstrated. The data allow a description of the lower range of the dose-response relationship between ALAN intensities and the suppression of nocturnal melatonin levels, but a no observed effect level could not be identified. The rhythmic pattern of the circadian melatonin profile (low levels during the day, high levels during the night) was notably disturbed at an ALAN intensity of 1 lx. Melatonin production is directly suppressed by light and thus melatonin may be an important regulator of other light-sensitive physiological processes, such as reproduction, thyroid metabolism, immune responses, antioxidative responses, or general metabolism.

Chapter 2 concludes that skyglow can affect reproductive processes of female Eurasian perch under certain circumstances. In the field experiment, emulated skyglow (0.06 lx) reduced the gene expression of luteinizing hormone (as compared to natural nights) in mature females. Under controlled conditions with surface ALAN of 0.01, 0.1, or 1 lx, gonadotropin expression was not significantly reduced in smaller females, but strong suppression of follicle-stimulating hormone was observed for some individuals compared to control means. The data in Chapter 2 indicate skyglow effects on reproduction of Eurasian perch, but more data accounting for season, developmental stage of the fish and sex differences are needed for a conclusive picture. Effect thresholds of such sensitive response variables to ALAN exposure, such as melatonin and reproduction, can be important arguments for the conservational regulation of ALAN.

For the first time, a reduction of total plasma triiodothyronine (Chapter 3), as well as a reduction of relative liver weight (Chapter 4) were evident after exposure to ALAN. However, both effects were only significant at a high intensity of 100 lx. At low intensities (0.01, 0.1, 1 lx), no significant effects on thyroid hormones or body indices were measured under controlled conditions (Chapters 3 and 4). Parameters of the innate immune system and antioxidative defense system were not affected by ALAN at any tested intensity (0.01, 0.1, 1, 10, 100 lx) after two weeks under controlled conditions but long-term effects cannot be excluded (Chapter 4).

Overall, this thesis shows physiological changes already at very weak intensities of ALAN, like they occur over large areas of urban and suburban ecosystems in the form of skyglow. The most sensitive response variable to ALAN exposure is the nocturnal melatonin levels, which is congruent with research in other vertebrates. In this thesis, I discuss possible actions of ALAN on other physiological parameters – either by direct perception of light or indirectly via reduced melatonin. It is important to note that it might be the rhythmicity of melatonin production (i.e., relative difference between day and night levels) rather than the absolute blood melatonin concentrations that determines sensitivity to ALAN.

My thesis contributes to an understanding of effect thresholds for several physiological parameters that respond to ALAN exposure of several weeks. Future research should consider longer exposure with repeated measures since ALAN effects may accumulate over time or occur on different time scales. Such long-term experiments could allow estimates of the transferability of short-term ALAN effects at high intensities to long-term effects at lower intensities. Thresholds for ALAN intensities combined with an understanding of the temporal dynamics of ALAN effects, as well as consideration of different colors of ALAN, could provide the necessary descriptors for elaborating regulatory measures to reduce light pollution in the future.

Zusammenfassung

Künstliches Licht in der Nacht (artificial light at night – ALAN) entsteht in Zentren menschlicher Aktivität und erhellt zunehmend die Nacht, wodurch biologische Rhythmen von Menschen und Wildtieren gestört werden können. Skyglow (oder deutsch: Himmelsleuchten) ist eine diffuse Aufhellung des Nachthimmels aufgrund von Reflexion und Streuung von ALAN, welche indirekt große Bereiche städtischer und vorstädtischer Ökosysteme beleuchtet. Da sich die Zentren menschlicher Aktivitäten in der Regel in der Nähe von Flüssen und Seen befinden, kann sich Skyglow unverhältnismäßig stark auf wildlebende Tiere in Süßwassergebieten auswirken.

In dieser Arbeit untersuche ich die Auswirkungen von ALAN und insbesondere der schwachen Lichtintensität von Skyglow auf die Physiologie des Europäischen Flussbarsches *Perca fluviatilis*, einer weit verbreiteten Süßwasserfischart in den gemäßigten Breiten Eurasiens. Die Ergebnisse von drei Experimenten werden vorgestellt: Ein Experiment wurde unter kontrollierten Bedingungen in einer Klimakammer bei niedrigen Intensitäten durchgeführt, die eine Skyglow-Exposition nachahmen (nächtliche Beleuchtung von 0,01, 0,1, 1 lx an der Wasseroberfläche, alle Kapitel). Ein zweites Experiment wurde ebenfalls unter kontrollierten Bedingungen in einer Klimakammer durchgeführt, jedoch mit höheren Lichtintensitäten (1, 10, 100 lx), um Effekte zu untersuchen, für die aus der bisherigen Literatur nur wenig Information zu ALAN Effekten vorlagen (Kapitel 3 und 4). Das dritte Experiment war ein Freilandversuch in großen Versuchszylindern im Stechlin See, die einen Skyglow von 0,06 lx an der Wasseroberfläche nachahmten (Kapitel 2).

In Kapitel 1 wird eine signifikante Unterdrückung des nächtlichen Melatoninspiegels bei sehr schwachem ALAN (0,01, 0,1, 1 lx) unter kontrollierten Bedingungen gezeigt, wodurch das Potenzial von Skyglow, biologische Rhythmen zu stören, deutlich wird. Im Vergleich mit der bestehenden Literatur gehört die Verringerung von Melatonin bei nur 0.01 lx zu den niedrigsten Lichtintensitäten, bei denen ALAN-Effekte nachgewiesen wurden. Die Daten erlauben eine Beschreibung des unteren Bereichs der Dosis-Wirkungs-Beziehung zwischen ALAN-Intensitäten und der Unterdrückung des nächtlichen Melatoninspiegels, aber ein unterer Grenzwert ohne Effekte konnte nicht identifiziert werden. Das rhythmische Muster des zirkadianen Melatoninprofils (niedrige Werte am Tag, hohe Werte in der Nacht) war bei einer ALAN-Intensität von 1 lx deutlich gestört. Die Melatoninproduktion wird direkt durch Licht unterdrückt, sodass Melatonin ein wichtiger Regulator für weitere lichtempfindliche physiologische Prozesse sein kann, wie z. B. der Fortpflanzung, des Schilddrüsenhormonstoffwechsels, der Immunreaktion, der antioxidativen Prozesse oder des allgemeinen Stoffwechsels.

Kapitel 2 zeigt auf, dass Skyglow unter bestimmten Umständen die Fortpflanzungsprozesse von weiblichen Flussbarschen beeinflussen kann. In dem Feldexperiment mit emulierten Skyglow (0,06 lx) war die Genexpression des luteinisierenden Hormons (im Vergleich zu natürlichen Nächten) bei geschlechtsreifen Weibchen reduziert. Unter kontrollierten Bedingungen mit

Oberflächen-ALAN von 0,01, 0,1 oder 1 lx war die Gonadotropin-Expression bei kleineren Weibchen nicht signifikant reduziert, aber bei einigen Individuen wurde eine starke Unterdrückung des follikelstimulierenden Hormons im Vergleich zur Kontrolle beobachtet. Die Daten in Kapitel 2 deuten auf Effekte von Skyglow auf die Fortpflanzung des Flussbarsches hin, aber für ein schlüssiges Bild sind weitere Daten erforderlich, die die Jahreszeit, das Entwicklungsstadium der Fische und die Geschlechtsunterschiede berücksichtigen. Schwellenwerte für solch empfindliche Reaktionsvariablen in Bezug auf ALAN-Exposition, wie Melatonin und Reproduktion, können wichtige Argumente für regulierende Naturschutzmaßnahmen von ALAN sein.

Zum ersten Mal wurden eine Verringerung des Gesamtplasma-Trijodthyronins (Kapitel 3) sowie eine Verringerung des relativen Lebergewichts (Kapitel 4) nach ALAN-Exposition nachgewiesen. Beide Effekte waren jedoch nur bei einer hohen Intensität von 100 lx signifikant. Bei niedrigen Intensitäten (0,01, 0,1, 1 lx) wurden unter kontrollierten Bedingungen keine signifikanten Auswirkungen auf Schilddrüsenhormone oder Körperindizes gemessen (Kapitel 3 und 4). Parameter des angeborenen Immunsystems und des antioxidativen Abwehrsystems wurden durch ALAN bei keiner der getesteten Intensitäten (0,01, 0,1, 1, 10, 100 lx) nach zwei Wochen unter kontrollierten Bedingungen beeinflusst, aber Langzeiteffekte können nicht ausgeschlossen werden (Kapitel 4).

Insgesamt zeigt diese Arbeit, dass physiologische Veränderungen bereits bei sehr schwachen ALAN-Intensitäten auftreten, wie sie in großen Bereichen städtischer und suburbaner Ökosysteme in Form von Skyglow vorkommen. Übereinstimmend mit der Forschung bei anderen Wirbeltieren, ist die empfindlichste Reaktionsvariable auf die Belastung durch ALAN bei Fischen der nächtliche Melatoninspiegel. In dieser Arbeit diskutiere ich mögliche Wirkungen von ALAN auf andere physiologische Parameter – entweder durch direkten Lichteinfall oder indirekt über reduziertes Melatonin. Es ist wichtig zu beachten, dass die Empfindlichkeit gegenüber ALAN möglicherweise eher durch die Rhythmik der Melatoninproduktion (d.h. den relativen Unterschied zwischen Tages- und Nachtwerten) als durch die absolute Melatoninkonzentration im Blut bestimmt wird.

Meine Arbeit trägt zum Verständnis der Effektschwellenwerte für verschiedene physiologische Parameter nach einer mehrwöchige ALAN-Exposition bei. Künftige Forschungsarbeiten sollten längere Expositionen mit wiederholten Messungen in Betracht ziehen, da die Auswirkungen von ALAN akkumulieren oder in unterschiedlichen Zeitabständen auftreten können. Solche Experimente könnten Schätzungen der Übertragbarkeit von kurzfristigen ALAN-Effekten bei hohen Intensitäten auf langfristige Effekte bei niedrigeren Intensitäten ermöglichen. Schwellenwerte für ALAN-Intensitäten in Kombination mit einem Verständnis für die zeitliche Dynamik von ALAN Effekten, aber auch die Berücksichtigung verschiedener Farben von ALAN, könnten zukünftig die notwendigen Deskriptoren für die Ausarbeitung von regulierenden Maßnahmen zur Reduzierung von Lichtverschmutzung liefern.

Introduction

Artificial light at night and light pollution

The nights on earth have been getting brighter since humans introduced artificial light at night (ALAN). Particularly, since technological advancements have made light more efficient, ALAN is financially affordable to many people and the extent of artificial illumination of the night is increasing every year (Hölker *et al.*, 2010a, Kyba *et al.*, 2017a). While humans make use of artificial light to expand their temporal activity range to a "24 h society", possible negative side effects of ALAN include increased incidence of cancer, metabolic syndrome, and mood disorders in humans (Walker *et al.*, 2020) as well as an unintended impact on nature (Rich and Longcore, 2006). Most dramatically, the "vacuum cleaner effect" describes how insects are attracted to light and circle around artificial light sources to exhaustion and death (Eisenbeis, 2006, Frank, 2006). Other tragic effects of ALAN include the attraction of migrating birds to bright lights, which can result in mass die-offs due to exhaustion (Witherington, 1997), or the disorientation of hatching sea turtles, which crawl towards artificial lights instead of the sea and often die in road traffic or are easy prey for predators (Lorne and Salmon, 2007, Salmon and Witherington, 1995, Witherington, 1992).

During most of the time of evolution, almost all organisms on earth have adapted to a daily cycle of light at day and darkness at night until humans started to regularly light up the night. For a long time, the full moon was the brightest regularly occurring light source at night. All species have adapted to specific ecological niches, which has required an optimization of physiology by spatially and temporally adjusting biochemical processes to their environment. For example, day-active species often rely on their visual sense whereas night-active species have generally higher sensitivities in other sensory systems (olfactory, auditory) and special adaptations of the eyes for capturing low light incidence. Flora and fauna constantly adapt to environmental changes, but light pollution has been increasing by rates of 2 – 6% globally, with rapidly developing areas of up to 20% more illuminated areas each year (Hölker *et al.*, 2010a, Kyba *et al.*, 2017a). Like other anthropogenic impacts, such dramatic changes may be too fast for most species to adapt. Currently, the world faces a global biodiversity crisis due to overuse of the environment by humans (e.g., climate change, deforestation, chemical pollution, overfishing) associated to losses of ecosystem services (IPCC, 2014, OECD, 2019). In this biodiversity crisis, freshwaters are disproportionally affected by manmade stressors and show particularly severe declines in biodiversity (Reid *et al.*, 2018). Light pollution may be another factor adding up on the biodiversity crisis (Hölker *et al.*, 2021, Hölker *et al.*, 2010b). Anthropogenically introduced factors which alter natural ecosystems beyond its natural limits of variation are called environmental stressors (Carrier-Belleau *et al.*, 2021). ALAN can therefore be considered an environmental stressor and in this context, ALAN is often referred to as light pollution. Light pollution is listed as one of the pollution categories under "excess energy" in the IUCN threat classification scheme (IUCN, 2021). For further classification of the severity of this type of pollution to wildlife, a

better understanding of the range of exposure and description of threshold light intensities at which population declines can be expected, will be necessary.

Skyglow in freshwater ecosystems

Most ALAN sources originate from above water surfaces, but light penetrates into water and ALAN can affect aquatic organisms. Humans rely on a good ecological status of freshwaters which is the basis of our lives. ALAN mainly derives from centers of human activities, namely cities, which are usually located near bodies of freshwater (Kummu *et al.*, 2011). Therefore, freshwaters are frequently exposed to ALAN and freshwater organisms, which are adapted to natural nocturnal darkness in the same way as terrestrial species, are prone to a disruption of biological rhythms by ALAN.

Currently, most of the available literature on ALAN effects in aquatic ecosystems focuses on light intensities as they would occur in immediate proximity to bright light sources such as streetlamps (see next section, Figure 1). However, only small fractions of water bodies are exposed to direct glare and large parts of the water surface of lakes, rivers or coastal areas is rather exposed to indirect illumination that is reflected by clouds and particles in the air and causes a brightening of the sky over large areas – a phenomenon called skyglow (Kyba *et al.*, 2011). Typically, it occurs as a homogenous luminance of the sky at rather low intensities that vary depending on the amount of light-reflecting aerosols and particles in the air, such as clouds, but also on the ground, e.g., snow or wet roads (Jechow and Hölker, 2019b). Skyglow can therefore intensify ten- to hundred-fold depending on the weather condition as measured in industrial regions of Europe (Jechow *et al.*, 2016, Kyba *et al.*, 2011, Kyba *et al.*, 2015, Puschnig *et al.*, 2014a, Puschnig *et al.*, 2014b) and Asia (Pun and So, 2012). The light intensity of skyglow alone without direct illumination on the surface of urban water typically ranges from 0.001 lx to 0.065 lx in clear nights and from 0.03 lx to 0.55 lx in cloudy nights (Hänel *et al.*, 2018), sometimes reaching up to 1 lx (Jechow *et al.*, 2020, Kyba *et al.*, 2015). For comparison, on a bright clear day surface illuminance reaches up to 120,000 lx and still up to 10,000 lx on a cloudy day (Figure 1). In contrast, the brightest natural light source at night is the full moon, which typically results in illuminance up to 0.1 lx in temperate latitudes but never larger than 0.4 lx (Kyba *et al.*, 2017b), whereas typical natural illuminance during moonless nights is below 0.001 lx (Hänel *et al.*, 2018, Hölker *et al.*, 2018). Skyglow can therefore vary within the range of lunar variation of illumination or even exceed it but is less predictable for aquatic wildlife. It can thus blur (i.e., pollute) natural circalunar rhythms of night sky brightness (Puschnig *et al.*, 2014a). The spectral composition of skyglow has not often been described and depends on the spectrum of the predominant light-emitting sources in the surroundings. With predominant use of sodium vapor pressure lamps, skyglow has a typical peak around 580 nm (Hänel *et al.*, 2018, Spitschan *et al.*, 2016). The correlated color temperature (CCT) of skyglow ranges from neutral-white light (3500 – 5000 K) in clear nights to warmer white light (2100 – 4000 K) with increasing cloud cover (Jechow *et al.*, 2020). Illumination by the moon has a CCT of approximately 4000 K with a spectral shift towards red wavelengths at lower elevation (Ciocca and Wang, 2013).

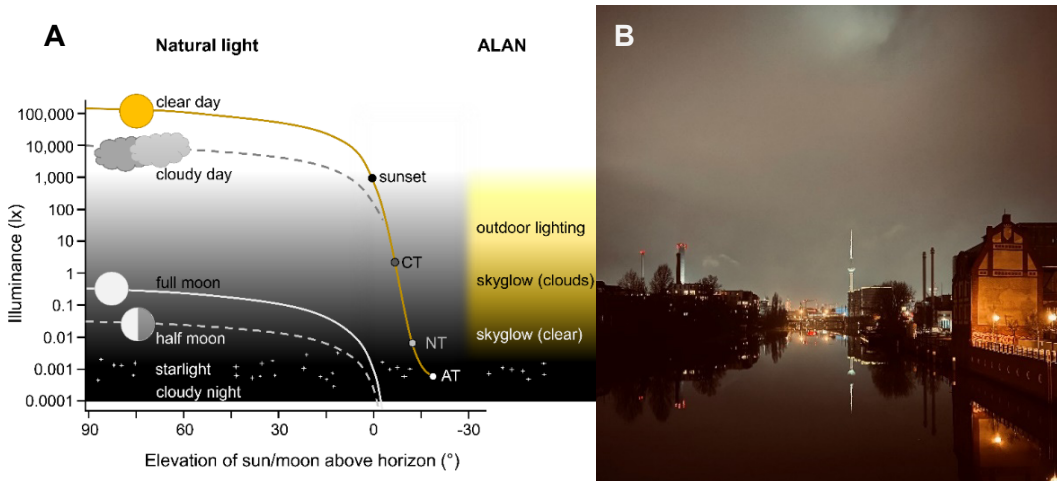

Figure 1 A) Illuminance (lx) on earth's surface by the sun (solid line), moon (dotted line) or artificial light at night (right hand side, yellow to orange color) over the altitude of the sun's or moon's position above or below the horizon (0° is at the level of the horizon). CT – lower boundary of civil twilight; NT – lower boundary of nautical twilight; AT – lower boundary of astronomical twilight. Modified from Grubisic *et al.* (2019) and Gaston *et al.* (2014). **B)** View from the Schilling bridge towards the city center of Berlin with direct illumination by city lights and indirect illumination by skyglow in a cloudy night on the water surface of the Spree River (photo by Franziska Kupprat).

Ecophysiological consequences of ALAN in freshwater ecosystems

In the past 10 – 15 years, numerous effects of ALAN on the physiology or ecology of freshwater organisms have been reported. These effects reach from hormonal changes in freshwater fish, like Eurasian perch (*Perca fluviatilis* L., 1758) and roach (*Rutilis rutilus* L., 1758) (Brüning *et al.*, 2016, 2018a, Brüning *et al.*, 2015, Brüning *et al.*, 2010, Brüning *et al.*, 2018b) to behavioral changes of guppies (*Poecilia reticulata* Peters, 1859) (Kurvers *et al.*, 2018) or Atlantic salmon (*Salmo salar* L., 1758) fry and smolts (Riley *et al.*, 2012, Riley *et al.*, 2013). Furthermore, crayfish reduced their activity (Thomas *et al.*, 2016), zooplankton diel vertical migration was attenuated (Moore *et al.*, 2001) and aquatic insects were drawn towards the lights resulting in increased mortality (Perkin *et al.*, 2014a). Moreover, benthic primary producers (Grubisic *et al.*, 2017) and phytoplankton (Diamantopoulou *et al.*, 2021) are affected by ALAN in terms of biomass and community composition. Phytoplankton (Stephan, 2021) and cyanobacteria (Poulin *et al.*, 2014) also showed impaired photophysiology after exposure to ALAN. Even the composition of bacterial communities in freshwater sediments was shown to be impaired by ALAN (Hölker *et al.*, 2015).

Marine ecosystems, especially the coastlines, which are the most productive zones of marine ecosystems, can be affected by ALAN similarly to freshwater ecosystems. ALAN induced changes in predator-prey interactions (Bolton *et al.*, 2017) and changes in sessile assemblages (Davies *et al.*, 2015). Further, coral reef fish (which often live close to the shore) are affected by ALAN in reproductive behavior and success (Fobert *et al.*, 2019) as well as larval recruitment and settlement (O'Connor *et al.*, 2019) and growth and survival (Schligler *et al.*, 2021). Even corals of the reefs themselves were impaired by ALAN in terms of

gametogenesis and spawning (Ayalon *et al.*, 2021), expression of genes involved in cell cycle, cell proliferation, cell growth, protein synthesis (Rosenberg *et al.*, 2019), and photosynthesis of symbionts as well as oxidative damage on lipids (Levy *et al.*, 2020).

To minimize the ecological consequences of ALAN, regulatory measures will require a description of the dose-response relationship of ALAN intensities and negative ecological consequences. It is not feasible to turn off all lights and thus, it is important to know, how much light is too much light. Most of the above-mentioned studies used rather high light intensities of ca. 1 – 100 lx, mimicking mostly direct exposure to ALAN by strong light sources which is in most cases limited to a certain time window in the day or night. There is a rather large data gap to the effects of ALAN exposure at low intensities < 1 lx to which larger areas are exposed continuously in the form of skyglow.

Some of the above-described outcomes can be indirectly induced by ALAN through effects on other organisms and inter-species interactions. Especially changes on a population or community level might be the result of top-down or bottom-up effects (Hölker *et al.*, 2015). However, other effects are a direct result of the incidence of light. Directly light-dependent processes in teleost fish include vision (and associated feeding and locomotor activity, predator-prey interactions), resting, biological rhythms (molecular clock and melatonin, associated to reproductive processes and potentially many other physiological processes). In the following, I will first briefly describe how ALAN (or light in general) is perceived and then how different physiological variables respond to light during particular times of day and year.

Light perception in fish

All vertebrates, including fish, possess a variety of photoreceptors, i.e., pigments in photoreceptor cells that are capable of light harvesting. Photoreceptors can detect ALAN and transduce signals at night which would naturally only be transduced throughout the day or prevent signals which would naturally be transduced in the darkness of the night. The best-known photoreceptor cells of vertebrates are rods and cones in the retina of the lateral eyes with their image-forming photoreceptors cone-opsins and rod-opsins. In the retina and in the pineal gland, there are also non-image forming photoreceptors, such as melanopsin, an opsin involved in photoentrainment (Eilertsen *et al.*, 2014, Falcón *et al.*, 2020, Grubisic *et al.*, 2019). Furthermore, non-retinal photoreceptors in fish can be found in deep-brain regions, or in the skin, mainly for adjustment of pigmentation (Kelley and Davies, 2016, Peirson *et al.*, 2009). An illustration of different photoreceptive tissues and types of photoreceptors (image-forming and non-image forming) is given in Figure 2.

The non-image forming photoreceptors in the pineal gland (red structure in Figure 2) will be most important in my thesis. Besides excitatory neurotransmission upon light perception, the cone-like photoreceptor cells produce melatonin during darkness, which is subsequently transported to the cerebrospinal fluid and circulates throughout the body (Falcón *et al.*, 2010).

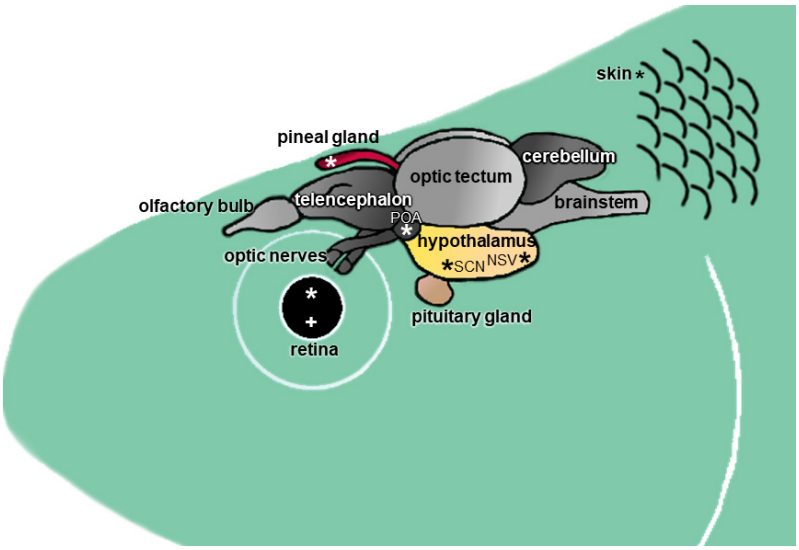

Figure 2 Fish brain and neuroendocrinological key structures for the action of ALAN on fish physiology. NSV – nucleus of the *saccus vasculosus* (structure of the inferior lobe of the hypothalamus); POA – preoptic area; SCN – suprachiasmatic nucleus; * – non-image forming photoreception (pineal gland, retina, *saccus vasculosus*, skin); + – image-forming photoreception (retina). Illustration by Franziska Kupprat.

The pineal circadian system of fish – photoreception, the molecular clock and melatonin

Every circadian system within a fish consists of three components: input, intrinsic oscillators, and output (Cowan *et al.*, 2017).

Photoreception (Input)

Light serves as an input parameter to the circadian system. It informs the body about the time of day in the surrounding environment. Light is perceived by fish through the retina in the eyes, some deep-brain regions, and also by the pineal gland, which is located on top of the diencephalon of the brain underneath a less-pigmented window in the skull (Figure 2). Pineal photoreceptor cells have a similar organization to retinal photoreceptor cells and the photoreceptors harvesting the light are also similar to retinal photoreceptors. They include primarily rod-like opsins but also cone-like opsins, melanopsin, or vertebrate-ancient like opsin (Peirson *et al.*, 2009, Philp *et al.*, 2000).

Spectral sensitivity of fish is generally adapted to the surrounding light conditions in the water which can vary more greatly than in terrestrial animals, e.g., by density and composition of phytoplankton communities and organic particles. Whereas terrestrial animals usually have a sensitivity peak in the blue spectral range, freshwater fish are usually more susceptible to green or red light as these are the dominating colors in their natural habitat (Carleton *et al.*, 2020). Marine fish, in contrast have higher spectral sensitivities in the blue range.

Similar to retinal cells, pineal cells contain a full set of molecular clock components (the oscillator) as well as all components to synthesize melatonin (the output) (Saha *et al.*, 2019).

The molecular clock (Oscillator)

Four genes build the core of the molecular clock: CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL (Brain and Muscle ARNT-Like) are rhythmically expressed and build a heterodimer in the late afternoon (Cahill, 2002). The CLOCK:BMAL heterodimer binds to promoters of the PER (Period) gene, thereby activating its transcription. The activation potential of the fourth gene – CRY (Cryptochrome) – is not so well-established in the literature. PER and CRY repress the transcription of CLOCK and BMAL with PER peaking in the morning and CRY peaking slightly delayed (Fig.3 in Cahill, 2002). By the repression of the CLOCK:BMAL heterodimer, the activation of PER (and CRY) also stops and PER and CRY are degraded by hyperphosphorylation which again allows CLOCK and BMAL translation. Post-translational control also involves a second loop of REV-ERB (nuclear receptor family of intracellular transcription factors) and ROR (retinoic acid receptor-related orphan receptors) as well as casein kinases CA Iε and CA Iδ (Cahill, 2002, Reppert and Weaver, 2002). The oscillators are intrinsic although some components are also directly affected by light, entraining the circadian system and "resetting the clock". For example, transcription of PER2 (one of the isoforms of PER) is directly activated by light in the morning and also CRY1a can be directly activated by light (Cahill, 2002, Isorna *et al.*, 2017, Reppert and Weaver, 2002). The intrinsic nature of the molecular clock is the reason why some output parameters (sometimes called overt rhythms) are maintained even under constant light or constant darkness. However, the components directly responding to the daily change of light and darkness might be the reason output rhythms are only maintained for a few days.

Besides the pineal circadian system, oscillators can be found in numerous other tissues, including the retina, other brain regions, the pituitary gland, liver, gut, gonads, and head kidney (Isorna *et al.*, 2017).

Interestingly, the CLOCK:BMAL heterodimer also activates Arylalkyl-N-aminotransferase (AANAT), which is the rate-limiting enzyme for melatonin synthesis, by binding to an E-box of the promoter (Appelbaum *et al.*, 2006, Isorna *et al.*, 2017). However, AANAT2 abundance and activity are also directly regulated by light, i.e., hyperpolarization of pineal photoreceptor cells at light (low AANAT2 levels and activity) or depolarization at darkness (high levels of AANAT2).

Melatonin (Output)

Melatonin secretion is the main output of the circadian system. Whereas retinal melatonin acts mainly locally in fish, pineal melatonin is mainly secreted into the blood. The blood plasma melatonin levels of fish resemble AANAT2 mRNA abundance and activity in the pineal gland. Pineal photoreceptor cells mainly express AANAT2 and secrete melatonin into the blood stream; retinal photoreceptor cells express AANAT1, produce retinal melatonin, and the same cells also express melatonin receptors. This suggests an autocrine (local) action of retinal melatonin and its functions include retinomotor movements, neurotransmitter release and melanosome aggregation (Falcón *et al.*, 2010).

Here it should be noted that the gastrointestinal tract as well as the liver also contain full circadian systems with clock components and melatonin as output

parameters (but also e.g., ghrelin and leptin, Isorna *et al.*, 2017). However, the entraining input signal for these systems might not be light, but rather feeding-fasting cycles (Delgado *et al.*, 2017, Isorna *et al.*, 2017) and therefore, I will not go into more detail on this aspect.

Targets of the endocrine melatonin message – Melatonin receptors

In fish (similar to other vertebrates), the hormonal message of pineal melatonin is received by a variety of neuronal and peripheral tissues through melatonin receptors. Melatonin receptors belong to the superfamily of G protein-coupled receptors and are subdivided into four subtypes with varying terminology in the literature (Mel 1a 1.7 = MT1, Mel 1b = MT2, Mel 1c, Mel 1a-like = Mel 1ab = Mel 1d), e.g., reviewed by Sakai *et al.* (2019). Melatonin receptors were identified in a variety of tissues in diverse teleost species, e.g., Atlantic salmon (Ekström and Vaněcek, 1992), carp (*Cyprinus carpio* L., 1758) (Kepka *et al.*, 2015), goldfish (*Carassius auratus* L., 1758) (Ikegami *et al.*, 2009), Northern pike (*Esox lucius* L. 1758) (Gaildrat *et al.*, 2002, Gaildrat and Falcón, 1999), rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) (Falcón *et al.*, 2003, Kulczykowska *et al.*, 2006), European seabass (*Dicentrarchus labrax* L., 1758) (Herrera-Pérez *et al.*, 2010, Kulczykowska *et al.*, 2006, Sauzet *et al.*, 2008), Senegalese sole (*Solea senegalensis* Kaup, 1858) (Confente *et al.*, 2010), medaka (Japanese rice fish, *Oryzias latipes* Temminck and Schlegel, 1846), Amazon molly (*Poecilia formosa* Girard, 1859), three-spined stickleback (*Gasterosteus aculeatus* L., 1758), and Nile tilapia (*Oreochromis niloticus* L., 1758) (Sakai *et al.*, 2019), and zebrafish (*Danio rerio* Hamilton, 1822) (Yumnamcha *et al.*, 2017) to name only a few. In the following, I will focus on the identified melatonin receptors that are relevant for the research in this thesis.

Important neuronal target tissues include, for instance, the melatonin receptors in the pituitary gland, which were identified in Northern pike, rainbow trout, European seabass, goldfish, Senegalese sole, and medaka (Confente *et al.*, 2010, Falcón *et al.*, 2003, Gaildrat *et al.*, 2002, Ikegami *et al.*, 2009, Sakai *et al.*, 2019, Sauzet *et al.*, 2008). Melatonin can additionally act on the pituitary gland via melatonin receptors in other diencephalic brain areas (e.g., rostral preoptic area and the ventromedial thalamic nucleus), which regulate projections to the pituitary gland via dopamine and peptides (Falcón *et al.*, 2010). In goldfish and zebrafish, melatonin receptors were also expressed in ovaries (Ikegami *et al.*, 2009, Yumnamcha *et al.*, 2017) and embryonic expression of melatonin receptors started at 18 h post-fertilization (hpf) in zebrafish (Danilova *et al.*, 2004). Melatonin receptors were further located in the livers of European seabass (Sauzet *et al.*, 2008), golden rabbitfish (Park *et al.*, 2006), and in Senegalese sole (Confente *et al.*, 2010). With respect to the immune system, melatonin receptors were expressed in peripheral blood leucocytes, in head kidney leucocytes (monocytes/macrophages, granulocytes, lymphocytes) of carp (Kepka *et al.*, 2015) and in blood cells in European seabass (Sauzet *et al.*, 2008). Moreover, lymphatic tissues such as head kidneys and thymus also expressed melatonin receptors in carp (Kepka *et al.*, 2015). Splenic melatonin receptors were reported for carp (Kepka *et al.*, 2015), golden rabbitfish (Park *et al.*, 2006), goldfish (Ikegami *et al.*, 2009), and Senegalese sole (Confente *et al.*, 2010).

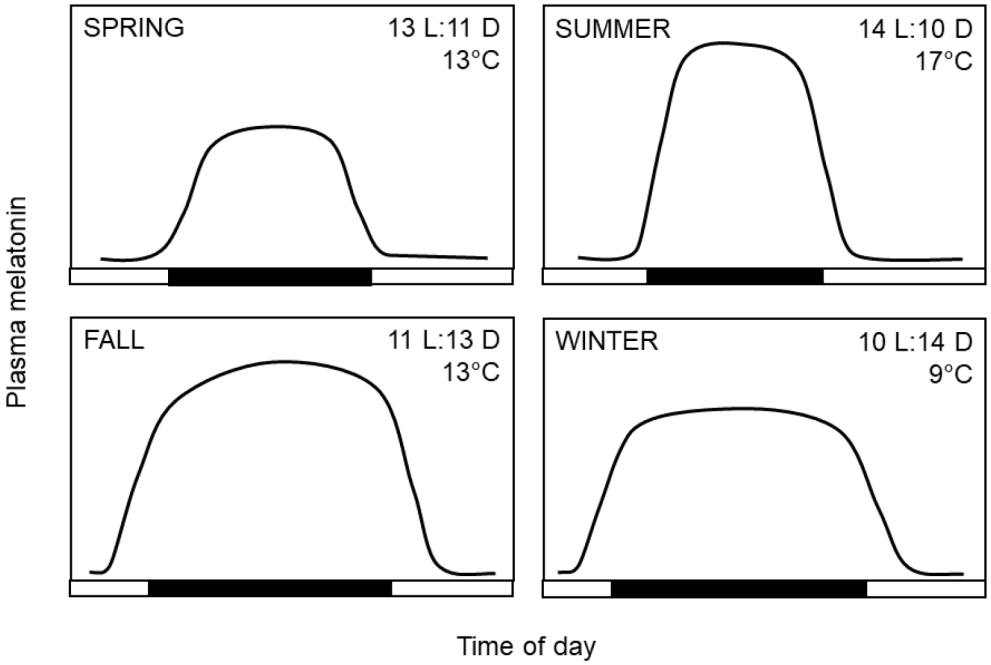

Figure 3 Daily rhythms of plasma melatonin in temperate fish species throughout different seasons. Varying photoperiod indicated by black and white bars underneath each panel and written in hours for light (L) and darkness (D) along with temperature variation. This graph is a simplified version of data published by Masuda *et al.* (2003)for rainbow trout (*Oncorhynchus mykiss*). Similar seasonal variations are assumed for other temperate freshwater fishes, including Eurasian perch (*Perca fluviatilis*).

Overall, regarding ALAN research, the pineal circadian system and its output of circulating melatonin are the most interesting physiological parameters, as both are directly light sensitive. Melatonin has been shown to be reduced by ALAN in a variety of species from invertebrates to vertebrates and the suppression of plasma melatonin by ALAN seems to be a consistent feature within fishes (Grubisic *et al.*, 2019, Sanders *et al.*, 2021). However, a clear description of the dose-response relationship has not been described for freshwater fish, yet. Moreover, circulating melatonin acts on a variety of other physiological processes via melatonin receptors, meaning that direct light effects on pineal melatonin may cascade to secondary effects in other cells and organs. Furthermore, melatonin not only entrains circadian rhythms but also conveys the information on photoperiod, i.e., seasonal variations of light (Nakane *et al.*, 2013) (Figure 3). Seasonal variation of light is particularly important for reproduction of temperate fishes.

Reproduction of fish

A detailed introduction into ALAN effects on reproductive processes of teleost fish is given in Chapter 2.

The following mainly refers to temperate teleosts, which experience four seasons. The basic principles of hormonal control can be expected to be similar in lunar spawners in tropical ecosystems or reproduction in polar regions, but

timescales of processes may be fundamentally different, e.g., lunar instead of annual rhythms.

The age when the first full reproductive cycle is completed depends on the species and sex, males often start to reproduce earlier than females. For example, male Eurasian perch reach reproductive maturation at an age between 1 – 2 years, females at 2 – 4 years; male roach reproduce for the first time at an age of 2 – 3 years, and females typically one year later (Kottelat and Freyhof, 2007). Hormones play a crucial role in the reproductive annual cycle. After spawning (in spring/early summer for many temperate freshwater fishes), which requires a large amount of energy, most species have a short period of low concentrations of sexual hormones (for temperate freshwater fishes typically summer), in which feeding and strengthening of condition is a major goal. At the end of summer, production of reproductive hormones starts. Reduction of photoperiod (shortening of days) induces an increase in gonadotropin-releasing hormone (GnRH) in the hypothalamus which leads to increased gene expression of gonadotropins (follicle-stimulating hormone, FSH; luteinizing hormone, LH) from gonadotropic cells in the pituitary gland. Gonadotropins are transported via the bloodstream to the gonads (ovaries or testes) to induce production of sexual steroids (in females, mainly 17-*β*-estrogen (E2); in males, mainly 11-keto testosterone (11 KT), and testosterone). This hormonal cascade is often referred to as the hypothalamic-pituitary-gonadal axis (HPG axis). In females, E2 induces production of vitellogenin in the liver, which is transported to, taken up by and incorporated into oocytes (vitellogenesis). Ovarian maturation culminates in ovulation of fertilizable eggs (ova) and finally in the spawning event (Reading *et al.*, 2017). In males, gonadotropins (mainly FSH) and 11 KT induce proliferation of spermatogonia as an initiating step in spermatogenesis. After mitosis, spermatogonia become spermatocytes followed by meiosis to spermatids and with ongoing spermatogenesis to spermatozoa which become ready to fertilize an egg when they gain full motility (Schulz *et al.*, 2010, Schulz and Miura, 2002). After or during completion of gonadogenesis throughout fall, winter, and early spring, reproductive behavior can include migration to spawning grounds, species-specific mating behavior, or gathering at suitable spawning grounds. After successful spawning and fertilization by males, embryos develop in a few days to weeks in most temperate freshwater teleosts before larvae hatch and develop to a new cohort of the population (see next section on development).

Several of these processes are prone to impairment by ALAN as they are directly or indirectly regulated by light. The onset of gonadogenesis by production of gonadotropins is one sensitive point in the annual reproductive cycle because it is related to decreasing photoperiod and this environmental cue can be blurred by artificial illumination. Although many reproductive processes like spawning are co-regulated by temperature, photoperiod is a more reliable zeitgeber without notable natural variation.

Brüning and colleagues showed a complex impact of ALAN on the reproductive processes in two temperate freshwater fish species – Eurasian perch and roach (Brüning *et al.*, 2016, 2018a, Brüning *et al.*, 2018b). Melatonin is thought to mediate the photoperiodic information and regulation of gonadotropin production via

melatonin receptors in the hypothalamus acting on GnRH, gonadotropin- inhibitory hormone (GnIH), kisspeptin and via melatonin receptors in the pituitary gland acting directly on FSH and LH production (Falcón *et al.*, 2010, Migaud *et al.*, 2010).

Direct effects of light on reproductive processes of fishes and indirect effects of light via melatonin suppression have been known for a long time because it has been applied in fish farming to counteract early maturation and control reproduction. For example, in Senegalese sole sexual steroids were reduced under continuous illumination indicating impaired gonadogenesis (García-López *et al.*, 2006). Likewise, 11 KT and gene expression of gonadotropins were reduced after exposure to continuous illumination in juvenile male European seabass, which can be applied to prevent precocious maturation in aquaculture (Felip *et al.*, 2008, Rodríguez *et al.*, 2005). In Eurasian perch, the gonadosomatic index was reduced by long photoperiod (16 L:8 D) or continuous illumination compared to a balanced photoperiod (12 L:12 D) (Migaud *et al.*, 2004). Many more examples are available from different fish species with varying outcomes depending on species, sex, time of the year and experimental setup. These applications in aquaculture are realized with high light intensities, usually in the form of continuous light with the same intensities as daylight illumination and therefore, comparability to ALAN exposure is limited. Expression of gonadotropins from the pituitary gland was impaired in Eurasian perch (Brüning *et al.*, 2016) associated to reduced melatonin levels (Brüning *et al.*, 2015) in experiments with ALAN exposure (high intensities during daytime, dimmed light mimicking twilight and lower illuminance of 1, 10, 100 lx during nighttime).

If spawning was successful despite ALAN, negative impacts of ALAN can be expected for further reproductive processes, like embryonal development, hatching, and early life stages of fish. For example, dispersal of Atlantic salmon fry was delayed after experimental exposure to ALAN of 12 lx so that fry did not only disperse throughout the night but was prolonged to the morning and even throughout the day (Riley *et al.*, 2013). As this increases chances for predators like birds, this ALAN effect might as well induce a reduction of overall reproductive success in terms of reduced number of viable offspring. Moreover, in tropical coral reef fish, hatching was completely inhibited in clownfish after exposure to 25 lx (Fobert *et al.*, 2019). Moreover, even after successful hatching, larval settlement and survival of convict surgeonfish larvae (*Acanthurus triostegus* L., 1758) was decreased by ALAN exposure of 20 – 25 lx, mainly due to higher susceptibility to predators (O'Connor *et al.*, 2019). Such a reduction of viable offspring is a clear sign for reduced reproductive success. Interestingly, in convict surgeonfish larvae in the same study, thyroid hormone levels were also altered by ALAN exposure with a significant decrease of T3 on day 2 without changes in T4 and non-significant increase in T4 after day 5 without changes in T3 (O'Connor *et al.*, 2019).

Development, growth, and metabolism of fish

Thyroid hormones (TH) can play a crucial role in the development, growth, and metabolic rate of teleost fish. A detailed introduction into thyroid metabolism of fish and its regulation by light is given in Chapter 3.

The synthesis of thyroid hormones in thyroid follicles, which are typically located loosely distributed in the subpharyngeal region in teleost fish (Geven and Klaren, 2017), is regulated via a hypothalamic-pituitary cascade, very similar to the regulation of reproductive hormones. Thyrotropin-releasing hormone (TRH) is produced in the hypothalamus which projects onto the pituitary thyrotropic cells that produce TSH (differing from gonadotropins only in the *β*-subunit). TSH is released into the blood and induces synthesis of thyroid hormones in thyroid follicles. This hormonal cascade is generally referred to as the hypothalamic-pituitary-thyroid axis (HPT axis). The most biologically active thyroid hormone is triiodothyronine (T3), which is mainly produced from thyroxine (T4) via deiodination by specific enzymes (deiodinases) in various target tissues. Depending on the type of deiodinase, thyroid hormones can be activated or inactivated by conversion from T4 to T3 or from T4 to the less active reverse triiodothyronine (rT3) or by conversion from T3 to the less active diiodothyronine (T2). Whereas, in mammals, mainly T4 circulates in the blood (T3:T4 ratio is ca. 1:14 in humans) and T3 is produced from T4 mainly by outer-ring deiodination in the target tissues, ratios vary greatly between fish species and are often 1:1 or even have higher T3 values than T4 values. In fish, a large portion of outer-ring deiodination may occur in the liver as it was reported for some teleost species (Adams *et al.*, 2000, Morin *et al.*, 1993). Subsequently, T3 could be circulated to peripheral target tissues that do not express deiodinases themselves, which may explain a more balanced T3:T4 ratio in fish.

As thyroid hormones are known to display circadian rhythms (although not as pronounced as the ones of melatonin), light might play a role in the regulation of thyroid hormones. Light is thought to act on the *saccus vasculosus*, a brain structure of fish, which was suggested to play a role in physiological sensing of seasons via TSH production as a functional equivalent to the *pars tuberalis* of the pituitary gland of mammals and birds (Nakane *et al.*, 2013, Nakane and Yoshimura, 2014, O'Brien *et al.*, 2012).

Almost no experiments have been performed to test for ALAN effects on thyroid hormones in fish. T3 was significantly lowered by ALAN exposure of 20 – 25 lx in larvae of convict surgeonfish associated to faster and heavier growth but with decreased survival probabilities (O'Connor *et al.*, 2019). Specific growth rates of a coral reef fish were reduced after long-term exposure (18 – 23 months) to ALAN of approximately 4 lx but TH were not measured in this study (Schligler *et al.*, 2021). Continuous light had effects on thyroid hormones in rainbow trout, but feeding times or reproductive status (e.g., sexual hormones) are also known to (co-)regulate thyroid hormone rhythmicity (Boeuf and Le Bail, 1999, Cyr and Eales, 1996). Therefore, regulation of TH rhythmicity might depend on various factors and is not directly controlled by light. Moreover, melatonin is known to be reduced directly by light and might also mitigate ALAN effects on TH. In fish, no data are available for melatonin effects on TH (e.g., by melatonin administration or pinealectomy), but in rodents and amphibians for instance, melatonin is known to be a TH antagonist (Wittkowski *et al.*, 1988, Wright, 2002). ALAN of 3 – 15 lx impaired amphibian metamorphosis in the American toad (*Anaxyrus americanus* Holbrook, 1836) but no link to TH has been established (Dananay and Benard, 2018).

The immune system and antioxidative defense system – overall health condition of fish

The general health and physiological condition of fish is guaranteed by a complex network of mechanisms like the innate immune system, protecting the organism from bacterial, viral, or fungal infections as a first line of defense, the acquired immune system, which builds up long-term resistance against infections in the form of antibodies, but also the antioxidative defense system as a protection against oxidative stress induced by naturally and unnaturally occurring radicals. Various indices can be used as quick markers for the condition of a fish. For example, the condition factor is a ratio between length and weight of the animals, or the hepatosomatic index the ratio between liver weight and body weight. A detailed introduction to health parameters can be found in Chapter 4.

Some studies showed effects of continuous light or long photoperiod on the innate immune system (Burgos *et al.*, 2004, Valenzuela *et al.*, 2007) or antioxidative capacity (Corona-Herrera *et al.*, 2018, Sreejith *et al.*, 2007) of fish (for details see Chapter 4). Moreover, melatonin itself is a potent antioxidant (Reiter *et al.*, 2000) and is also known to modulate several parameters of the innate immune system in vertebrates (Carrillo-Vico *et al.*, 2013, Esteban *et al.*, 2013). In fish, this is evident for example by the presence of melatonin receptors on the cell membrane of leucocytes (Kepka *et al.*, 2015). For amphibians, low ALAN levels (0.1 and 5 lx) affected the transcriptome of common toad (*Bufo bufo* L., 1758) tadpoles, especially genes related to the immune system (Touzot *et al.*, 2021). Hence, some indication is given that light might play a role in immune functioning and antioxidative defense regulating overall body condition. However, effects of realistic ALAN exposure on the immune system, antioxidative defense or body condition have not been investigated in fish, yet.

Eurasian perch *Perca fluviatilis* L., 1758

Eurasian perch is considered an opportunistic diurnal feeder with activity peaks in twilight (Kottelat and Freyhof, 2007, Wang and Eckmann, 1994) and strongly reduced activity during the night, especially in the presence of predators (Hölker *et al.*, 2007). The freshwater fish inhabits a variety of habitats from medium-sized streams to all types of lakes and from freshwater to brackish estuaries (Kottelat and Freyhof, 2007). Gonadogenesis is initiated in late summer and spawning occurs between March and April in German freshwaters depending on photoperiod and temperatures need to be at least 6°C. Eggs develop in a few weeks until larvae hatch dependent on water temperature, e.g., 15 days after collection in blastula stage at 12°C (Brüning *et al.*, 2010). They feed on pelagic zooplankton in the first months before they return to the littoral zones in late summer (Wang and Eckmann, 1994). Larvae are known to be positively phototactic, but adults also seem to show some behavioral responses to light at night. Nakayama *et al.* (2018) showed correlations of shallower water depth and less swimming distance in full moon nights compared to new moon nights, potentially linked to increased feeding. Bergman (1988) and Flik *et al.* (1997) showed that Eurasian perch feed at very low light intensities of 0.2 lx (infra-red light) and 0.01 lx, so the visual acuity would allow feeding under the illumination of a full moon. Eurasian perch undergo a food niche

shift from zooplanktivorous juveniles to piscivorous adults, but generally Eurasian perch are opportunistic and feed on any available prey (Kottelat and Freyhof, 2007, Persson, 1986, Wang and Eckmann, 1994).

Research objectives

The main objective of this thesis was to determine whether the low light intensities of skyglow are enough to induce physiological effects on melatonin, reproductive processes, thyroid hormones, and health of Eurasian perch. Cortisol was not in the focus of this thesis since thresholds were identified already in a previous study. Thresholds were high and effects did not seem to be adverse (reduced cortisol at 100 lx, Brüning *et al.*, 2015). Understanding the effect levels in the lower range of light intensities is of high relevance because skyglow is not limited to certain locations. Instead, skyglow brightens up the night sky over vast areas spreading also into suburban areas and affecting not only individual fish (e.g., fish swimming underneath a streetlamp) but could affect entire populations.

As effects have been described at higher intensities for melatonin and reproductive processes in previous studies (Brüning *et al.*, 2016, Brüning *et al.*, 2015, Brüning *et al.*, 2018b), this thesis focused on testing realistic skyglow intensities (0.01, 0.1, 1 lx), as they occur over many natural habitats of the Eurasian perch. Effects on circadian melatonin rhythms and reproductive processes were investigated.

There were no studies regarding the effects of ALAN on thyroid metabolism or the health status of fish. However, several studies suggest that ALAN could induce significant alterations, either directly by light itself or indirectly via reduced melatonin. Hence, for investigations on thyroid hormones and health of Eurasian perch, a wide range of light intensities were investigated for these endpoints (0.01, 0.1, 1 lx and 1, 10, 100 lx).

Accordingly, this thesis is divided into four chapters:

1. Chapter: Effects of skyglow on melatonin production of Eurasian perch
2. Chapter: Effects of skyglow on reproductive processes of Eurasian perch
3. Chapter: Effects of artificial light at night on thyroid hormones of Eurasian perch
4. Chapter: Effects of artificial light at night on the immune system, antioxidative system and body indices of Eurasian perch

The aim was to determine the no-observed-effect levels (NOEL) and lowest-observed-effect levels (LOEL) for each parameter to describe the dose-response relationship between ALAN and physiological responses.

List of publications and manuscripts

1. Chapter:

   Kupprat F, Hölker F, Kloas W (2020) Can skyglow reduce nocturnal melatonin concentrations in Eurasian perch? *Environ. Pollut.* 262: 114324. https://doi.org/10.1016/j.envpol.2020.114324

   - Author contributions
     - F. K.: conceptualization, data acquisition, data analyses, interpretation of results, manuscript writing
     - F. H.: conceptualization, interpretation of results, manuscript writing
     - W. K.: conceptualization, interpretation of results, manuscript writing
   - Published: July 2020

2. Chapter:

   Kupprat F, Kloas W, Berger SA, Bittmann S, Gessner MO, Jechow A, Kyba CCM, Mahlow P, Nejstgaard JC, Scharfenberger U, Singer GA, Wuertz S, Hölker F (*draft*) Dim artificial light at night affects reproductive processes of Eurasian perch (*Perca fluviatilis*) – climate chamber and outdoor enclosure experiments

   - Author contributions
     - F. K.: conceptualization, data acquisition, data analysis, interpretation of results, manuscript writing
     - W. K.: conceptualization, interpretation of results, manuscript writing
     - S. W.: conceptualization, data acquisition, interpretation of results, manuscript writing
     - S. B.: data acquisition, data analysis, manuscript writing
     - U. S.: data analysis, interpretation of results, interpretation of results, manuscript writing
     - A. J.: conceptualization, data acquisition, manuscript writing
     - C. C. M. K.: conceptualization, data acquisition, interpretation of results, manuscript writing
     - M. O. G.: conceptualization, data acquisition, manuscript writing
     - S. A. B.: conceptualization, data acquisition, interpretation of results, manuscript writing
     - J. C. N.: conceptualization, data acquisition, manuscript writing
     - P. M.: conceptualization, data acquisition, manuscript writing
     - G. A. S.: conceptualization, data acquisition, manuscript writing
     - F. H.: conceptualization, data acquisition, interpretation of results, manuscript writing

3. Chapter:

Kupprat F, Kloas W, Krüger A, Schmalsch C, Hölker F (2021b) Misbalance of thyroid hormones after two weeks of exposure to artificial light at night in Eurasian perch *Perca fluviatilis*. *Conserv. Physiol.* 9: coaa124. https://doi.org/10.1093/conphys/coaa124

- Author contributions
  - F. K.: conceptualization, data acquisition, data analyses, interpretation of results, manuscript writing
  - W.K.: conceptualization, interpretation of results, manuscript writing
  - A.K.: data acquisition, data analyses, manuscript writing
  - C.S.: data acquisition, data analyses, manuscript writing
  - F.H.: conceptualization, interpretation of results, manuscript writing
- Published: January 2021

4. Chapter:

Kupprat F, Hölker F, Knopf K, Preuer T, Kloas W (2021a) Innate immunity, oxidative stress, and body indices of the Eurasian perch *Perca fluviatilis* after two-week exposures to artificial light at night. *J. Fish Biol.* 99: 118-130. https://doi.org/10.1111/jfb.14703

- Author contributions
  - F. K.: conceptualization, data acquisition, data analyses, interpretation of results, manuscript writing
  - F. H.: conceptualization, manuscript writing
  - K. K.: conceptualization, data analyses, interpretation of results, manuscript writing
  - T. P.: conceptualization, data acquisition, manuscript writing
  - W. K.: conceptualization, manuscript writing
- Published: February 2021

List of co-authored publications

1. Grubisic M, Haim A, Bhusal P, Dominoni DM, Gabriel KMA, Jechow A, **Kupprat F**, Lerner A, Marchant P, Riley W, Stebelova K, van Grunsven RHA, Zeman M, Zubidat AE, Hölker F (2019) Light pollution, circadian photoreception, and melatonin in vertebrates. *Sustainability* 11: 6400. https://doi.org/10.3390/su11226400
2. Jechow A, Schreck G, Kyba CCM, Berger SA, Bistarelli LT, Bodenlos M, Gessner MO, Grossart H-P, **Kupprat F**, Nejstgaard JC, Pansch A, Penske A, Sachtleben M, Shatwell T, Singer GA, Stephan S, Walles TJW, Wollrab S, Zielinska-Dabkowska KM, Hölker F (2021) Design and implementation of an illumination system to mimic skyglow at ecosystem level in a large-scale lake enclosure facility. *Sci. Rep.* 11: 23478. https://doi.org/10.1038/s41598-021-02772-4

Material & Methods

Ethical statement

The care and use of all experimental animals complied with German animal welfare laws, guidelines and policies as approved by the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16) for the climate chamber experiments (all chapters), and the State Office for Occupational Safety, Consumer Protection and Health of Brandenburg (LAVG reference number 2347-17-2016) for the field experiment at the LakeLab within Lake Stechlin (in Chapter 2).

The experiments in a nutshell

This thesis includes data from three experiments – two experiments in a climate chamber under controlled conditions and one experiment in an open field enclosure setup (LakeLab) under close-to-natural environmental conditions. Details are given in the Material and Methods section of each Chapter and this section here serves as a quick overview.

Climate chamber experiments

Eurasian perch from Lake Müggelsee (Berlin, Germany, 52° 26′ 0″ N, 13° 39′ 0″ O) and from Lake Stechlin (Neuglobsow, Germany, 53° 9′ 6″ N, 13° 1′ 34″ O) were studied. According to the "new world atlas of artificial night sky brightness" the surface of Lake Müggelsee experiences an illumination of ca. 0.003 lx in moonless clear nights (Falchi *et al.*, 2016), which lies in the lower range of suburban skyglow (Hänel *et al.*, 2018). Lake Stechlin experiences little to no light pollution and belongs to the darkest regions in Germany (Jechow *et al.*, 2016). In both locations, experimental fish (juvenile and adults) were kept in large indoor tanks before transfer to the experimental setup. During this pre-acclimation, fish experienced natural photoperiod (sunlight through windows and dark nights) and were fed twice a day with the food source of the respective experiment (frozen blood worms or commercial fish feed).

Experimental setup in the climate chamber

Aquaria with 80 L tap water were illuminated with 2900 lx during daytime by three fluorescent tubes. An additional fluorescent tube was used to realize nighttime illumination (1, 10, 100 lx in the "high ALAN experiment" and 0.01, 0.1, 1 lx in the "low ALAN experiment"). Controls had a fluorescent tube installed but there was no illumination during the night (< 0.00167 lx, "0 lx"). The photoperiod was adjusted to the natural photoperiod at the time of year (December – January in the "high ALAN experiment" and October – November in the "low ALAN experiment") and was controlled by an automatic time switch. The spectral composition was the same in all treatments and thus, lux is a suitable unit for comparison of light intensities (1 lx ~ 3.7 mW m^{-2}). The spectral sensitivity of Eurasian perch is covered by the spectrum of the fluorescent tubes except for higher sensitivities for red light (Cameron, 1982). More details on the experimental setup are available in

Franke *et al.* (2013). See Figure 4 for an illustration of the climate chamber experiments.

High ALAN experiment

Eurasian perch were raised from fertilized egg ribbons collected from Lake Müggelsee (Berlin, Germany) and distributed to twelve identical 80-L aquaria with a tap water flow-through of 10 L h^{-1} and a water temperature of approximately 16°C. Six adult fish (2.5 years old) per aquarium were acclimated for two weeks of bright days (2900 lx) without illumination during night ("0 lx") followed by two weeks of experimental conditions with the same bright daylight illumination and the respective nocturnal light intensity (1, 10, 100 lx) or controls without illumination. The experimental fish in the high ALAN experiment had an average body mass of 69.0 ± 18.4 g and an average standard length of 15.3 ± 1.3 cm (mean ± standard deviation, n = 120). Fish were fed twice a day with commercial fish feed at a rate of 0.5% of their body mass except starving of 24 h prior to sampling. Full nighttime illumination was from 18:00 till 06:00 and full daylight was realized from 09:00 to 15:00 with a simulated dawn or dusk period over 3 h each starting at 06:00 or 15:00, respectively. There were two runs of the experiment – one in December 2016 and a second run in January 2017. During the first run, each treatment was duplicated, and triplicated in the second run (i.e., n = 5 for each treatment). All animals were sampled in the mornings on two consecutive days after 13 – 14 days of exposure. Besides measuring length and body mass, blood was sampled form the caudal vein, the masses of liver and spleen were recorded, and the sex was determined by visual inspection of the gonads. Additionally, the head kidneys were dissected for preparation of primary cell cultures of head kidney leucocytes.

Low ALAN experiment

Eurasian perch (juveniles and young adults) were caught from Lake Müggelsee and fed twice a day with frozen blood worms during pre-acclimation and two weeks of acclimation in the experimental 80-L aquaria of the experimental setup (30 fish per aquarium, same acclimation conditions as in high ALAN experiment). The water temperature was approximately 16°C and the photoperiod was adjusted to October conditions with full daylight from 09:30 to 18:30 with a 3 h dawn or dusk period starting at 06:30 and 18:30, respectively. Fish had an average body mass of 16.8 ± 4.1 g and an average standard length of 10.6 ± 0.9 cm (mean ± standard deviation, n = 720 fish). Full nighttime illumination of the treatments (0.01, 0.1, 1 lx) was from 21:30 till 6:30 and controls were not illuminated during night ("0 lx"). At 18:30 or 6:30, dimming of 3 h allowed a smooth transition from darkness (controls) or nocturnal illumination (treatments) into bright daylight (2900 lx), which was on from 9:30 till 18:30. The water flow-through was reduced to 4 L h^{-1} during the two weeks of experimental conditions to enable water-based melatonin measurements (Chapter 1). Animals were fasted during the two weeks of exposure to maintain good water quality. The experiment consisted of two runs – one in October and a second one in November 2017 – and each treatment was triplicated in both runs (i.e., n = 6 for each treatment). Water samples for water-based melatonin measurements were taken every 3 h from day 10 to day 11 over a full 24 h period. After 13 – 14 days of exposure, all animals were sampled on two consecutive days during the night. Length and body mass were taken from all experimental fish, and

blood was sampled form the caudal vein from the first 15 fish. From the first ten fish, the pituitary glands were excised and frozen in liquid nitrogen for later analysis, and masses of livers, spleens, and gonads were recorded. The sex was determined by visual inspection of the gonads. Additionally, the head kidneys were dissected for preparation of primary cell cultures of head kidney leucocytes.

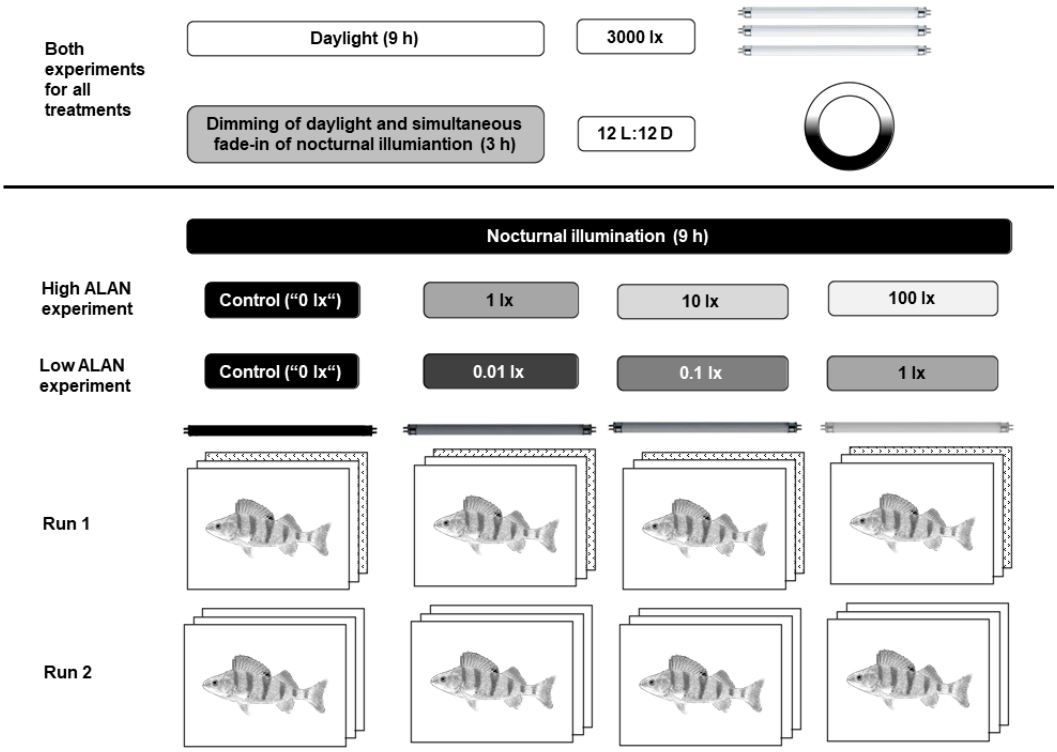

Figure 4 Experimental setup of the climate chamber experiments ("high ALAN experiment" and "low ALAN experiment"). Fluorescent tubes had a correlated color temperature of 6000 K (broad spectrum white light, 1 lx ≈ 3.8 mW m^{-2}) with three fluorescent tubes for illumination throughout the day (9 h of full daylight) and one fluorescent tube for nocturnal illumination (9 h of darkness or ALAN). High ALAN experiment: Six adult Eurasian perch (*Perca fluviatilis*) per aquarium with two aquaria in the first run and three aquaria in the second run (n = 5). Low ALAN experiment: Thirty juvenile Eurasian perch per aquarium, both runs with three aquaria (n = 6). Sampling of melatonin from the water in the "low ALAN experiment" after 10 – 11 days; blood and tissue sampling after 13 – 14 days of experimental conditions. Picture of Eurasian perch modified from a photo by Andreas Hartl.

Field experiment

Eight Eurasian perch (juveniles and adults) were each released into ten large lake enclosures (9 m diameter, 20 m depth, ca. 1300 m^3) at LakeLab located in Lake Stechlin (www.lake-lab.de; Figure 5A). Five enclosures were used as controls with natural nights with no ALAN exposure and five enclosures were illuminated with low ALAN intensities of 0.06 lx at the surface (n = 5 replicates, Figure 5B – D). The lighting is described in detail by Jechow *et al.* (2021) and the LEDs had warm-white light (CCT of 2700 K), which is representative for skyglow. Similar to the climate chamber experiment, the spectral sensitivity of Eurasian perch was covered by the spectrum with a higher relative red portion of the light than in the climate chamber experiments. Experimental fish were divided in two size groups and tagged with

two different methods for individual identification in smaller fish and tags with depth and temperature logger in larger fish (see Material and Methods in Chapter 4). Smaller fish had a body mass of 53 ± 16 g and a standard length of 15.1 ± 1.5 cm (mean ± standard deviation, n = 50 fish, tagged and some untagged fish). Larger fish had a body mass of 166 ± 41 g and a standard length of 21.1 ± 1.7 cm (mean ± standard deviation, n = 18 fish, only re-captured, large, tagged fish). The water temperature in the upper 8 – 10 m (epilimnion) was approximately 20°C. The experimental fish were not fed but they likely fed on natural zooplankton and some benthic organisms on the walls of the enclosures. The photoperiod ranged from 15 L:9 D in the beginning of the experiment (early August) to 12 L:12 D on the final sampling day in early October. Half of the fish were re-captured from the enclosures on two consecutive days and sampled during daytime. As some fish were re-stocked after ca. four weeks due to high mortalities after the first stocking event, exposure ranged from 27 – 58 days. For all re-captured tagged fish (n = 29), body mass and lengths were measured, pituitary glands were excised and frozen in liquid nitrogen for gene expression analysis, and gonads were fixed in a formaldehyde solution for histological analysis. Sex was determined by visual inspection of the gonads. Gene expression was only determined for mature females, which had an average body mass of 129.6 ± 62.2 g and a standard length of 19.3 ± 3.5 cm (n = 14 fish).

Visualization

Scientific illustrations were made in R (R Core Team, 2020) with the ggplot2 package (Wickham, 2016), and GraphPad Prism (version 4.03, GraphPad Software Inc., La Jolla, CA, USA) was partially used in Chapter 1. All other figures and graphs in the general Introduction and Discussion were created in paint.net (version v4.3.3, © 2021 dotPDN LLC, Rick Brewster, and contributors) or PowerPoint (version 2111, Microsoft Office 365).

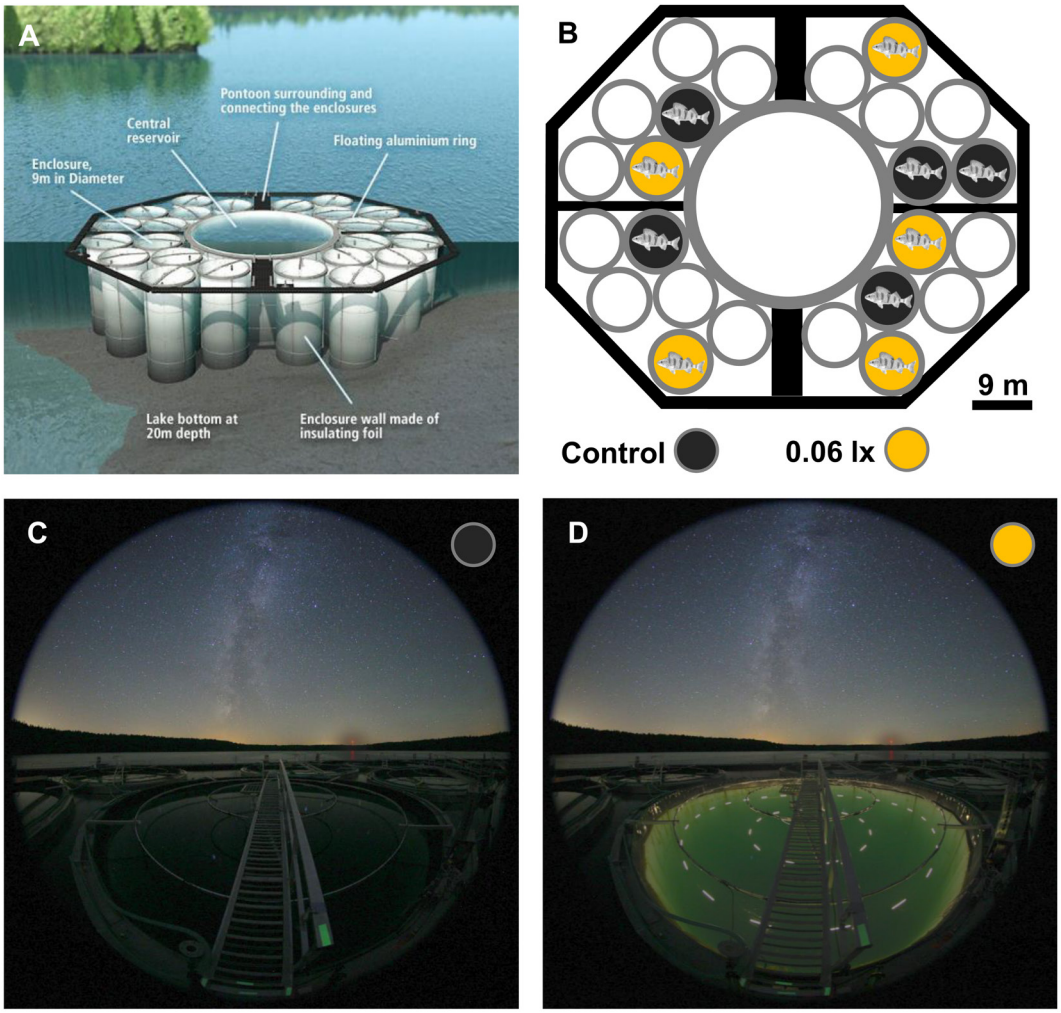

Figure 5 Illustration of the enclosure facility at Lake Stechlin (A, LakeLab, Graphic by Holger Klimek), distribution of controls and skyglow treatments (0.06 lx nocturnal illumination) across enclosures of the LakeLab (B), and photos of controls (C) and skyglow treatment (D) at night (photo courtesy of Andreas Jechow).

Chapter 1

Effects of skyglow on melatonin production of Eurasian perch

Can skyglow reduce nocturnal melatonin concentrations in Eurasian perch?

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Abstract
Artificial light at night (ALAN) changes the natural rhythm of light and darkness and can impair the biorhythms of animals, for example the nocturnal melatonin production of vertebrates, which serves as a proxy for daily physiological rhythms. Freshwater fish are exposed to ALAN in large urban and suburban areas in the form of direct light or in the form of skyglow, a diffuse brightening of the night sky through the scattered light reflected by clouds, atmospheric molecules, and particles in the air. However, investigations on the sensitivity of melatonin production of fish towards low intensities of ALAN in the range of typical skyglow are rare. Therefore, we exposed Eurasian perch (*Perca fluviatilis*) to nocturnal illumination levels of 0.01 lx, 0.1 lx and 1 lx and a control group with dark nights and daylight intensities of 2900 lx in all groups. After ten days of exposure to the experimental conditions, tank water was noninvasively sampled every 3 h over a 24 h period and melatonin was measured by ELISA. Melatonin was gradually reduced in all treatments with increasing intensity of ALAN whereas rhythmicity was maintained in all treatment groups although at 1 lx not all evaluated parameters confirmed rhythmicity. These results show a high sensitivity of Eurasian perch towards ALAN indicating that low light intensities of 0.01 lx and 0.1 lx as they occur in urban and suburban areas in the form of skyglow can affect the physiology of Eurasian perch. Furthermore, we highlight how this may impact perch in their sensitivity towards lunar rhythms and the role of skyglow for biorhythms of temperate freshwater fish.

Keywords: Light pollution; Dose-response; Circadian rhythm; Freshwater fish; *Perca fluviatilis*

1.1. Introduction

The natural darkness of nocturnal environments is disturbed by increasing levels of artificial illumination deriving from human activities (Hölker *et al.*, 2010a, Kyba *et al.*, 2017a). The daily recurring change from brightness to natural darkness is used as a source of information by most organisms to synchronize daily rhythms of metabolic and behavioral processes (Gaston *et al.*, 2013, LeGates *et al.*, 2014, Whitmore *et al.*, 2000). These biorhythms are altered by artificial light at night (ALAN) because it introduces light at times when it had been naturally dark throughout the entire time course of evolution and thereby changes the information about the environmental nighttime context (Gaston *et al.*, 2015b, Kurvers and Hölker, 2014, Rich and Longcore, 2006). A key factor in the regulation of biological rhythms is the rhythmic production of melatonin, which can be directly inhibited by light and is therefore low during day and high during natural dark nights (Navara and Nelson, 2007, Reiter *et al.*, 2007). Melatonin thereby translates the environmental information about light or darkness into a hormonal signal for cells and organs by which behavioral and physiological rhythms can be synchronized. The daily rhythm of melatonin production is of particular importance when studying the effects of light pollution by exposure to ALAN because the main production of melatonin occurs during the night and can be inhibited directly by the exposure to ALAN. The natural nocturnal melatonin production has been shown to be suppressed in invertebrates (e.g., Durrant *et al.*, 2015, Jones *et al.*, 2015) and vertebrates (e.g., Brüning *et al.*, 2015, de Jong *et al.*, 2016, Firth *et al.*, 2006, Grubisic *et al.*, 2019, Tapia-Osorio *et al.*, 2013, Wright and Bruni, 2004).

ALAN mainly derives from centers of human activities, namely cities, which typically occur close to water and especially close to bodies of freshwater (Kummu *et al.*, 2011). Therefore, freshwaters are frequently exposed to ALAN and freshwater organisms, such as fish, are prone to a disruption of melatonin rhythms by ALAN. Plasma melatonin has been experimentally shown to be reduced by ALAN in fish species from different habitats and taxonomic groups, from tropical marine species (e.g., Carazo *et al.*, 2013, Nikaido *et al.*, 2009, Park *et al.*, 2014, Rahman *et al.*, 2004) to temperate freshwater species (e.g., Brüning *et al.*, 2018a, Brüning *et al.*, 2015, Porter *et al.*, 2001, Vera *et al.*, 2005). The circulating plasma melatonin of fish is mainly produced in the pineal gland, which is located underneath the skull and detects light information by direct photosensitivity (Ekström and Meissl, 1997, Falcón *et al.*, 2009, Falcón *et al.*, 2010). In most teleost species, endogenous clock systems (mainly *clock*, *bmal*, *per* and *cry* genes) anticipate light changes and control circadian melatonin production (Falcón *et al.*, 2010). The photoperiod information entrains clock systems and synchronizes the circadian rhythm of melatonin with the daily environmental rhythm (Falcón, 1999). In some teleost species the endogenous control of melatonin seems to be weak and environmental light information is considered the main factor controlling its production in these species, for example in salmonids (Iigo *et al.*, 2007). The rhythmic production of melatonin in the pineal gland of fish is generally considered to be a major source of information on photoperiod and light (Falcón *et al.*, 2009, Grubisic *et al.*, 2019, Underwood, 1989), although other tissues are also known to play an important role such as deep brain photoreceptors, the *saccus vasculosus*, or photoreceptors in the retina (Falcón *et al.*, 2010, Kojima *et al.*, 2000, Nakane *et*

al., 2013, Peirson *et al.*, 2009, Philp *et al.*, 2000). Furthermore, melatonin is also produced rhythmically in the gastrointestinal tract with a peak during day in catla (Mukherjee and Maitra, 2015) and a peak during the night in three-spined sticklebacks (Kulczykowska *et al.*, 2017) and rainbow trout (Muñoz-Perez *et al.*, 2016) but there is little evidence for light-controlled mechanisms. Circulating melatonin is partially released into the surrounding water via the gills, which can be used for non-invasive water-based measurements of melatonin (Ellis *et al.*, 2005, James *et al.*, 2004).

Currently, most of the available literature on melatonin suppression under ALAN conditions focuses on light intensities as they would occur in immediate proximity to bright light sources such as streetlamps. However, only small fractions of freshwater bodies are exposed to direct glare and most of the water surface is rather exposed to indirect illumination that is reflected by clouds and particles in the air and causes a brightening of the sky over large areas – a phenomenon called skyglow (Kyba *et al.*, 2011). Typically it occurs as a homogenous luminance of the sky at rather low intensities that varies depending on the amount of light-reflecting aerosols and particles in the air, e.g., clouds, but also on the ground, e.g., snow or wet streets (Jechow *et al.*, 2019). Skyglow can therefore intensify ten-to hundred-fold dependent on the weather condition as measured in industrial regions of Europe (Jechow *et al.*, 2016, Kyba *et al.*, 2011, Kyba *et al.*, 2015, Puschnig *et al.*, 2014a, Puschnig *et al.*, 2014b) and Asia (Pun and So, 2012). The light intensity of skyglow alone without direct illumination on the surface of urban water typically ranges from 0.007 lx to 0.065 lx in clear nights and from 0.03 lx to 0.55 lx in cloudy nights (Hänel *et al.*, 2018). In contrast, the brightest natural light source at night is the full moon, which can produce maximum illuminance between 0.05 lx and 0.1 lx in temperate latitudes (Kyba *et al.*, 2017b), whereas typical natural illuminance during moonless nights ranges from < 0.0006 lx to 0.0009 lx (Hänel *et al.*, 2018, Hölker *et al.*, 2018). Skyglow can therefore vary within the range of lunar variation of illumination or even exceed it but is less predictable for wildlife. It can thus blur (i.e., pollute) natural circalunar rhythms of night sky brightness (Puschnig *et al.*, 2014a). The spectral composition of skyglow has not often been described and depends on the spectrum of the pre-dominant light-emitting sources in the surroundings. With predominant use of sodium vapor pressure lamps, skyglow has a typical peak around 580 nm (Hänel *et al.*, 2018, Spitschan *et al.*, 2016). The correlated color temperature (CCT) of skyglow ranges from neutral-white light (3500 – 5000 K) in clear nights to warmer white light (2100 – 4000 K) with increasing cloud cover (Jechow *et al.*, 2020). Illumination by the moon has a CCT of ca. 4000 K with a spectral shift towards red wavelengths at lower elevation (Ciocca and Wang, 2013). Whether skyglow already causes a reduction of nocturnal melatonin and thereby disturbs circadian rhythms of fish is not known since evidence on the effects of ALAN at these low intensities is rare. For some marine fish species it has been shown that the low illuminance of a full moon night significantly reduces melatonin (e.g., Fukunaga *et al.*, 2019, Oliveira *et al.*, 2010, Park *et al.*, 2014, Takemura *et al.*, 2006), but for freshwater fish this has not been studied so far. An enhanced understanding of the dose-response relationship between the intensity of ALAN and nocturnal melatonin production is desirable. Previous studies on Eurasian perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) attempted to determine dose-response relationships, but missed the lower

threshold of ALAN because they found a very strong suppression of melatonin under all applied intensities of ALAN down to 1 lx (Brüning *et al.*, 2016, Brüning *et al.*, 2015).

Therefore, we aimed to find a no observed effect level (NOEL) of ALAN by studying intensities of 0.1 lx and 0.01 lx while reproducing the controls with dark nights and the 1 lx treatments from Brüning *et al.* (2015) in order to better describe a dose-response relationship of ALAN and the suppression of melatonin in Eurasian perch. We studied the changes of melatonin in the tank water under controlled laboratory conditions with bright daylight and a simulated dusk and dawn period. We hypothesized a NOEL of ALAN at 0.01 lx and thus no reduction of nocturnal melatonin compared to control levels. Furthermore, we expected a partial reduction at 0.1 lx and – based on earlier findings – a strong reduction at 1 lx. We hypothesized a maintained rhythmicity of melatonin levels in all ALAN treatments. Overall, we aimed to estimate the impact of skyglow on melatonin production and to discuss ecological implications of skyglow for temperate freshwater ecosystems in and around cities.

1.2. Material and methods

The experiment was approved by the legal review of animal testing of the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16).

The experiment was done according to the setup and procedure of Brüning et al. (2015). The light intensities were further dimmed down to 0.1 lx and 0.01 lx by partially covering the light source with tape and neutral density filter foil. Everything else was replicated with great care to ensure comparability of the results. The setup was originally described by Franke *et al.* (2013) with more details on the physical properties of the light and the underlying mechanisms of water-based melatonin measurements (Ellis *et al.*, 2005, James *et al.*, 2004).

1.2.1. Experimental fish

Juvenile Eurasian perch (*Perca fluviatilis*) were obtained from Lake Müggelsee (Berlin, Germany) and kept in indoor tanks (ca. 600 L) with natural photoperiod and dark nights for at least two weeks prior to moving into the aquaria of the experimental setup. According to the “new world atlas of artificial night sky brightness” the surface of Lake Müggelsee experiences an illumination of ca. 0.003 lx in moonless clear nights (Falchi *et al.*, 2016), which lies in the lower range of suburban skyglow (Hänel *et al.*, 2018). Individual fish mass was 16.83 ± 4.08 g with a standard length of 10.6 ± 0.9 cm and a total length of 12.3 ± 1.0 cm (mean ± standard deviation (SD), n = 720).

1.2.2. Experimental setup

Twelve equal aquaria with 80 L tap water were set up in a climate chamber at ca. 17°C (see below for more details). Each aquarium was equipped with four fluorescent tubes (Biolux, Osram, Munich, Germany) of which three realized daylight with average intensities of 2900 lx on the water surface (measured at 25 equally distributed points on the water surface) and up to 7000 lx at the brightest spot. The fourth fluorescent tube realized experimental nighttime illumination.

Specifications on the spectrum and physical properties of the lamps have been described in an earlier publication using the same setup (Franke *et al.*, 2013) and can be used for conversion to irradiance units (1 lx ≈ 3.7 mW m^{-2}). The experimental photoperiod was based on the natural photoperiod in October and controlled over a time switch and control unit. Daylight started at 6:30 a.m. with a dawn period in which light intensities increased over 3 h until full daylight, which started at 9:30 a.m. and lasted until 6:30 p.m. followed by a 3 h dusk period in which daylight was dimmed and night conditions lasted from 9:30 p.m. until 6:30 a.m.

1.2.3. Experimental procedure

Thirty fish were moved to each aquarium and allowed to acclimate for two weeks with bright days (around 2900 lx) and dark nights, i.e., non-detectable light intensities below 0.00167 lx with the used luxmeter (ILT1700, International Light Technologies, Peabody, MA, USA). During acclimation the flow-through with tap water was 12.4 ± 2.2 L h^{-1} and the fish were fed twice per day with frozen blood worms and the aquaria were cleaned once per day. After the acclimation period, average nocturnal light intensities of 0.01 lx, 0.1 lx and 1 lx were experimentally applied for 10 d. Control tanks were dark at night as in the acclimation period and daylight was the same for all experimental groups. The controls are labeled “0 lx” in the figures and tables. Each treatment and the control were simultaneously replicated three times in each run. During the experimental period, the flow-through was reduced to 3.9 ± 0.2 L h^{-1} in order to increase melatonin accumulation in the tank water. This was necessary in order to meet the sensitivity range of the method of measurement (0.3 – 50 pg mL^{-1}). To ensure water quality under this low flow-through conditions fish were not fed during the experimental period. Fish had no opportunity to avoid light exposure. Tanks were cleaned once in the beginning of the experimental period and two days prior to sampling. After ten days of experimental conditions 1 L of holding water from each aquarium was sampled every 3 h over a 24 h period (11 a.m., 2 p.m., 5 p.m., 8 p.m., 11 p.m., 2 a.m., 5 a.m., 8 a.m., 11 a.m.). The water samples were pumped into 1-L bottles from outside the climate chamber with a 12-channel pump using Tygon® tubing (MHSL 2001, ismatec, Cole-Parmer, Wertheim, Germany). By this procedure the fish were not disturbed by the sampling.

The entire experiment was repeated once with a new set of fish directly after the first run to achieve a sufficient amount of replicates for each treatment (n = 6). Hence, there was about a month in between the temporal replication. The experiments were run in October and November 2017. Temperature was measured 5 – 6 times in each run in each of the twelve aquaria and differed by ca. 1°C between runs due to technical difficulties with the air conditioning in the first run. Hence, temperature was 17.4 ± 0.9°C (mean ± SD, n = 60; median = 17.6) in October and 16.3 ± 0.6°C (mean ± SD, n = 72; median = 16.3) in November. The biomass per tank (summed up mass of thirty fish per tank) was 535 ± 23 g (mean ± SD, n = 12; median = 538) in October and 475 ± 16 g (mean ± SD, n = 12; median = 470) in November.

1.2.4. Melatonin extraction and analysis

Each water sample was pre-filtered (glass fiber filters, 0.7 mm) and pumped through SPE cartridges (Oasis HBL, Waters, Milford, MA, USA) at a rate of 25 mL min^{-1}. Each cartridge was activated beforehand with 5 mL methanol and washed with 5 mL distilled water. After pumping the sample, cartridges were washed once more with 5 mL distilled water and eluted with ethyl acetate. Extracts were evaporated under a stream of nitrogen at 45°C and the dried extracts were stored at –20°C. For analysis the dried extracts were re-dissolved in 0.1 M PBS buffer containing 0.1% bovine serum albumin and 5% ethanol. Melatonin concentrations were determined in duplicates according to manufacturer's protocol with a commercially available ELISA kit (Melatonin Saliva ELISA, IBL, Hamburg, Germany). All 216 samples were randomized across 6 assays, which were run one after another within one week. A control (provided in the kit) with low concentrations of 2.87 ± 0.4 pg mL^{-1} (n = 6) was assayed on each 96-well plate, leading to an inter-assay coefficient of variation of 13.9%. Coefficients of variation for intra- and inter-plate variation reported by the manufacturer are 10.8% or 12.7% for concentrations around 2 pg mL^{-1}, 6.1% or 7.6% for concentrations around 5 pg mL^{-1}, and 8.7% or 13.0% for concentrations of 33 pg mL^{-1} or 15 pg mL^{-1}, respectively. All samples were in the range of functional sensitivity and were > 1 pg mL^{-1} and < 30 pg mL^{-1}. As shown in the supplementary material of an earlier publication using the same setup, melatonin in the tank water was relatively stable under experimental daylight conditions of up to 7000 lx over 7.5 h and therefore differences in melatonin most likely derive from production and subsequent secretion of melatonin rather than photodegradation (Brüning *et al.*, 2015).

1.2.5. Data handling and statistical analyses

The melatonin concentrations were normalized to 1 L tank water and 1 kg fish biomass. For an additional analysis of the circadian melatonin rhythmicity the melatonin concentrations at 8 p.m. were set to 100% for each tank and all other values were calculated relative to the respective baseline. The 8 p.m. values were chosen as a baseline since it had the lowest melatonin concentrations in the control treatments (Brüning *et al.*, 2016, Brüning *et al.*, 2015). For comparisons of melatonin concentrations between day and night, the values between 11 a.m. and 8 p.m. or the values between 11 p.m. and 8 a.m. were summed up, respectively.

Differences in distribution between the two runs for temperature and biomass per tank were tested by Mann-Whitney U test.

Linear mixed models (LMM) were fit to the absolute, the relative or the summed up data, respectively, with fixed terms for treatment and time in an interaction and tanks nested in run as random factors (Pinheiro *et al.*, 2018, Zuur *et al.*, 2009). The relationship of melatonin and time was linearized by adding a cosine function as covariate to time (costime). Post-hoc tests compared the slopes of the linearized relationship between melatonin and costime across treatments. A weight term was added for treatment to account for heterogeneous variances across treatments. All models were validated by confirming the normality and homogeneity of residuals. Marginal and conditional R^2 values were calculated for each model (Barton, 2018). All post-hoc comparisons were calculated by comparing every treatment with every

other using Tukey's adjustment of *p*-values (Lenth, 2019). The complete LMM specifications and post-hoc results can be obtained from the supplementary material. The level of significance for all tests was set at $p = 0.05$.

Additionally, a cosine function was fitted on the relative melatonin data for each treatment with a single-component non-linear regression model (Equation (1.1)) (GraphPad Prism 4.03, GraphPad Software Inc., La Jolla, CA, USA).

$$mel\ (time) = M + A * \cos\left(\frac{2 * 3.14159}{P} * \ (time - C)\right) \tag{1.1}$$

where *mel* is the melatonin concentration in tank water relative to the 8 p.m. value in that treatment, *time* is the point of sampling time, *M* is the MESOR (mean melatonin concentration of all sampling times), *A* is the amplitude (maximum difference between MESOR and cosine function), *P* is the period (phase of the cosine function), and *C* is the acrophase (point in time when cosine function reaches maximum). The goodness of fit was defined as sufficient when $R^2 > 0.3$ as previously described (Brüning *et al.*, 2015) and rhythmicity was rejected when the 95% confidence intervals of amplitude or period contained zero (Cornelissen, 2014, Halberg *et al.*, 1967, Refinetti *et al.*, 2007).

1.3. Results

1.3.1. Circadian melatonin rhythm under ALAN

Melatonin concentrations decreased during bright daylight hours and increased during night conditions with a significant time effect dependent on the treatment (LLR = 37.58, $p < 0.0001$, Table 1.1, Figure 1.1). There was significantly less melatonin in the tank water of each ALAN treatment compared to the control (0 lx vs. 0.01 lx: $p = 0.0003$, 0 lx vs. 0.1 lx and 0 lx vs. 1 lx: $p < 0.0001$) and the 1 lx treatment was significantly lower than the other treatments (0.01 lx vs. 1 lx: $p = 0.003$, 0.1 lx vs. 1 lx: $p = 0.0265$). Mean melatonin concentrations were lower at all sampling times of the 0.1 lx treatment compared to the 0.01 lx treatment but were not statistically different ($p = 0.5034$). Melatonin in the tank water gradually decreased with increasing ALAN intensity not only during nighttime, but also during daytime (Figure 1.1).

For normalization to a common baseline and comparison of relative amplitudes, melatonin concentrations were calculated relative to the 8 p.m. values, which had the lowest concentrations in the control. The same LMM was run on these relative data with likewise significant time effects depending on treatment (LLR = 17.83, $p = 0.0005$). The differences across treatments were less pronounced for the relative melatonin data in which only the 0.1 lx and the 1 lx treatment were significantly lower than control levels (0 lx vs. 0.1 lx: $p = 0.0077$; 0 lx vs. 1 lx: $p = 0.0003$). The 0.01 lx treatment was not significantly lower than the control ($p = 0.2055$) and there were no significant differences across the three treatments ($p > 0.05$).

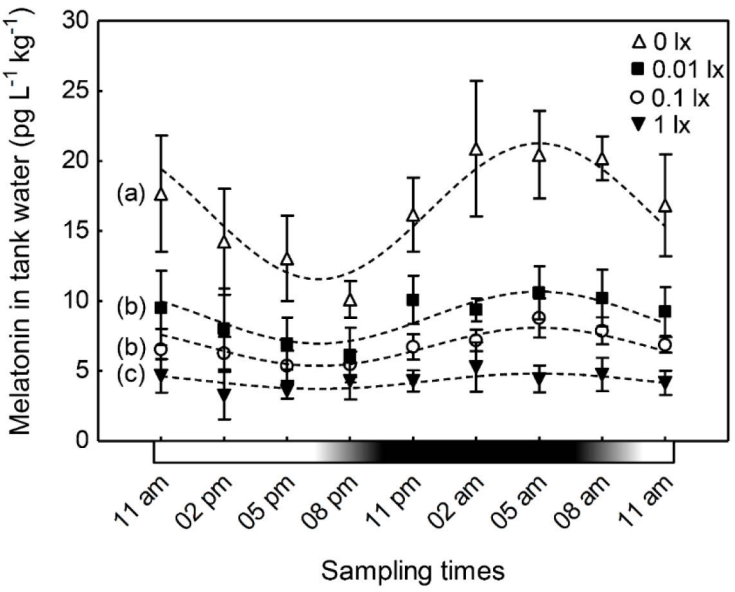

Figure 1.1 Melatonin concentrations in the tank water of Eurasian perch (mean ± SD, n = 6) at nine different times of a 24 h period under control conditions (triangles) with 2900 lx at day and dark nights (0 lx) and under three different light pollution scenarios with night-time illuminance of 0.01 lx (solid squares), 0.1 lx (circles) and 1 lx (solid triangles). The photoperiod is indicated by the black and white bar underneath the x-axis including 3 h of emulated dusk or dawn. Dotted lines represent the predictions of the LMM and letters indicate significant differences across treatments detected by a LMM followed by comparisons of every treatment to every other (Tukey's post-hoc, $p < 0.05$).

Table 1.1

Main results of the LMM analyses of A) melatonin concentrations in the tank water, B) relative melatonin concentrations normalized to a baseline (8 p.m.), and C) summed up diurnal and nocturnal melatonin concentrations in the tank water of Eurasian perch. For A) and B) a cosine function as a covariate of time was included to linearize the relationship (costime). LLR – Log-likelihood ratio. Detailed model specifications are attached in the supplementary material.

	LLR	*p*-value	$R^2_{marginal}$	$R^2_{conditional}$
A) Melatonin in tank water			0.6772	0.7139
Fixed effects (treatment * costime)	37.58	<0.0001		
Random effects (tanks nested in runs)	47.43	<0.0001		
B) Melatonin relative to baseline at 8 pm			0.4430	0.5578
Fixed effects (treatment * costime)	17.83	0.0005		
Random effects (tanks nested in runs)	28.35	<0.0001		
C) Sums of diurnal and nocturnal melatonin in tank water			0.8671	0.9222
Fixed effects (treatment * time of day)	27.02	<0.0001		
Random effects (tanks nested in runs)	24.05	<0.0001		

Table 1.2
Best-fit values (95% confidence intervals) of the non-linear regression using the single-component cosine analysis (Equation 1.1) for relative amplitudes and periods of daily melatonin rhythms in the tank water of Eurasian perch under control conditions with dark nights (0 lx) and three different nocturnal light intensities. The fits are graphically displayed in Figure 1.2.

	Amplitude (%)	**Period (h)**
Initial values	100	24
0 lx	49.96 (36.83 to 63.09)	22.72 (20.54 to 24.90)
0.01 lx	34.00 (20.65 to 47.34)	23.73 (20.09 to 27.37)
0.1 lx	26.61 (19.48 to 33.75)	24.53 (21.81 to 27.26)
1 lx	16.84 (3.08 to 30.60)	19.81 (16.70 to 22.92)

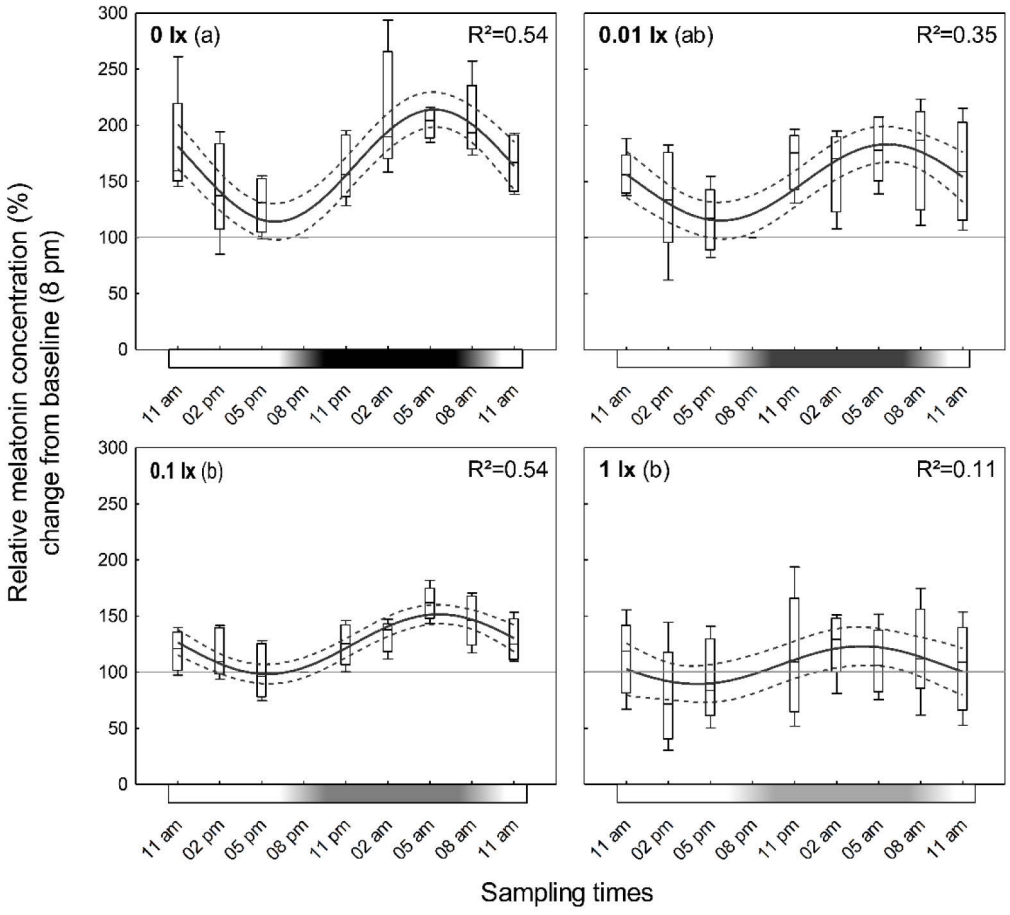

Figure 1.2 Relative melatonin concentrations in the tank water of Eurasian perch in the control (0 lx) and three different light pollution scenarios with nocturnal light intensities of 0.01 lx, 0.1 lx and 1 lx (n = 6). Concentrations at each sampling time are relative to the 8 p.m. value of the respective treatment (8 p.m. mean as baseline at 100%). The concentrations are presented as boxplots (box: median and interquartile range (IQR); whiskers: range from minimum to maximum values). Solid lines represent the non-linear regressions calculated with a single-component cosine analysis (1) (best-fit values ± 95% confidence bands). Letters indicate significant differences across treatments detected by a LMM followed by comparisons of every treatment to every other (Tukey's post-hoc, $p < 0.05$).

For analysis of rhythmic parameters a non-linear regression using the single-component cosine analysis was applied on the relative melatonin data (Figure 1.2).

The cosine function was fitted to the data with $R^2 = 0.54$ in the control and the 0.1 lx treatment and with a lower goodness of fit in the 0.01 lx treatment ($R^2 = 0.35$) whereas the goodness of fit at 1 lx nocturnal illumination was further reduced to $R^2 = 0.11$ (Figure 1.2). Best-fit values for amplitude decreased with increasing ALAN intensity and 95% confidence intervals of the 0.1 lx and the 1 lx treatments did not overlap with the confidence intervals of the control (Table 1.2). Best-fits for the parameter period were between 19 h and 25 h with an expanded range of confidence intervals at 1 lx. Confidence intervals for neither amplitude nor period contained zero for any of the treatments (Table 1.2). The results for MESOR and acrophase are attached in the supplementary material.

1.3.2. Daily vs. nocturnal melatonin production

To facilitate comparisons between day and night, melatonin concentrations were added up for the night from 11 p.m. to 8 a.m. and for the day from 11 a.m. to 8 p.m. (Figure 1.3). Treatments were significantly different dependent on the time of day (LLR = 27.02, $p < 0.0001$). At day and night melatonin levels decreased with increasing nocturnal light intensity whereas the decrease was slightly stronger for nocturnal melatonin (Figure 1.3). Diurnal as well as nocturnal melatonin was significantly higher in the control compared to every treatment and also every treatment was different from each other ($p < 0.005$). In every treatment melatonin in the night was significantly elevated compared to daytime ($p < 0.005$), whereas the difference became smaller with increasing nocturnal light intensities (Figure 1.3).

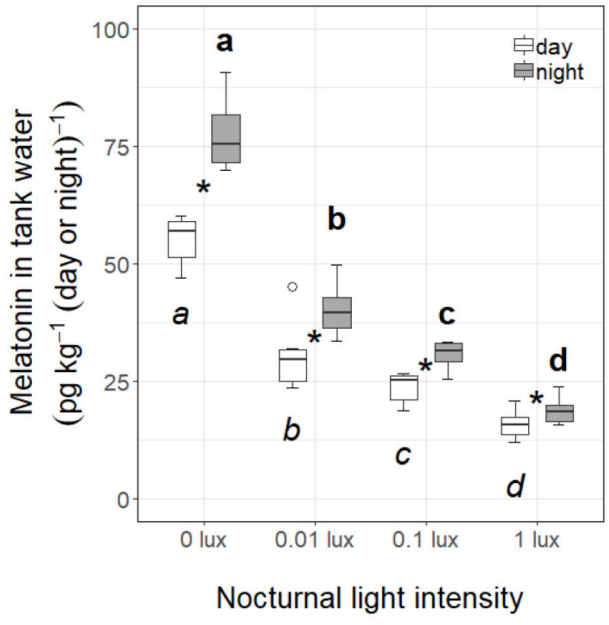

Figure 1.3 Sums of melatonin concentrations in the tank water of Eurasian perch for day (11 a.m. – 8 p.m.) and night (11 p.m. – 8 a.m.) in the control with dark nights (0 lx) and three light pollution scenarios with nocturnal light intensities of 0.01 lx, 0.1 lx and 1 lx (n = 6). Sums are presented as boxplots (box: median and IQR; whiskers: 1.5x IQR, circles: outliers > 1.5 IQR). A significant interaction between treatments and the time of day was detected with a LMM (Table 1.1) and Tukey's post-hoc tests revealed the differences for night (bold letters), day (italic letters) and differences between day and night for each treatment (asterisks) ($p < 0.05$ for every comparison).

1.3.3. Random effects

The distributions differed significantly between runs for temperature (Mann-Whitney U: 50030, $n_{Dec} = 60$, $n_{Jan} = 72$, $p < 0.0001$, two-tailed) and biomass per tank (Mann-Whitney U: 2000, $n_{Oct} = n_{Nov} = 12$, $p < 0.0001$, two-tailed). Overall in October melatonin concentrations in the tank water were slightly higher than in the November run. For the absolute and the relative melatonin data as well as the daily and nocturnal sums the random effects (tanks nested in run) significantly contributed to explaining variance ($p < 0.0001$, Table 1.1). For the absolute data and the sums, runs had a higher variance than tanks nested in runs. In the relative data, however, tanks nested in run had a much higher variance than the runs (Table 1.1). The random structure of the data was not taken into account in the figures for better graphical display.

1.4. Discussion

In all ALAN treatments – even at the lowest intensity of 0.01 lx - melatonin concentrations were significantly lowered in the tank water compared to the controls with dark nights. However, differences between treatments were not as pronounced when melatonin concentrations were normalized to a common baseline. A rhythmic pattern was detected in all treatments although some rhythmicity parameters were reduced in the 1 lx treatment.

1.4.1. Reduced melatonin under ALAN

The melatonin measured from the water samples decreased gradually with increasing ALAN intensities. When daily or nocturnal melatonin levels are added up the differences are even more pronounced and every treatment is significantly different from each other. When normalizing the treatments to the same diurnal baseline nocturnal melatonin is still gradually reduced with increasing nocturnal illumination but differences are less pronounced compared to the absolute data and only the 0.1 lx and 1 lx treatments are significantly reduced compared to the control, but not the 0.01 lx treatment. Additionally, the confidence intervals of amplitudes of the cosine fits on the relative data overlap for the control and the 0.01 lx treatment, but not for the 0.1 lx or the 1 lx treatment. Therefore, we conclude that 0.01 lx was enough to reduce melatonin at night due to significant differences in the absolute measurements but might be closely above a NOEL due to the small and non-significant differences in the relative data.

The majority of data variance is explained by the fixed effects (treatment * time) expressed by the marginal R^2 values of the LMM results. However, a small portion is also explained by the random structure of the data, which results from slightly higher melatonin levels in October compared to those in November, which may be explained by the slight differences in the water temperature between October and November as melatonin production is temperature dependent in fish (Porter *et al.*, 2001, Vera *et al.*, 2007, Zachmann *et al.*, 1992). Despite normalization of melatonin values to the biomass per tank, small differences in biomass between October and November may have additionally influenced variation over runs. However, the scope of interpretation of the random effects is limited since there are just two levels of run and hence only two measurements for each tank.

1.4.2. Rhythmicity of melatonin in tank water

To analyze the rhythmic pattern of melatonin in the tank water we analyzed the goodness of the fits (R^2 values) of the non-linear regressions as well as the confidence intervals of the fitted parameters amplitude and period. The time effects of the LMM analyses give additional information on rhythmicity. The goodness of fit in the control and the lower two ALAN treatments was $R^2 > 0.3$ indicating a sufficient fit of a rhythmic dataset according to the threshold defined in an earlier study (Brüning *et al.*, 2015). The 1 lx treatment, however, had a clearly lowered goodness of fit indicating disturbed rhythmicity. The confidence intervals of the parameter amplitude did not contain zero for any of the treatments indicating rhythmicity (Cornelissen, 2014, Halberg *et al.*, 1967, Refinetti *et al.*, 2007). However, the lower confidence interval of the 1 lx treatment is very close to zero with a minimum of only 3%. In general, the period of the melatonin rhythm seems to reflect the 24 h period given by the experimental setup with best-fits for period between 19 h and 25 h indicating a circadian rhythm in every treatment. The confidence intervals of the best-fits for period did not contain zero for any treatment but are expanded to the lower end at 1 lx compared to the control and the other treatments, which points towards a prospective shortening of the melatonin rhythm due to nocturnal illumination. All in all, the melatonin rhythm at 1 lx is strongly flattened and slightly shortened. Nevertheless, there is still a significant time effect in the LMM analysis at 1 lx and a significant difference between diurnal and nocturnal sums of melatonin at 1 lx so the melatonin rhythm is not entirely inhibited as it was shown for Eurasian perch at 10 lx or 100 lx in the same system (Brüning *et al.*, 2015).

1.4.3. Dose-response relationship between ALAN and melatonin levels in Eurasian perch

The results in this study are overall consistent with the findings in an earlier study from the same setup with a strong suppression of melatonin at 1 lx compared to control levels and a rhythmic pattern at 1 lx to some extent (Brüning *et al.*, 2015). The goodness of fit was comparably low at 1 lx in the previous study and the confidence intervals for period were also wider at 1 lx compared to the control, although they were overall narrower in the current study for both the control and the 1 lx treatment.

In general, the current study confirms the results from the previous one and expands the understanding on the dose-response relationship, although a definite NOEL could still not be defined and seems to be lower than originally expected for this species. The lower threshold for an onset of melatonin suppression seems to be below 0.01 lx but might be closely below this intensity due to the small differences in the relative data. Between 0.01 lx and 1 lx melatonin decreases gradually with an intact rhythmicity although at 1 lx first signs of disturbed rhythmicity were detected by wider confidence intervals for period and confidence intervals close to zero for amplitude and a low goodness of fit of the cosine regression. Hence, the upper threshold of ALAN allowing rhythmicity might be closely above 1 lx. Melatonin was almost entirely suppressed at 10 lx and 100 lx with no detectable rhythmic pattern (Brüning *et al.*, 2015). For Eurasian perch, the combination of the current study and the previous study on higher intensities

(Brüning *et al.*, 2015) sum up to a thorough understanding of the sensitivity towards ALAN with respect to circadian melatonin rhythms, although a definite NOEL remains to be determined. For another temperate European freshwater fish, roach (*Rutilus rutilus*), a similar pattern was found for the higher ALAN intensities between 1 lx and 100 lx (Brüning *et al.*, 2018a). This overall pattern of strongly suppressed melatonin in all ALAN treatments indicates that similar responses to ALAN can be expected for other fish species in the same habitat although upper and lower thresholds of the dose-response curves might differ.

1.4.4. Potential *in situ* effects of skyglow on melatonin in Eurasian perch

The light intensities in this study resemble realistic illumination levels at water surfaces of rivers and lakes in urban and suburban regions. Only a few underwater measurements are available for light polluted waters (Hölker *et al.*, 2018, Perkin *et al.*, 2014b). In an agricultural ditch with relatively transparent water illuminance at 50 cm water depth was roughly half of the illuminance at the surface (Brüning *et al.*, 2018b). Based on this, a typical urban skyglow of 0.03 – 0.55 lx (Hänel *et al.*, 2018) would result in half the illuminance at 50 cm depth in similar transparent waters, which is in the range of our studied intensities. Therefore, our results suggest that skyglow can partially suppress nocturnal melatonin when Eurasian perch live in transparent shallow water. Further interpretation for more turbid or deeper habitats requires more underwater measurements of nocturnal illuminance by skyglow.

Apart from light intensity, the spectral composition also depends on turbidity and composition of particles. Our experimental illumination is not fully comparable with real-world illumination by skyglow or moonlight because our experimental light source has a higher CCT than skyglow or moonlight. However, melatonin production in Eurasian perch is equally sensitive to green and red light and only slightly less sensitive to blue light (Brüning *et al.*, 2016). Therefore, melatonin suppression under exposure to real skyglow or moonlight with less blue light and more green and red light is not expected to differ substantially from the results reported here. Future experiments exclusively addressing skyglow effects should take care to illuminate at a CCT < 5000 K.

1.4.5. Research gaps

A better understanding of the mechanisms underlying melatonin production and secretion in Eurasian perch would give further insights into the susceptibility towards ALAN. For example, it would be of great importance to understand to which extent an endogenous circadian clock regulates melatonin rhythms in Eurasian perch apart from the external light information. Furthermore, despite the numerous advantages of water-based measurements, the results here require ultimate validation by measurements of daily rhythms of circulating melatonin in Eurasian perch. It has also rarely been studied to which extent different sources of melatonin (pineal melatonin, retinal melatonin, gut melatonin etc.) contribute to the circulating levels and finally the secreted levels measured in the water.

1.4.6. Eco-physiological implications of reduced melatonin at night

If melatonin production is suppressed by skyglow in natural ecosystems this will lead to physiological changes of individuals, populations or indirectly even ecosystem processes such as food web interactions. Potential negative impacts of reduced or lacking melatonin for individuals include reduced immune function, since melatonin acts as an antioxidant and as a modulator for several immune parameters as it is well-known for mammals (e.g., reviewed by Carrillo-Vico *et al.*, 2013) but only occasionally studied in fish (Esteban *et al.*, 2006, Jung *et al.*, 2016a, Jung *et al.*, 2016b, Kepka *et al.*, 2015, Morgan *et al.*, 2008).

Furthermore, reduced or lacking nocturnal melatonin might affect other endocrine signals such as sexual hormones. Melatonin can act as a regulatory mediator for reproduction processes to synchronize gonadogenesis within a population (Maitra and Hasan, 2016). For seasonally reproducing Eurasian perch it was shown that strong intensities of ALAN did not only suppress nocturnal melatonin production (Brüning *et al.*, 2015) but also suppressed mRNA expression of gonadotropins in females (Brüning *et al.*, 2016). In a field experiment, mRNA expressions of gonadotropins as well as concentrations of sexual steroids in the blood plasma were reduced in female and male perch and roach under illumination by streetlamps, but melatonin was not reduced in this study (Brüning *et al.*, 2018b). Further, a relation of melatonin to the thyroid system has been discussed in a few studies in fish (Dolomatov *et al.*, 2013, Jung *et al.*, 2016b, Nayak and Singh, 1987). For now, it remains unclear if and at which timescale permanently reduced nocturnal melatonin by exposure to skyglow would affect endocrine or immune function.

Moreover, melatonin might act on activity patterns of fish comparable to the antagonistic effects of melatonin on activity and sleep patterns in mammals (Datta and King, 1980, Golombek *et al.*, 1996, van der Heijden *et al.*, 2007) and birds (Raap *et al.*, 2016, Sun *et al.*, 2017). If this relationship was similar in fish, reduced melatonin under ALAN would lead to increased activity at night (e.g., predation, schooling or mating behaviors), which could cascade to ecosystem-scaled effects of ALAN. Diurnal fish species, such as the Eurasian perch, might be particularly affected by these shifts of activity patterns (Aulsebrook *et al.*, 2018). For guppies it was demonstrated that skyglow light levels (0.5 lx) can also affect diurnal behavioral processes associated with risk-taking (Kurvers *et al.*, 2018). Prey detection and feeding was observed at light intensities as low as 0.02 lx in Eurasian perch (Bergman, 1988, Flik *et al.*, 1997), 0.05 lx in coregonids (Ohlberger *et al.*, 2008) or at less than 0.005 lx in freshwater bream (Townsend and Risebow, 1982). However, the link of behavioral activities at these low light intensities to circulating melatonin is unknown. Investigations on the mediating effect of melatonin on behavioral patterns in fish would facilitate estimates of ecosystem-scaled effects of ALAN.

1.4.7. Lunar rhythms

Especially the lower intensities of ALAN in this study are in the range of nocturnal illuminance that is covered by lunar phases. Full moon can create a maximum

illumination of 0.3 lx at the surface of waters but illumination is lower in temperate latitudes (Kyba *et al.*, 2017b). In a rural area close to Berlin (Westhavelland) the water surface of an agricultural ditch was illuminated with 0.1 lx in a full moon night whereas new moon illumination of the water surface was 0.002 lx (Brüning *et al.*, 2018b). In the nearby Lake Döllnsee such differences in nocturnal light levels were significantly correlated to changes in swimming activities of perch (Nakayama *et al.*, 2018). Typical urban skyglow can blur the light signal of the moon as it was measured in Berlin, Germany, or Vienna, Austria (Kyba *et al.*, 2011, Puschnig *et al.*, 2014a). Our results suggest that Eurasian perch are capable of physiologically detecting the full moon and potentially even half-moon light. Brüning *et al.* (2018b) did not find significant differences between control and a strong ALAN treatment in a field study and argued that the illumination by the half-moon light during the sampling (up to 0.02 lx) was probably enough to reduce melatonin also in the controls. Our results confirm that half-moon light may indeed have been sufficient to reduce melatonin in perch, but also suggest that melatonin levels should have been different between animals exposed to half-moon light in the control treatment and ca. 15 lx at the water surface in the ALAN treatment.

For tropical fish species rhythmic changes in lunar illuminance lead to circalunar melatonin rhythms facilitating the synchronization of reproduction within a population that does not experience strong changes in temperature or photoperiod in tropical and subtropical regions. For tropical rabbitfish and grouper it has been postulated that reduced melatonin levels during full moon are used to synchronize spawning (Fukunaga *et al.*, 2019, Ikegami *et al.*, 2014, Takemura *et al.*, 2010) and for the temperate Senegalese sole full moon illumination significantly reduced plasma melatonin potentially linked to an increase in sex steroids at full moon (Oliveira *et al.*, 2010). Still, for temperate freshwater fish, such as the Eurasian perch, further research is required to understand the role of lunar rhythms and it is unclear if lunar rhythms are linked to reproductive processes as most of these species reproduce annually. As argued in previous studies it is not well understood yet if the melatonin concentration at night or the rhythmicity alone drives physiological and behavioral processes (Brüning *et al.*, 2015). In Eurasian perch phase shifts seem to occur only at ALAN intensities of more than 1 lx, which is much brighter than the variation of natural illumination. This can explain why ALAN leads to an impairment of reproductive processes, but lunar rhythms do not.

Apart from reproductive control, the potential sensitivity to lunar rhythms might also be an evolutionary adaptation towards an expansion of the feeding niche under the assumption that decreased nocturnal melatonin would increase behavioral activity. This hypothesis is supported by the significant correlation of swimming depth and lunar phase as well as the use of low light levels for feeding in laboratory experiments (see discussion above).

1.5. Conclusion

In conclusion, the production of nocturnal melatonin in Eurasian perch is sensitive to very low artificial light levels at night. In our experiment, which mimicked intensities of skyglow on urban and suburban water surfaces, even the lowest intensity of 0.01 lx was enough to reduce the concentration of melatonin at night while maintaining circadian rhythmicity. The NOEL of ALAN affecting melatonin

production could not be determined in this study but is below 0.01 lx. As melatonin acts as the main mediator between environmental time cues and physiological timing, skyglow can impair the physiology of Eurasian perch in urban freshwaters. In order to assess the eco-physiological implications of skyglow and reduced melatonin levels, linkages of melatonin to other physiological processes (immune function, thyroid system and reproduction) as well as behavioral traits need to be considered.

Declaration of competing interest
None.

CRediT authorship contribution statement
Franziska Kupprat: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft, Writing - review & editing. Franz Hölker: Conceptualization, Funding acquisition, Project administration, Resources, Writing - review & editing. Werner Kloas: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

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Chapter 2

Effects of skyglow on reproductive processes of Eurasian perch

Dim artificial light at night affects reproductive processes of Eurasian perch (*Perca fluviatilis*) – climate chamber and outdoor enclosure experiments

– DRAFT VERSION –

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The manuscript in this chapter is a draft version and has not been published before.

Supplementary material
Supplementary material is attached in Appendix C in this dissertation.

Abstract
Reproduction of freshwater fishes from temperate zones is partially regulated by photoneuroendocrine processes, which can be disrupted by artificial light at night (ALAN). Skyglow illuminates large portions of urban and suburban areas and the freshwater ecosystems within them, yet little is known about the effects of such low ALAN intensities on the reproduction of freshwater fish. We studied the effects of ALAN on the *β*-subunits of the follicle stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in Eurasian perch in a controlled climate chamber experiment of two weeks, and a field enclosure experiment of two months. In the climate chamber experiment, ALAN (0.01 lx, 0.1 lx, 1 lx) resulted in a tendency for reduced *fshβ* expression in the pituitaries of females at all intensities but no changes in *lhβ*. In the field experiment, ALAN (0.06 lx) resulted in a reduction of female *lhβ*, but not of *fshβ*. Males in the climate chamber experiment did not show notable changes either in gonadotropin expression or plasma 11-keto testosterone after ALAN exposure. Overall, our results indicate that even low intensities of ALAN interfere with the gene expression of gonadotropins in female Eurasian perch.

Keywords: enclosure; fish; freshwater; FSH; LH; 11 KT; light pollution; mesocosm; reproduction; skyglow; vitellogenesis

2.1. Introduction

Most fish species from temperate zones reproduce in annual rhythms, and spawning occurs synchronously in both sexes at the same time of the year. These annual rhythms require a delicate timing of energy allocation in terms of food acquisition, onset and progression of gonadogenesis, maturation of gonads, mating behavior and spawning (Cowan *et al.*, 2017, Migaud *et al.*, 2010). The synchronization of these processes is physiologically orchestrated by the endocrine system integrating changes in photoperiod and temperature in order to optimize reproductive success and ultimately survival of the population (Migaud *et al.*, 2010). Both photoperiod and temperature have predictable annual patterns, and both are used for seasonal timing of annual reproductive processes. Of the two environmental zeitgeber (temporal cues), photoperiod is the more reliable (e.g., discussed by Gaston *et al.*, 2014, Migaud *et al.*, 2010). Artificial light at night (ALAN) is increasingly changing the environmental light information as natural nighttime light levels no longer occur, and the night is instead replaced by an extended twilight (Hölker *et al.*, 2010a). In particular, the skyglow caused by scattering of artificial light by atmospheric molecules and clouds, brightens the night sky over large areas (Aube, 2015, Falchi *et al.*, 2016) and illuminates urban and suburban freshwater surfaces (Jechow and Hölker, 2019a) at illuminances ranging from ca. 0.001 to 0.5 lx, with urban extremes reaching more than 1 lx (Jechow *et al.*, 2020, Kyba *et al.*, 2015). For comparison, natural nocturnal illumination is below 0.001 lx in starlit, moonless nights; moonlight is usually below 0.01 lx and never larger than 0.4 lx (Kyba *et al.*, 2017b). In contrast, typical streetlights can reach tens to hundreds of lux, and daylight ranges up to 120,000 lx (Hänel *et al.*, 2018, Hölker *et al.*, 2018). As ALAN prolongs twilight periods, many animals that rely on the photoperiod as external trigger for the synchronization of reproduction exhibit impaired reproductive processes (Gaston *et al.*, 2015b, Hölker *et al.*, 2010a, Ouyang *et al.*, 2018).

Reproductive hormones, which are key regulators of the annual reproductive cycle in temperate fish species, show circannual rhythms regulated by light (Cowan *et al.*, 2017, Migaud *et al.*, 2010). In fish, this is co-regulated by light sensitive hormones such as melatonin derived from the pineal organ (Falcón *et al.*, 2010) or thyroid-stimulating hormones (TSH) secreted from the pituitary gland and – more important for seasonal rhythms – from the *saccus vasculosus* (Nakane *et al.*, 2013). A better understanding of these processes led to an application in fish farming of economically interesting species, such as Eurasian perch (*Perca fluviatilis* L. 1758) (Migaud *et al.*, 2004, Szczerbowski *et al.*, 2009), Yellow perch (*Perca flavescens* M. 1814) (Kolkovski and Dabrowski, 1998), Pink salmon (*Oncorhynchus gorbuscha* W. 1792) (MacQuarrie *et al.*, 1979), Atlantic salmon (*Salmo salar* L. 1758) (Thrush *et al.*, 1994), and European seabass (*Dicentrarchus labrax* L. 1758) (Felip *et al.*, 2008, Rodríguez *et al.*, 2005). Photoperiods are regularly manipulated in order to induce gonad development and advance or postpone maturation for out-of-season reproduction (Rodríguez *et al.*, 2019). These light treatments in aquaculture are often more effective when applied in a particular time of the year – a so-called photo-sensitive or photo-labile period in which the animals are particularly susceptible to changes in environmental light information (Falcón *et al.*, 2010, Rodríguez *et al.*, 2019). Recently, continuous

illumination (LL) during the sensitive period successfully prevented maturation for an improved growth (Carrillo *et al.*, 2009, Rodríguez *et al.*, 2012).

It is therefore not surprising that ALAN interferes with reproductive processes in fish targeting fertilization, hatching or changes in reproductive hormones affecting the gonad development. Such effects were reported in clownfish (*Amphiprion ocellaris* C. 1830) (Fobert *et al.*, 2019), Eurasian perch (Brüning *et al.*, 2016, Brüning *et al.*, 2018b), roach (*Rutilus rutilus* L. 1758) (Brüning *et al.*, 2018a, Brüning *et al.*, 2018b), bleak (*Alburnus alburnus* L. 1758), and chub (*Squalius cephalus* L. 1758) (Brüning *et al.*, 2010). However, the existing studies focused on rather bright ALAN while illuminance comparable to skyglow has been less well-studied so far (e.g., Kupprat *et al.*, 2020, Kupprat *et al.*, 2021a, Kupprat *et al.*, 2021b, Liu *et al.*, 2019).

Reproductive processes are mainly regulated via a hormonal cascade known as the hypothalamic-pituitary-gonadal axis (HPG axis). Briefly, gonadotropin releasing hormone (GnRH) is produced and released from the hypothalamus. Subsequently, gonadotropes in the pituitary gland secrete gondaotropins into the blood stream, i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins then stimulate the synthesis of sexual steroids in the gonadal tissue, mainly testosterone (T), 11-keto testosterone (11 KT), and 17*β*-estradiol (E2), which in turn induce a negative feedback on GnRH production (van der Kraak, 2009, Weltzien *et al.*, 2004, Zohar *et al.*, 2010). For instance, 11 KT inhibited pituitary FSH in New Zealand shortfinned eel (*Anguilla australis* R. 1841) (Setiawan *et al.*, 2012). In addition, E2 triggers the synthesis of vitellogenin in the liver of female fish, initiating the transition from previtellogenesis to vitellogenesis, often referred to as puberty (Wuertz *et al.*, 2007).

In several previous studies, ALAN impaired reproductive processes of Eurasian perch (Brüning *et al.*, 2016, Brüning *et al.*, 2018b). At nocturnal surface illuminance of 1, 10, and 100 lx with white light for two weeks, the gene expression for the *β*-subunits of FSH (*fshβ*) and LH (*lhβ*) were strongly and significantly reduced (< 20% of controls) compared to individuals exposed to dark nights (Brüning *et al.*, 2016). These effects were only measurable in females and only under broad-spectrum white light under laboratory conditions whereas gonadotropin expression did not differ in males. Furthermore, gonadotropin expression was not reduced in both sexes after nocturnal exposure to 1 lx of red, blue, or green LED illumination (Brüning *et al.*, 2016). In a field study in August by Brüning *et al.* (2018b), a nocturnal illumination of 15 lx (white light) at the water surface of agricultural ditches reduced gene expression of both gonadotropins in females and to a lesser extent in males. Moreover, the sexual steroids 11 KT and to a lesser extent E2 were significantly reduced in the blood. Similar effects for gonadotropins and sex steroids were reported for roach (Brüning *et al.*, 2018b). A controlled climate chamber study with roach did not show effects on gene expression of gonadotropins in December (Brüning *et al.*, 2018a). Overall, these findings suggest complex contextual effects of ALAN on reproductive hormones of freshwater fishes, which depend not only on the light intensity and quality but also on season and sex. Brüning *et al.* (2018b) suggested a photo-labile period for reproductive processes in summer for males and in late summer/fall for female Eurasian perch.

This is in accordance with the seasonal reproductive cycle in Eurasian perch which starts earlier in males than in females (Migaud *et al.*, 2006). However, the illuminance thresholds could not be identified in the previous studies as all applied treatments showed strong responses (Brüning *et al.*, 2016, Brüning *et al.*, 2018b).

We hypothesized that ALAN reduces mRNA expression of the gonadotropins (*lhβ* and *fshβ*) in females and males in the field in late summer, and in females but to a lesser extent in males in the climate chamber in fall (based on Brüning *et al.*, 2016, Brüning *et al.*, 2018b). However, we did not expect the ratio of *fshβ*-change/*lhβ*-change to alter, as both hormones were reduced to a similar extent under ALAN in earlier studies (Brüning *et al.*, 2016, Brüning *et al.*, 2018b). We further expected 11-keto-testosterone (11 KT) in the plasma of male and female Eurasian perch to be reduced by ALAN (based on Brüning *et al.*, 2018b). Finally, we assumed a slower vitellogenesis and spermatogenesis under ALAN due to the reduced gene expression of gonadotropins and reduced sex steroids (based on Brüning *et al.*, 2016, Brüning *et al.*, 2018b).

Because illuminance as low as 1 lx suppressed gonadotropin expression in Eurasian perch (Brüning *et al.*, 2016), we aimed to determine the threshold for gonadotropin suppression in a climate chamber experiment with a similar setup to Brüning *et al.* (2016) but with illuminance reduced to skyglow levels (0.1 lx and 0.01 lx). For this, we measured the mRNA expression of *lhβ* and *fshβ* from the pituitary gland of males and females as well as 11 KT from blood plasma.

We further exposed perch to an average surface illuminance of 0.06 lx and under quasi *in situ* conditions in the LakeLab during August and September 2018. The LakeLab is a large-scale experimental enclosure facility installed in Lake Stechlin, Germany. Details on the light installation mimicking skyglow are published by Jechow *et al.* (2021). After 1 – 2 months exposure, we measured mRNA expression of gonadotropins and analyzed the histological status of gonadogenesis (spermatogenesis and vitellogenesis).

2.2. Results

2.2.1 Gonadotropins in the climate chamber experiment

Mean gene expression of *fshβ* in ALAN-exposed females was consistently decreased for all treatments compared to control levels (2.3-fold at 0.01 lx and 0.1 lx and 2.4-fold at 1 lx), yet none of the differences were statistically significant (LM, ALAN-factor: $p = 0.65$, n = 3 – 6 fish, Table 2.1, Figure 2.1). However, some individual females showed strongly reduced *fshβ* expression with a 12-fold (0.01 lx), 16-fold (0.1 lx), or 10-fold (1 lx) decrease compared to the mean of the control group (Figure 2.1). Changes in mean *lhβ* expression were less consistent and smaller than for *fshβ*, and also not significant (LM, ALAN-factor: $p = 0.06$, n = 3 – 6, Figure 2.1, Table 2.1). The largest mean reduction of *lhβ* expression was a 1.2-fold decrease at 0.01 lx, yet at 1 lx *lhβ* expression was slightly increased with a 0.2-fold mean increase compared to the mean of the control group. The female individuals with the lowest *fshβ* expression were the same individuals who had the lowest *lhβ* expression at exposures to 0.01 lx and 0.1 lx (Figure 2.1, details in Supplementary material). In ALAN-exposed males, mean *fshβ* and *lhβ* expression

levels were both higher (0.01 lx, 0.1 lx) and lower (1 lx) compared to the control (Figure 2.1) without a significant group difference (LMM, ALAN-factor, *fshβ*: $p = 0.11$; *lhβ*: $p = 0.25$, n = 4 – 16, Figure 2.1, Table 2.2). Still, also in the ALAN-exposed males, there were individuals with considerably lower levels of mRNA expression compared to control means (one male each at 0.01 lx, 0.1 lx or 1 lx with 6-, 5.6-, or 3-fold decrease in *fshβ*, and one male each at 0.1 lx or 1 lx with 5.6-, or 5.9-fold decrease in *lhβ*). Male individuals with lowest *fshβ* levels were not the same individuals who had the lowest *lhβ* expression (Supplementary material). However, for *fshβ* there was also considerable variance in males in the control treatment (Figure 2.1).

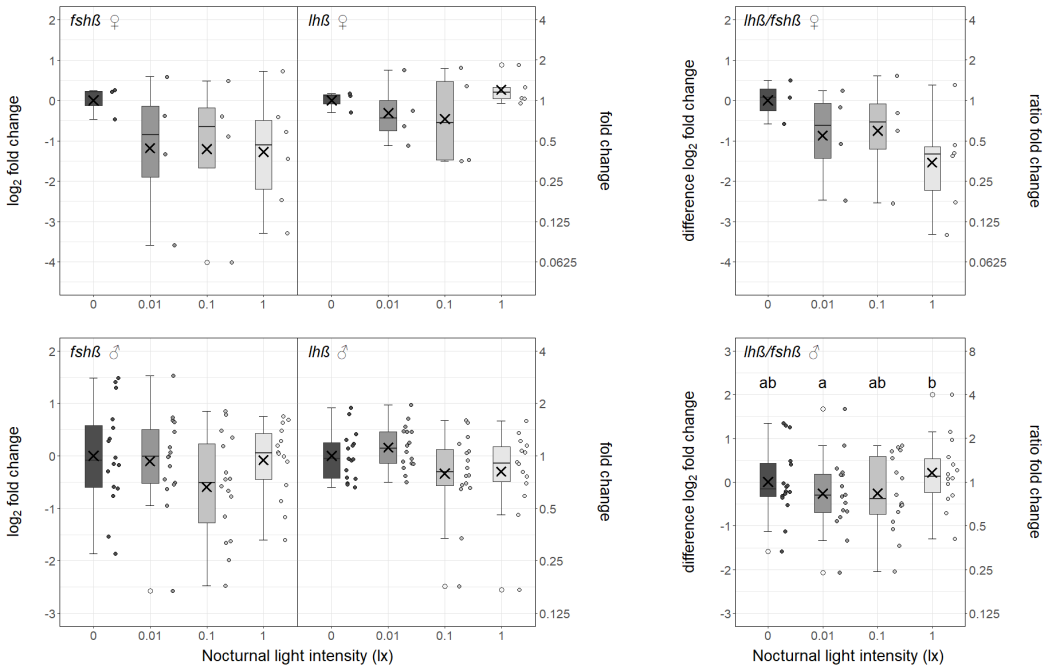

Figure 2.1 Relative mRNA expression of the *β*-subunits of the gonadotropins follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in female (upper row) and male (lower row) Eurasian perch (*Perca fluviatilis*) exposed to different levels of nocturnal illumination (0.01, 0.1, 1 lx) or controls (< 0.00167 lx, "0") for two weeks. Gene expressions were normalized to mRNA expression of ribosomal protein (rpL8) and are presented as change from the mean of the control. Pituitary glands were sampled throughout the night. Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5x interquartile ranges (IQR); outliers > 1.5x IQR are indicated by circles, and "X"s indicate the mean. Small points to the right of each boxplot represent values for each individual (females: n = 3 for 0 lx and 0.1 lx, n = 4 for 0.01 lx, and n = 6 for 1 lx; males: n = 16 for 0 lx, 0.01 lx, 0.1 lx, n = 14 for 1 lx). The right column shows the ratio of *fshβ*-change/*lhβ*-change. Different letters in the lower right graph (*fshβ*-change/*lhβ*-change, males) indicate statistical significance in post-hoc testing (Tukey's correction, $p < 0.05$) after LMM analysis with ALAN and body mass as fixed factors and runs and aquaria as random terms including a weight term for variation across aquaria. Boxplots serve as a visualization of data and do not represent statistical analyses.

For females and males, both *fshβ* and *lhβ* expression was positively and significantly correlated with body mass (Table 2.1, Table 2.2, Supplementary material). Expression of *fshβ* in females showed the steepest increase with body mass increase (0.22-fold increase per gram), and expression of *lhβ* in the male

control group the weakest increase (0.02-fold increase per gram, Supplementary material). An interaction between ALAN and body mass was not significant in the model selections (data not shown). The mean ratio of *fshβ*-change/*lhβ*-change decreased gradually with increasing ALAN illuminance in females (–45% at 0.01 lx, –41% at 0.1 lx and –66% at 1 lx, Figure 2.1), but neither ALAN nor body mass were statistically significant (LM, ALAN-factor: $p = 0.39$, Body mass-factor: $p = 0.07$, Table 2.1). In males, the mean ratio decreased slightly at 0.01 lx and 0.1 lx and increased at 1 lx compared to controls (–17% at 0.01 lx, –16% at 0.1 lx, +16% at 1 lx, Figure 2.1). Both, ALAN and body mass significantly explained variance in the data (LMM, ALAN-factor: $p = 0.03$, Body mass-factor: $p < 0.0001$, Table 2.2). Post-hoc comparisons for the ALAN-factor showed that the mean ratios at 1 lx were significantly higher than at 0.01 lx (+40%, Tukey's post-hoc test: $p = 0.03$, Table 2.2). The increase of the ratio per gram body mass increase was rather low with a predicted estimate of 0.09 (Supplementary material).

2.2.2 Plasma 11 KT in the climate chamber experiment

In ALAN-exposed females, mean 11 KT levels were slightly lower for 0.01 lx treatment but otherwise higher compared to the mean of the control (Figure 2.2, Table 2.1). Neither ALAN nor body mass significantly explained variance in the data (LM, ALAN-factor: $p = 0.67$, body mass-factor: $p = 0.19$, Table 2.1). In ALAN-exposed males, 11 KT levels decreased slightly with increasing ALAN intensity compared to the mean of the control group (–5% at 0.01 lx, –13% at 0.1 lx, –12% at 1 lx, Figure 2.2), and both ALAN and body mass significantly explained variance of the data (LMM, ALAN-factor: $p = 0.048$, body mass-factor: $p < 0.0001$, Table 2.2). 11 KT was predicted to increase by 186 pg mL^{-1} per gram body mass increase (Supplementary material). Post-hoc analysis showed that 11 KT levels at 0.1 lx and 1 lx were significantly lower than at 0.01 lx, but not significantly lower than control levels (post-hoc tests in Table 2.2, 0.01 lx vs. 0.1 lx: $p = 0.03$, 0.01 lx vs. 1 lx: $p = 0.02$, $p > 0.5$ for comparisons with controls). Male 11 KT levels had a considerable range of variance (10 – 3360 pg mL^{-1}, min – max of all treatments), and naturally higher levels of plasma 11 KT than females (ca. 10 times higher in males).

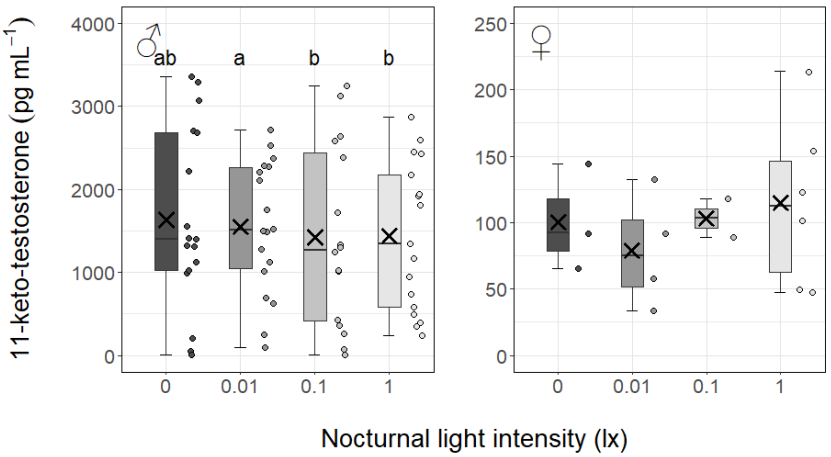

Figure 2.2 Plasma concentrations of 11-keto-testosterone (11 KT) in male and female Eurasian perch (*Perca fluviatilis*) exposed to different levels of nocturnal illumination (0.01, 0.1, 1 lx) or controls (< 0.00167 lx, "0") for two weeks. Plasma samples were taken throughout the night. Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5x interquartile ranges (IQR); outliers > 1.5x IQR are indicated by circles, and "X"s indicate the mean. Small points to the right of each box represent values for each individual (males: n = 17 for 0 lx and 1 lx, n = 18 for 0.01 lx, n = 16 for 0.1 lx; females: n = 3 for 0 lx, n = 4 for 0.01 lx, n = 2 for 0.1 lx and n = 6 for 1 lx). Different letters in the left graph (males) indicate statistical significance in post-hoc testing (Tukey's correction, $p < 0.05$) after LMM analysis with ALAN and body mass as fixed factors and runs and aquaria as random terms including a weight term for variation across aquaria. Boxplots serve as a visualization of data and do not represent the statistical analysis.

Table 2.1
Linear model (LM) specifications for females of the climate chamber experiment. LM analysis of relative mRNA expression of the *β*-subunits of follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in the pituitary gland as well as the ratio of *fshβ*-change/*lhβ*-change, and plasma 11-keto-testosterone (11 KT) in female Eurasian perch (*Perca fluviatilis*) exposed to different illuminance of artificial light at night (ALAN; 0.01, 0.1, 1 lx) or controls (< 0.00167 lx). Random effects could not be included due to small numbers of females in the climate chamber experiment. See supplementary material for full model specifications.

Parameter	Variables	*F*-statistic	DF	*p*-value	Multiple R^2
fshβ	ALAN	0.11	15	0.96	0.65
	Body mass	18.75	13	0.001	
lhβ	ALAN	3.21	15	0.06	0.77
	Body mass	30.66	13	0.0001	
Ratio *fshβ*-change/ *lhβ*-change	ALAN	0.91	15	0.39	0.41
	Body mass	4.11	13	0.07	
11 KT	ALAN	0.54	13	0.67	0.24
	Body mass	1.93	11	0.19	

Table 2.2
Linear mixed model (LMM) specifications for males of the climate chamber experiment. LMM analysis of relative mRNA expression of the *β*-subunits of follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in the pituitary gland as well as the ratio of *fshβ*-change/*lhβ*-change, and plasma 11-keto-testosterone (11 KT) in male Eurasian perch (*Perca fluviatilis*) exposed to different intensities of artificial light at night (ALAN; 0.01, 0.1, 1 lx) or controls (< 0.00167 lx, "0 lx"). Each LMM included ALAN and body mass as fixed factors (without interaction), runs and aquaria as random terms, and a weight term for variation across aquaria. For significant ALAN effects, post-hoc tests with Tukey's correction were calculated. See supplementary material for full model specifications.

Parameter	Fixed variable	Fixed effects		Random effects		Goodness of fit	
		LLR†	*p*-value	LLR	*p*-value	$R^2_{marginal}$	$R^2_{conditional}$
fshβ	ALAN	6.08	0.11	$1.4*10^{-08}$	1	0.1512	0.1512
	Body mass	22.37	<0.0001				
lhβ	ALAN	4.12	0.25	0.26	0.88	0.1045	0.2208
	Body mass	7.07	0.01				
Ratio *fshβ*-change/ *lhβ*-change	ALAN	9.09	0.03 ‡	$8*10^{-09}$	1	0.3272	0.3272
	Body mass	17.70	<0.0001				
11 KT	ALAN	7.92	0.048 §	$8*10^{-08}$	1	0.0156	0.3271
	Body mass	29.81	<0.0001				

‡ Post-hoc test for ALAN effect in the ratio of *fshβ*-change/*lhβ*-change

contrast	estimate	standard error	df	t-ratio	*p*-value
0 lx – 0.01 lx	0.39	0.21	19	1.85	0.28
0 lx – 0.1 lx	0.23	0.27	19	0.87	0.82
0 lx – 1 lx	–0.15	0.17	19	–0.86	0.83
0.01 lx – 0.1 lx	–0.16	0.27	19	–0.59	0.93
0.01 lx – 1 lx	–0.54	0.17	19	–3.12	0.03
0.1 lx – 1 lx	–0.38	0.24	19	–1.60	0.40

§ Post-hoc test for ALAN effect in 11 KT

contrast	estimate	standard error	df	t-ratio	*p*-value
0 lx – 0.01 lx	374	241	19	1.56	0.43
0 lx – 0.1 lx	–193	265	19	–0.73	0.88
0 lx – 1 lx	–377	308	19	–1.23	0.62
0.01 lx – 0.1 lx	–568	178	19	–3.19	0.03
0.01 lx – 1 lx	–752	236	19	–3.18	0.02
0.1 lx – 1 lx	–184	254	19	–0.72	0.89

†Log-likelihood ratio

2.2.3 Gonadotropins in the field experiment

As there were only two male fish among the re-captured fish for each treatment in the field experiment, no data evaluation or interpretation was possible for males. In the skyglow group, gene expression was roughly at the same level as gene expression in control males after 1 – 2 months (Supplementary material). Premature females (n = 3 for Control, n = 8 for Skyglow) had undifferentiated

gonads, for which gene expression was not analyzed because it was presumed to be very low.

In maturing females, the mean *fshβ* expression was increased (+26%) after 1 – 2 months of exposure to an average surface illumination of 0.06 lx compared to control females which were not exposed to additional illumination (Figure 2.3). However, three individuals also had lower values than the mean expression in controls (Figure 2.3) and the mean increase was not statistically significant (U-test: $p = 0.22$, Table 2.3). Mean *lhβ*-expression was 1.4-fold decreased (–27%) and the lowest *lhβ*-expression of an individual female in the skyglow treatment was 2.2-fold decreased from the control mean (Figure 2.3). The reduced expression of *lhβ* was statistically significant (U-test: $p = 0.045$, Table 2.3). The mean ratio of *fshβ*-change/*lhβ*-change increased significantly by 84% as compared to controls (U-test: $p = 0.03$, Figure 2.3, Table 2.3). For data evaluation, it should be remembered that the power of the experiment and informative value is limited due to the small sample size. Although the uncertainty caused by few data remains, we also evaluated a Bayesian approach to provide an alternative perspective on the data.

In the Bayesian model for maturing females, the most likely expected value (maximum a posteriori probability, MAP) for mRNA expression ratio of *lhβ* in the skyglow treatment was 0.73, i.e., a 1.4-fold decrease compared to controls (Table 2.4). The percentile intervals with 89% posterior probability did not overlap for the modelled posterior distributions of the control and skyglow treatment (Figure 2.3, solid lines in middle row; Table 2.4). The probability mass to have on average no or a positive group difference is 0.2%. These results support our hypothesis of reduced *lhβ* expression after skyglow exposure. However, the 89% prediction intervals overlap, indicating that individuals from both groups with comparable *lhβ* expression can be expected (Figure 2.3, Table 2.4).

For *fshβ* expression, the MAP for females in the skyglow treatment was 1.26, i.e., a 0.3-fold increase but the percentile intervals with 89% posterior probability or both groups largely overlap (Figure 2.3, Table 2.4). The probability mass to have no or a negative group difference is 10%, and in line with this, also the 89% prediction intervals overlap considerably. Hence, based on our data, and contrary to our hypotheses, an average difference between dark controls and skyglow treatment is unlikely and it is also likely that further data would overlap between treatments.

For the ratio of *fshβ*-change/*lhβ*-change, the MAP for the skyglow treatment is 73% higher compared to the control treatment (Table 2.4). The percentile intervals with 89% posterior probability do not overlap for the modelled posterior distributions of the control and skyglow treatment. The probability mass to have no or a negative group difference is 0.4%. Therefore, contrary to our hypothesis, the results suggest that a group difference of zero or a negative group difference are highly incompatible with our data. However, since 89% prediction intervals overlap, it is to be expected to find individuals with similar ratio of *fshβ*-change/*lhβ*-change in both groups (Figure 2.3, Table 2.4).

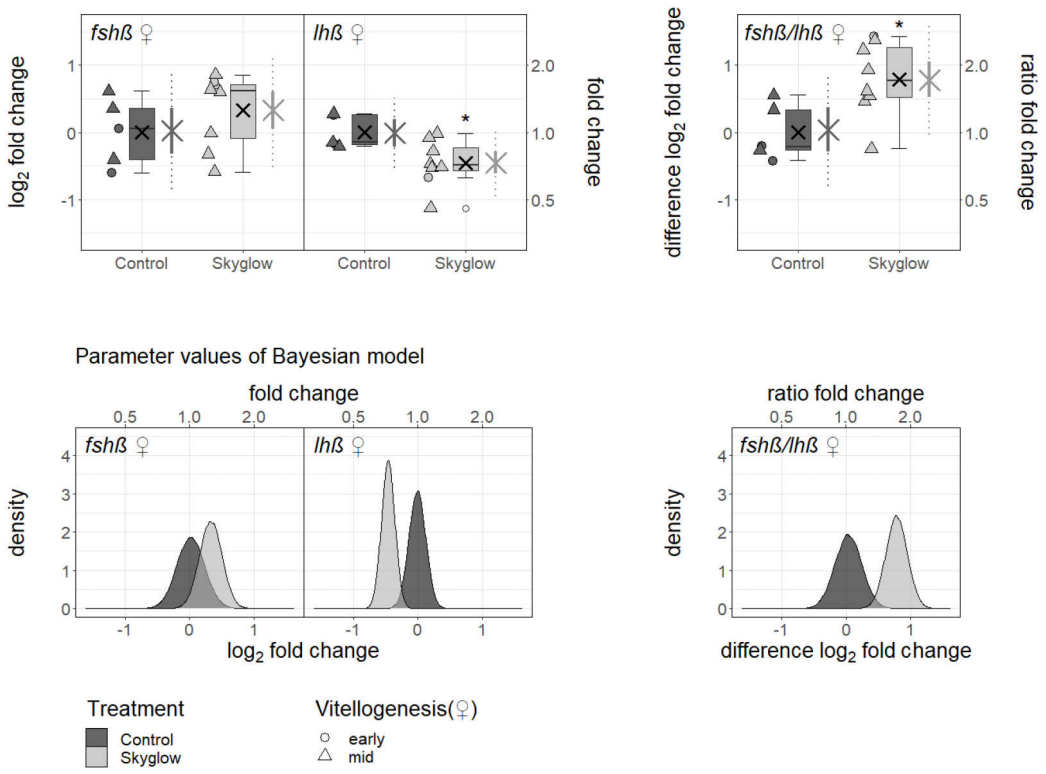

Figure 2.3 Relative mRNA expression of the *β*-subunits of the gonadotropins follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) of female Eurasian perch (*Perca fluviatilis*) exposed to natural conditions (Control, dark gray) or artificial illumination (Skyglow; light gray) for 1 – 2 months in the field experiment (outdoor enclosures). Pituitary glands were sampled throughout the day. Upper row: Boxplots are limited by the 25% and 75% quartile, horizontal lines marks the 50% quartile, and black "X"s indicate the mean. Boxplots serve as a visualization of data and do not represent the statistical analysis. Asterisks mark statistical significance at $p < 0.05$ in Mann-Whitney U-test analyses. Individual points to the left of boxplots represent values for each individual (n = 5 for Control, n = 8 for Skyglow) with circles or triangles indicating early or mid-vitellogenesis, respectively. Vertical solid lines through the gray "X"s indicate the percentile interval of the 89% posterior probability mass (5.5% and 94.5% quantiles), and dotted lines represent the prediction intervals at 89% credibility of the Bayesian regression. On the left, both y-axes refer to both genes. The right graphs show the ratio of *fshβ*-change/*lhβ*-change. Lower row: Density plots represent the posterior distribution of the Bayesian regression analysis.

Table 2.3
Mann-Whitney U-test results of relative mRNA expression of the *β*-subunits of follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in the pituitary gland as well as the ratio of *fshβ*-change/*lhβ*-change in female Eurasian perch (*Perca fluviatilis*) comparing natural conditions (Control) with artificial illumination of 0.06 lx (Skyglow) for 1 – 2 months in the field experiment.

Parameter	**U**	***p*-value**
fshβ	11	0.22
lhβ	34	0.045
Ratio *fshβ*-change/*lhβ*-change	5	0.03

Table 2.4
Summary of Bayesian analysis of relative mRNA expression of the *β*-subunits of follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) in the pituitary gland as well as the ratio of *fshβ*-change/*lhβ*-change in the Eurasian perch (*Perca fluviatilis*) comparing a control treatment exposed to natural nights and a treatment with simulated skyglow of ca. 0.06 lx average nocturnal illuminance on the water surface. Gaussian approximations for each model parameter's marginal distribution (A), and posterior distribution of the group means as well as the prediction intervals (B). Since the marginal posteriors and posteriors are Gaussian, the mean coincides with the mode of the distribution and thus with the maximum a posteriori probability (MAP). Values are given as ddCt-values and 2^{ddCt}-values in parentheses. The 5.5% and 94.5% quantiles are percentile interval boundaries, corresponding to an 89% of the probability mass.

A)	**Response variable**	**Model parameter**	**Mean/ MAP**	**5.5%**	**94.5%**
Marginal posterior distribution of each model parameter	***fshβ***	*α*	0.02	–0.33	0.36
		β	0.31	–0.13	0.75
		σ	0.49	0.34	0.65
	lhβ	*α*	–0.01	–0.22	0.2
		β	–0.45	–0.71	–0.18
		σ	0.29	0.2	0.39
	Ratio *fshβ*-change/ *lhβ*-change	*α*	0.04	–0.29	0.36
		β	0.75	0.32	1.16
		σ	0.47	0.33	0.62
B)	**Response variable**	**Treatment**	**Mean/ MAP** ddCt (2^{ddCt})	**5.5%**	**94.5%**
Posterior distribution of the group means	***fshβ***	Control	0.02 (1.01)	–0.33	0.36
		Skyglow	0.32 (1.25)	0.05	0.6
	lhβ	Control	–0.01 (0.99)	–0.22	0.2
		Skyglow	–0.46 (0.73)	–0.62	–0.29
	Ratio *fshβ*-change/ *lhβ*-change	Control	0.04 (1.03)	–0.29	0.36
		Skyglow	0.78 (1.72)	0.51	1.05
Prediction intervals	***fshβ***	Control	0.02 (1.01)	–0.85	0.89
		Skyglow	0.33 (1.26)	–0.53	1.17
	lhβ	Control	–0.01 (0.99)	–0.53	0.51
		Skyglow	–0.46 (0.73)	–0.96	0.04
	Ratio *fshβ*-change/ *lhβ*-change	Control	0.04 (1.03)	–0.79	0.87
		Skyglow	0.79 (1.73)	0.02	1.6

2.2.4 Histological analysis in the field experiment

After skyglow exposure in the field experiment, we found most of the female perch in pre- and mid-vitellogenesis (both 47%) and only a few in early vitellogenesis (6%). In the control group, the percentage distribution was slightly different (pre- and mid-vitellogenesis: 37.5%; early vitellogenesis: 25%), but both groups had a balanced distribution regarding females in pre- and mid-vitellogenesis (Figure 2.4). Unfortunately, due to low numbers of re-captured fish, further statistical evaluation of the distribution differences concerning the histological status and the influence of enclosures was not meaningful.

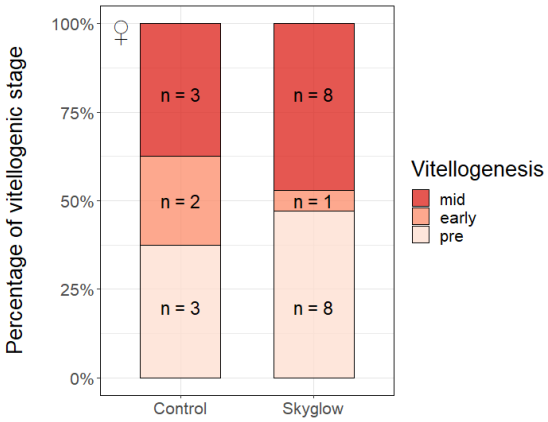

Figure 2.4 Relative differences of vitellogenic stages (%) in female Eurasian perch (*Perca fluviatilis*) exposed to natural conditions (Control) or ALAN of 0.06 lx (Skyglow) for 1 – 2 months in an outdoor enclosure experiment. Histological gonad samples were taken throughout the day. Samples were staged by light microscopy into pre-vitellogenic females with multiple nucleoli ("pre"), early vitellogenesis with vitellogenin globules and no separation in the zona radiata ("early"), and mid-vitellogenic females with partially fused vitellogenin globules and a prominent zona radiata ("mid").

2.2.5 Validation of ribosomal protein L8 as reference gene

The reference gene ribosomal protein L8 (*rpL8*) was not differentially expressed across treatments in both the climate chamber and the field experiment (climate chamber exp., Kruskal-Wallis test for males: $Chi^2 = 4.63$, df = 3, $p = 0.20$, Kruskal-Wallis test for females: $Chi^2 = 5.43$, df = 3, $p = 0.14$; field exp., Mann-Whitney U-test for females: U = 27.5, $p = 0.30$, Supplementary material). Furthermore, *rpL8* was not expressed differentially across sexes in the climate chamber experiment (Mann-Whitney U-test: U = 395, $p = 0.08$) or in the field experiment (Mann-Whitney U-test: U = 25, $p = 0.95$).

2.3. Discussion

2.3.1 Climate chamber experiment

In the climate chamber study, gene expression of *fshβ* was reduced in some female individuals in ALAN treatments of 0.01 lx, 0.1 lx, or 1 lx compared to dark controls after two weeks of exposure. For *lhβ*, such reductions in some individuals were not apparent. Mean ratios of *fshβ*-change/*lhβ*-change were gradually lowered with increasing ALAN intensity in females. There were only few females in this experiment and thus the power of statistical analysis was limited, and changes were not significantly different. In males, there were only small changes in means, with some statistically significant effects between treatments (but not between treatments and control groups) for the ratio of *fshβ*-change/*lhβ*-change and 11-keto-testosterone.

The trend of decreasing *fshβ* expression indicates that some individuals may have been affected by the low ALAN levels. Thus, the range between 0.01 lx and 1 lx might represent a transition zone of light intensities in which *fshβ* expression is already affected in some individuals, but not in others. For *fshβ* but not for *lhβ*,

this is in line with a well-comparable earlier experiment, where *fsh*β and *lhβ* expression was reduced at 1 lx or higher (5-fold decrease of means for both genes at 1 lx) in females (Brüning *et al.*, 2016). While the experimental setup and duration, the population, as well as the time of the year of the experiment were the same or similar, the most notable difference between our climate chamber experiment and the previous study (besides the two lower intensities), is the lower body mass of the experimental fish; 16.8 ± 4.1 g in our climate chamber experiment and 31.8 ± 10.1 g in Brüning *et al.* (2016). Therefore, a less advanced maturation status can be assumed in our experiment and might explain why females in our experiment were 52% less sensitive in *fshβ*-change and not sensitive in *lhβ*-change at 1 lx compared to results by Brüning *et al.* (2016). Due to the significant effects of body mass in the LM and LMM analyses of the climate chamber data, it cannot be excluded that the reduced expression of *fshβ* in some individuals is a result of body mass variation (Supplementary material). With respect to ALAN effects, more data are needed that consider correlations of gonadotropin expression with body mass. As a conclusion, our results hint to the fact that ALAN below 1 lx has the potential to elucidate ambiguous effects on female Eurasian perch depending on the sensitivity of the individual, while 1 lx represents a potential threshold for generalized gonadotropin reduction. It should be noted that a larger sample size would likely give clearer results and may allow clearer conclusions.

The trend of decreasing mean ratio of *fshβ*-change/*lhβ*-change might indicate that FSH was affected more by ALAN than LH in this experiment. This is in line with the well-documented endocrine regulation of the gonadogenesis, where FSH regulation precedes LH regulation in most teleost species. Another explanation could be a differential stimulation or inhibition by co-regulating factors of gonadotropin gene expression, e.g., the activin-follistatin ratio (Cheng *et al.*, 2007, Lau and Ge, 2005, Lin and Ge, 2009, van der Kraak, 2009, Yam *et al.*, 1999, Yuen and Ge, 2004).

A reduction of sex steroids was expected after ALAN exposure, but 11 KT was not altered consistently in females and no trend was apparent. Hence, 11 KT is less sensitive towards low ALAN intensities than the gonadotropin expression in the pituitary gland. Also, 11 KT is not a major sex steroid in female fish and other sex steroids like E2 or T might respond differently to slight changes in gonadotropin expression.

In males, in the climate chamber experiment, mRNA expressions of *lhβ* and *fshβ* after ALAN exposure did not change and is hence consistent with Brüning *et al.* (2016). The slight but significant increase in the ratio of *fshβ*-change/*lhβ*-change at 1 lx nocturnal illumination (compared to 0.01 lx) could still indicate a variation in gonadotropin expression pattern. Decreases in plasma 11 KT were small but significant at 0.1 lx and 1 lx compared to the lowest illuminance of 0.01 lx, but not to controls. This is surprising due to the lack of ALAN effects on gonadotropin expression in males at this time of the year. Hence, either the ratio of *fshβ*-change/*lhβ*-change or other co-regulating factors might be involved in ALAN effects on 11 KT.

As expected, 11 KT levels were generally much higher in males than in females. The large range of 11 KT concentrations in males (10 pg mL^{-1} – 3.4 ng mL^{-1}) was comparable to the range of 11 KT concentrations in male Eurasian perch (body mass 32.1 ± 13.2 g and few pg mL^{-1} – 1.5 ng mL^{-1}) from Lake Müggelsee in late summer in an earlier study (Brüning *et al.*, 2018b). In comparison, male Eurasian perch exhibited 3 – 5 ng mL^{-1} 11 KT in France between September and November (Sulistyo *et al.*, 2000), which might be explained by a higher body mass at that time. Histological analyses were not possible in our climate chamber experiment but as individuals were rather small compared to earlier experiments (Sulistyo *et al.*, 2000), it is possible that some males were still prepubertal and therefore produced less 11 KT than larger individuals in earlier experiments.

2.3.2 Field experiment (outdoor enclosures)

In the field experiment conducted in large-scale enclosures at Lake Stechlin, female *lhβ* mRNA expression decreased after 1 – 2 months exposure to a surface illumination of 0.06 lx as compared to natural nights in late summer. At the same time, *fshβ* showed almost no change and the ratio of *fshβ*-change/*lhβ*-change increased. The study did not have sufficient power to test for gonad maturation in the histological analysis, given the few individuals in each histological category due to low re-capture rates.

ALAN affected *lhβ* more than *fshβ* expression in our field experiment. The decrease of average *lhβ* expression in females indicates a weak effect of the skyglow treatment on reproductive physiology. Contrary to *lhβ*, we found no evidence for a decrease of *fshβ* expression with consequent increases in the ratio of *fshβ*-change/*lhβ*-change under skyglow conditions. This differential response of the two gonadotropins could be explained by potential differences in co-regulating factors of gonadotropin gene expression as outlined above. The differential response (change of only one gonadotropin) in the field experiment could also be related to dopamine, which inhibits *lhβ* but not *fshβ* expression in several teleost species (Saligaut *et al.*, 1998, van der Kraak *et al.*, 1986, Zohar *et al.*, 2010), and some studies indicate dopamine-driven LH suppression for *Perca* species (Costache *et al.*, 2017, Dabrowski *et al.*, 1996). Still, data variance of expression may also be explained by variance in exposure durations, body masses, tagging methods and differences in light exposure between individuals due to the large water column (ca. 20 m deep). Larger sample sizes would allow accounting for the complexity of these potentially influencing environmental and experimental parameters.

Histology does not seem to be overly sensitive, but if gonadotropin levels are suppressed by ALAN for a longer period, more dramatic changes are to be expected.

2.3.3 Both experiments

Interestingly, female expression of gonadotropins in the pituitary gland was differential in both experiments, i.e., only one gene was reduced while the other did not differ notably (climate chamber: *fshβ* tendency for reduction, *lhβ* less change; field: *lhβ* reduced, *fshβ* less change). In earlier experiments at higher

ALAN intensities both genes were equally reduced. Hence, differential effects on the expression of the two gonadotropins might be an ALAN effect which is limited to lower light intensities. In addition, one should keep in mind that the experimental fish in the two experiments came from different populations. Fish from Lake Stechlin can be considered light-naïve, but fish in the greater Berlin area might be adapted to dim nighttime light, even if the skyglow of Lake Müggelsee is in the lower range skyglow illuminance and the fish were acclimated to dark nights prior to the experiment. Moreover, experimental fish in the field experiment were larger and likely in a more advanced maturation status. This might further explain the opposing differential response of gonadotropins to ALAN exposure.

Both experiments are in line with the possibility of a photo-labile period in Eurasian perch discussed in earlier studies. It was postulated that the onset of gonadogenesis occurs earlier in the year in males than in females (Migaud *et al.*, 2006). This is consistent with the results of Brüning *et al.* (2016), who studied gene expression in October and found effects of ALAN (1 lx, 10 lx, 100 lx) in females, but not in males. Another experiment in Brüning *et al.* (2016) exposed male and female Eurasian perch to colored light at night (photon flux comparable to 1 lx of white light) in September and found no effect on gonadotropin expression at blue, green or red LED light. An earlier field study exposed male and female perch to 15 lx of white light at night and expression of both gonadotropins was suppressed in both sexes (Brüning *et al.*, 2018b). Except for the light color experiment in September (Brüning *et al.*, 2016), previous studies indicate a photo-labile period of males only in summer/late summer and ALAN-sensitivity in females ranging from late summer until fall, but the period could also start earlier and last longer or sensitivity of females towards ALAN might not be limited to a certain window at all. For males, there is considerable data that suggests a particular sensitivity towards ALAN exposure in summer/late summer and to a lesser extent in fall (October). Our results alone are not sufficient to prove the concept of a photo-labile period, but considering the broader context of the literature, the data are in line with the concept. A photo-labile period would explain why there were only weak ALAN effects in males but would not explain why the response in females was not clearer.

2.3.4 Conservational implications and research gaps

The reduced expression of gonadotropins at the beginning of the annual reproductive cycle (in the case of Eurasian perch, late summer), suggests a delay in gonadal development that could impair reproduction (Brüning *et al.*, 2016, Brüning *et al.*, 2018b). This is in line with the practice in aquaculture to suppress the initiation of the annual reproductive cycle by long photoperiod or continuous light to improve the productivity of fish farming (Davie *et al.*, 2007, Kissil *et al.*, 2001, Kolkovski and Dabrowski, 1998, MacQuarrie *et al.*, 1979, Migaud *et al.*, 2006, Rodríguez *et al.*, 2005, Thrush *et al.*, 1994). Hence, the results presented here justify some concern that the observed reduction in gonadotropin expression may induce a dysfunction of reproduction in wild populations. An adverse effect of ALAN on the reproduction of freshwater fishes would be a clear argument for regulatory measures to limit light pollution in aquatic ecosystems. Our experiments have indicated that some degradation is to be expected even at very low ALAN intensities, as it occurs over large urban and suburban areas in the form of skyglow.

The ALAN effects in our experiments are limited to indications on differential responses of only one of the gonadotropins in female Eurasian perch. For a decisive conclusion on skyglow effects on reproduction of freshwater fish, several research gaps remain to be filled. Future studies should clarify whether the suppression of only one gonadotropin – as it seems to be the case at exposure to skyglow – is indeed sufficient to suppress gonad maturation. For FSH, this can be expected because it is generally accepted to induce vitellogenesis in fish. Also, it is of utmost importance to assess whether the observed effects will result in a shift in spawning time and whether offspring fitness and survival will be affected. So far, few experiments have investigated ALAN effects on eggs and larval development in freshwater fishes (e.g., Brüning *et al.*, 2010, Riley *et al.*, 2013). Experiments over the full reproductive cycle (e.g., comparable to OECD, 2015) or even extended experiments over multiple generations, can be powerful tools to tackle these questions. Lastly, it will be important to understand which fish species are most sensitive with respect to ALAN-inhibited reproduction. It is already known that roach are similarly sensitive towards ALAN compared to Eurasian perch (Brüning *et al.*, 2018b). However, both roach and Eurasian perch are prominent species in urban and suburban areas and might not be the most sensitive species with regards to illuminated nights. Freshwater fishes primarily inhabiting rural areas without light pollution could be of particular interest to identify vulnerable species as illuminance and spatial distribution of skyglow is expected to increase and expand to more rural areas in the future. Obviously, demersal fish are not as exposed to skyglow, and pelagic species will presumably be more relevant. Future studies are needed to understand whether fish can compensate for skyglow by undertaking vertical movements, and what ecological consequences this may imply. Of course, several environmental stressors are responsible for loss of species in urban ecosystems, but it is unclear to which extent light pollution contributes to this decline.

2.4. Conclusion

Exposure of Eurasian perch to ALAN – even at an illuminance comparable to typical suburban skyglow – influences reproductive processes via the HPG axis, especially in females. Several factors seem to influence and potentially interact with ALAN-related effects on reproductive processes, for example, the intensity of ALAN, the time of year, the developmental/maturation stage and sex of investigated fish. Together with two previous studies (Brüning *et al.*, 2016, Brüning *et al.*, 2018b), the results from the two experiments presented here, suggest a rather complex influence of ALAN on reproductive processes in Eurasian perch.

1. The results of the climate chamber experiment indicate that the gonadotropin expression of female perch is reduced after ALAN exposure at low intensities in fall, whereas males showed little to no effects. Some individuals revealed relevant decreases of *fshβ* expression at intensities of 0.01 – 1 lx in the climate chamber experiment (although means were not significantly different) and *lhβ* expression was significantly reduced at 0.06 lx in the field experiment.

2. In the climate chamber experiment, *fshβ* seemed to be more sensitive than *lhß*, and in the field experiment *lhβ* was more sensitive. Our study indicates a

differential response in expression of the two gonadotropins in Eurasian perch after exposure to ALAN at low intensities, which might be explained by different sizes of the experimental animals.

3. A photo-labile period of male Eurasian perch can be expected between August and September (present, Brüning *et al.*, 2018b). For females, this period likely ranges from August at least till October (present, Brüning *et al.*, 2016, Brüning *et al.*, 2018b).

4. Due to significant effects of body mass on female gonadotropin expression and as effects on gonadotropin expression are less pronounced in females in our climate chamber experiment than in a previous study at comparable illuminance of 1 lx (Brüning et al., 2016), we assume that ALAN effects are more pronounced in larger, more mature individuals than in smaller Eurasian perch.

5. The observed effects on the endocrine system do not evoke a clear modulation of the gonad development in females as monitored by histological analysis in our study. Most females were in pre- and mid-vitellogenesis in controls and skyglow treatment in the field experiment.

2.5. Material and Methods

2.5.1 Ethical statement

The care and use of experimental animals complied with German animal welfare laws, guidelines and policies as approved by the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16, climate chamber experiment) and the State Office for Occupational Safety, Consumer Protection and Health of Brandenburg (LAVG reference number 2347-17-2016, field experiment).

2.5.2 The climate chamber experiment

We studied wild Eurasian perch (pubertal and young adults) originating from Lake Müggelsee (Berlin, Germany). All experimental fish were caught between July and September 2017 and kept in 600-L indoor tanks at 16°C for at least two weeks acclimation before transfer to the experimental aquaria. During this time fish experienced natural photoperiod (sunlight through windows on the side of the building) with dark nights (<0.00167 lx). Fish were fed twice a day with frozen chironomid larvae. According to the night sky brightness monitoring data, the surface of Lake Müggelsee experiences an illumination of ca. 0.008 lx in moonless clear nights, and 0.02 – 0.05 lx during cloudy nights (Jechow *et al.*, 2016), which lies in the lower range of suburban skyglow (Hänel *et al.*, 2018).

The climate chamber experiment was described in detail in previous publications (Kupprat *et al.*, 2020, Kupprat *et al.*, 2021a, Kupprat *et al.*, 2021b). Thirty fish with a mass of 17 ± 4 g and standard length of 10.6 ± 0.9 cm (mean ± standard deviation (SD), n = 720) were transferred to each of twelve 80-L aquaria in a climate chamber and fed twice a day with frozen blood worms. The temperature during acclimation and experimental exposure to ALAN was kept around 16.8 ± 0.9°C. During two weeks of acclimation, fish were fed twice a day and the water flow-through was adjusted to 10 L h^{-1}. The photoperiod was adjusted

to October conditions with full daylight (ca. 2900 lx) from 09:30 to 18:30 with a 3 h dawn or dusk period starting at 06:30 and 18:30, respectively. After acclimation, the nocturnal illumination from 21:30 till 6:30 was switched on with 0.01, 0.1, or 1 lx average illuminance on the water surface for two weeks. All aquaria were covered with black foil to ensure independence of the treatments. Controls were not illuminated during night (< 0.00167 lx, referred to as "0 lx" in figures and tables). Details on the experimental setup and the light source are given by Franke *et al.* (2013). During the two weeks of experimental illumination, the water flow-through was reduced to 4 L h^{-1} in order to allow water-based melatonin as described by Kupprat *et al.* (2020). To maintain good water quality, animals were not fed during the two weeks of exposure. The same experiment was performed twice – in October and November 2017 – with each treatment in triplicates for both runs (i.e., n = 6 for each treatment).

After two weeks of experimental illumination, fish were randomly sampled on two consecutive nights between 22:00 and 04:00. The body mass and length of all fish were measured, blood was only taken from the first fifteen fish, and blood sampling of each aquarium was completed within 35 min. Pituitaries were excised from the first 10 fish. Males (m) and females (f) were distinguished by visual inspection of the gonads. The experimental fish can be considered juvenile or pubertal, as some individuals showed maturing gonads and in others, male and female gonads could not be differentiated (nd, premature).

Blood (500 – 1000 µL) was taken from the caudal vein with heparinized syringes and transferred to a clean tube containing 1 – 2 mg Na_2EDTA (EDTA). Blood and EDTA were mixed by shaking and centrifuged at 7500 × *g* for 5 min at 4°C. The plasma was transferred to a new tube and immediately frozen in liquid nitrogen and then stored at –80°C until 11 KT was extracted. The pituitary gland was excised with sterilized tweezers by decapitation and removal of the skull cap and brain. Pituitary glands were transferred to RNA/DNA free tubes, immediately frozen in liquid nitrogen and then stored at –80°C until RNA was extracted. We aimed to analyze three plasma and pituitary gland samples from each aquarium and each run for each sex, i.e., n = 18 individuals for each sex for each experimental group. However, there were only few females in the experiment, and hence only one or two samples were analyzed. In some aquaria no females were present at all. Body mass of the animals analyzed for reproductive parameters was 19 ± 4 g and standard length was 11.2 ± 0.7 cm (mean ± SD).

2.5.3 The field enclosure experiment

Lake Stechlin (53°08'36" N, 13°01'41" E) offers excellent reference conditions for light pollution experiments due to its relatively natural dark nights (Jechow *et al.*, 2016). Eurasian perch (juveniles and adults) were caught from Lake Stechlin with fish traps between June and July 2018. They were kept in two 1000-L indoor tanks at 14 – 15°C prior to the experiment. Smaller fish (body mass 52 ± 14 g, standard length of 15.0 ± 1.4 cm, mean ± SD, n = 45) were injected with a passive integrated transponder (PIT) tag (HDX, Oregon RFID, Portland, USA). Larger fish (body mass 166 ± 41 g and a standard length for 21.1 ± 1.7 cm, n = 18, only re-captured fish) were anesthetized with 2-Phenoxyethanol (starting concentration of 0.5 mL L^{-1}) and a data storage tag (diameter = 8.3 mm, length = 25.4 mm) was

surgically implanted into the body cavity for recording depth every 2 min and temperature every 10 min (type micro-TD DST, Star-Oddi, Iceland). Surgery (anesthesia until waking) lasted ca. 15 min, and fish were observed after surgery until fully awake.

For the field experiment, eight Eurasian perch (mix of 3 – 5 PIT- and 2 – 3 DS-tagged fish per enclosure, and the rest untagged fish) were released to each of ten experimental enclosures of the LakeLab installed in Lake Stechlin (www.lake-lab.de). The number of fish were estimated to mimic natural fish densities in the epilimnion of Lake Stechlin. Five enclosures were used as control enclosures with exposure to natural sky lights but without artificial illumination, and five other enclosures were illuminated at night with 0.06 lx at the surface ("Skyglow" treatment, n = 5). The same procedure was followed for an additional ten experimental enclosures for a related experiment that is not part of this study such as a related study evaluating perch behavior of the DS-tagged fish (Mahlow, 2019).

The herein presented experiment was part of the ILES 2018 experiment (Illuminating Lake Ecosystems, https://www.lake-lab.de/index.php/iles-experiment-summer-2018.html), for which large illumination structures were installed above all enclosures and switched on at pseudo-randomly selected enclosures to achieve a diffuse and homogenous skyglow-like illumination with an illuminance of 0.06 lx at the water surface which is described in detail by Jechow *et al.* (2021). Briefly, the illumination was realized with 37 waterproof LED strips emitting spatially diffuse light (VarioLED Flex NIKE LD4 827 153SV, LEDlinear GmbH, Neukirchen-Vluyn, Germany) per enclosure were installed 0.4 m above the water surface arranged equidistantly on two ring structures with one central emitter (Jechow *et al.*, 2021). The color spectrum of the LED strips was warm-white (CCT of 2700 K), which is representative for typical CCTs reported for urban light-polluted skies (Jechow *et al.*, 2020). Although illuminance was slightly spatially inhomogeneous at the surface, the light distribution was calculated and validated to be homogenous from 1 m depth and below (Jechow *et al.*, 2021). Lights were switched on permanently.

PIT-tagged fish were released into the experimental enclosures on August 8th, 2018. DS-tagged fish had to be monitored after surgery and some were released on August 18th, 2018. A second round of tagging was necessary due to mortality of the DS-tagged fish in the first round; in the second round of DS-tagging mortality was low (3% in two days). Enclosures were re-stocked with these newly DS-tagged fish on September 7th, 2018. Average body mass of re-captured tagged fish (PIT and DST) was 119 ± 63 g with a standard length of 18.9 ± 3.0 cm (mean ± SD, n = 32). The enclosures of the research facility are 9 m in diameter and ca. 20 m deep (ca. 1300 m³). On the surface, each enclosure is encircled by a floating aluminum ring from which insulating foil goes down to the bottom and is buried in the sediment. Before the experiment started, the enclosures were cleaned by removing floating algae scums and installing a clean, white foil above the inner enclosure wall, which was turned several times during the experiment to keep the periphyton wall growth at low levels. Prior to addition of experimental fish, all remaining fish from previous experiments in the enclosures were removed by fishing with customized dip nets which were manufactured to fit the dimensions of

the enclosures (9 m diameter, 20 m long lines, handled by 8 people). Fishing was continued for several weeks to ensure all fish were removed. After that, the enclosure water was exchanged with water from the surrounding lake. First, by exchanging metalimnion water with the deeper layers of the stratified water column, and subsequently the epilimnion was exchanged from the upper layer of enclosure and lake. The exchange was performed in the last two weeks of July 2018 similar to the procedure described by Giling *et al.* (2017). To boost phytoplankton growth in the upper water layer (epilimnion), the phosphorus concentration was doubled by adding 15 µg P L^{-1} as ortho-phosphoric acid (and 105 µg N L^{-1} as ammonium nitrate to keep the N:P ratio similar to Lake Stechlin) into the well mixed upper water layer on 8th of August 2018. After nutrient addition, total phosphorus increased from 19.6 ± 1.6 µg P L^{-1} to 32.9 ± 3.1 µg P L^{-1} and total nitrogen from 586 ± 55 µg N L^{-1} to 737 ± 59 µg N L^{-1}, respectively (means ± SDs) and both returned to initial concentration towards the end of the experiment. Epilimnion chlorophyll-a concentration was initially below 1 µg L^{-1}, increased after the nutrient addition to 4 – 5 µg L^{-1} and declined to initial concentration without any differences between the treatments. The pH was on average 8.43 and did not differ between control and skyglow treatments. The mean water temperature in the epilimnion over the experimental period was 19.8 ± 3.0°C (mean ± SD, n = 9 weekly averages from hourly temperature profiles) with no differences between treatments. The temperature steadily declined over the experimental period from 24.0°C in the beginning of August over 19.9°C in the beginning of September until 14.6°C in the beginning of October. Perch stayed above the thermocline as determined by behavioral analyses from the DS-tagged fish (Mahlow, 2019). Stratification was comparable in all enclosures and the surrounding lake. Weekly mean thermocline depth was estimated from hourly temperature profiles. At the start of the experiment, the thermocline was highest at 7.1 ± 0.05 m in controls and skyglow enclosures (each n = 5) and descended gradually to 9.8 ± 0.05 m at the end of the experiment. Mean thermocline depth descended over time with negligible between-enclosure variance but did not differ between treatments. Overall mean thermocline depth in both treatments over the entire experimental period was 8.5 ± 0.8 m with a minimum of 7.0 m and a maximum of 9.9 m (n = 5 enclosures over 8 weeks). At the start of the experiment, Secchi depth (weekly measurements) was 6 ± 1 m in controls and 6 ± 0.4 m in skyglow enclosures (n = 5). Secchi depth decreased until the end of August with means of 3.7 ± 1.7 m in controls and 3.9 ± 1.2 m in skyglow enclosures. After this peak, the Secchi depth increased again but with larger between-enclosure variance with means of 5.6 ± 2.7 m in control enclosures and 7.3 ± 2.6 m in skyglow enclosures at the last measurement at the end of September. Means and variances of Secchi depth differed over time but did not differ between treatments. The overall mean Secchi depth was 4.8 ± 2.1 m in controls with minimum at 1.9 m and a maximum at 8.8 m, and 5.4 ± 1.8 m in skyglow treatments with a minimum at 2.1 m and a maximum at 10 m (n = 5 enclosures over 8 weeks). The extinction coefficient was 0.40 ± 0.07 m^{-1} in control enclosures and 0.37 ± 0.08 m^{-1} in skyglow enclosures. Natural zooplankton and some benthic organisms on the walls likely served as food source for the experimental fish. There was no additional feeding. The photoperiod at Lake Stechlin during the experimental period was 15 L:9 D (sunrise 5:36, sunset 20:49) on August 8th, 13 L:11 D (sunrise 6:27, sunset 19:42) on September 7th

and 12 L:12 D (sunrise 7:15 hours, sunset 18:35 hours) on 5th October. New moons were on August 11th, September 9th, and October 9th, 2018, whereas full moons were on July 27th, August 26th, and September 25th, 2018.

Fish were re-captured on two consecutive days (October 4th and 5th, 2018) with customized dip nets (see above). Although it would have been optimal to re-capture fish during the night, this was not possible for safety reasons due to the dimensions of the dip nets; instead, fish were re-caught throughout the day. Due to different release dates, re-captured fish were inside the enclosure for 27 – 58 days. Of the 80 fish, 29 were re-captured (45.3%), and re-capture rates among enclosures ranged from 12.5% (1/8) up to 62.5% (5/8) on the dates of sampling. All re-captured fish of one enclosure were quickly brought to land and samples were taken during 10:00 – 18:00. Body mass and length were measured for all fish. Fish were stunned by a blow to the head and killed by cutting through the neck. Animals were opened dorsally; in the case of DS-tagged fish, the DST was removed. Males (m) and females (f) were distinguished by visual inspection of the gonads, and the gonads were dissected for histological analysis (see below). Pituitary glands were sampled and stored as described above in the climate chamber experiment.

Body mass of re-captured experimental fish (control and skyglow treatment) ranged from 37 g to 222 g and the average body mass was 110 ± 58 g (mean ± SD, n = 29). Standard length ranged from 12.6 cm to 24.4 cm with a mean of 18.5 ± 3.2 cm. Maturing females (n = 14) had a mean body mass of 130 ± 62 g and a standard length of 19.3 ± 3.5 cm.

2.5.4 Sex steroid extraction and measurement

11 KT was extracted in 5 mL glass vials from 100 µL blood plasma with 500 µL ethyl acetate (Roth) according to the procedures described by Brüning *et al.* (2018b). In case less plasma was available, volumes were reduced accordingly. Plasma was mixed with ethyl acetate and vortexed for 30 s. Phases were allowed to separate for 5 min and then the samples were frozen at –80°C for 15 min, so that the lower watery phase was frozen, and the upper liquid phase could be transferred to a new vial. The extraction was performed twice, and extracts were dried at 45°C under a stream of nitrogen. Dried extracts were re-dissolved in EIA buffer (Cayman chemicals). 11 KT levels were measured by enzyme linked immunosorbent assay (ELISA) using a commercial kit (Item 582751, Cayman Chemicals).

2.5.5 Relative mRNA quantification by RT-qPCR

2.5.5.1 RNA extraction and reverse transcription

Total RNA was extracted from homogenized pituitaries (Tissue Lyser, Qiagen) by a commercial kit (RNeasy plus, Qiagen) following the manufacturer's protocol. RNA quality and quantity were determined by gel electrophoresis and by UV absorption spectrometry (Nanodrop ND-1000 spectrophotometer, Thermo Fisher Scientific). 1 µg of total RNA was transcribed into cDNA by Affinity Script Multiple Temperature Reverse transcriptase (Agilent Technologies, Cat: 600107) following the protocol by Brüning *et al.* (2016). In a reaction volume of 10 µL, a volume of

1.5 µL poly-dT-primer (CCTgAATTCTAgAgCTCA(T)17, Biometra, Göttingen) was mixed with 2 µL AffinityScript RT buffer (10x), 1 µL dNTP (each 10 nmol), 2 µL DTT (100 mM), and 1 µL Affinity Script Multiple Temperature Reverse Transcriptase.

2.5.5.2 Gene expression analysis by RT-qPCR

Ribosomal protein L8 (*rpL8*) was used as a reference gene (REF) because expression was stable across all data points in earlier studies (Brüning *et al.*, 2016, Brüning *et al.*, 2018b) which was again confirmed in our data (see results). The genes of interest (GOI) were *fshβ* and *lhβ*. Primer sequences for *rpL8* and *fshβ* were originally designed for pikeperch (*Sander lucioperca* L.) by Hermelink *et al.* (2011). Both primers were tested for Eurasian perch by Brüning *et al.* (2016) who also established the primer sequences for *lhβ* and verified the identity of all PCR products for Eurasian perch by direct sequencing. Primer efficiencies for all genes were determined in triplicates with a sample of pooled cDNA by a serial dilution series of pooled pituitary cDNA and ranged from 1.91 (91%) to 1.94 (94%) (Table 2.5).

cDNA was diluted 1:10 to yield concentrations of 1 µg 100 µL^{-1}. Real-time qPCR was performed in a Bio-Rad CFX Connect and CFX96 Touch Real Time PCR Cycler (Bio-Rad Laboratories) for the samples from the climate chamber experiment and in a Stratagene Mx3005 qPCR Cycler (Agilent Technologies) for the samples from the field experiment. Amplifications were carried out using hot start PlatinumTM Taq polymerase (Invitrogen by Thermo Fisher Scientific, Cat: 10966034) in a SYBR green-based assay with a reaction volume of 20 µL (0.2 µL polymerase, 2 µL diluted cDNA (final 0.02 µg per qPCR reaction), 0.17 µL dNTP-solution (10 mM, each), 2 µL reaction buffer, $MgCl_2$ in a final concentration of 2 mM, 0.1 µL of 200-fold diluted SYBR-green-I solution, 7.5 pmol µL^{-1} of each specific primer (Tib MolBiol) and PCR water). The PCR conditions were set to 95°C initial degradation for 7 min 40 s, followed by 40 cycles of 95°C for 17 s, 63°C (*rpL8*, *lhβ*) or 64°C (*fshβ*) for 25 s and 72°C for 25 s. The melting curves were analyzed between 60 – 95°C after 40 cycles.

Table 2.5
Overview of primer specific PCR conditions. Primer sequences, primer concentrations and product sizes are according to Brüning *et al.* (2016) and Hermelink *et al.* (2011). Annealing temperature (TA) and primer efficiencies (E) were optimized with our samples for the climate chamber experiment (cc) and the field experiment (field) only the optimized conditions were used.

Gene	Forward primer	Reverse primer	T_A	Primer conc.	Product size (bp)	E (cc)	E (field)
rpL8	GTTATCGCCTC TGCCAAC	ACCGAAGGGATG CTCAAC	63°C	375 nM	167	1.92	1.93
fshβ	CCTACTGGCA GGGAAGAAC	CTGACACCCACTG GACATC	64°C	375 nM	85	1.93	1.91
lhβ	GGCTGTCCAAA GTGTCACCT	GGGAGAACAGTCA GGGAGCTTAA	63°C	375 nM	158	1.94	1.92

2.5.5.3 Relative mRNA quantification

Samples were only considered for evaluation when RNA concentration in 15 µL resolved extract were ≥ 15 ng µL^{-1}, as no reliable qPCR could be obtained with < 15 ng µL^{-1}.

Samples were measured in duplicates. A sample of the pooled pituitary cDNA was run on each plate as a calibrator sample. Ct values were corrected for plate variations in the climate chamber experiment because samples were randomly distributed across plates. In the field experiments, all samples were run on one plate. The efficiency-weighted Ct-values were normalized to the efficiency of each gene by $Ct_{(w)} = Ct * \log_2(E)$, so that each weighted Ct-value ($Ct_{(w)}$) was normalized to optimal efficiencies (i.e., E = 2). This procedure is described by Ganger *et al.* (2017) except that for our values we use a $\log_2$ and not $\log_{10}$. After efficiency-weighting, we basically continued with the procedure described by Livak and Schmittgen (2001), except that dCt values were calculated so that lower values represent less cDNA, i.e., $dCt = Ct_{(w)\,REF} - Ct_{(w)\,GOI}$. The average dCt of all samples of the control treatment was subtracted from every Ct value (also from individual control samples) to obtain ddCt (lower ddCt values hence also represent less cDNA), i.e., $ddCt = dCt_{Sample\ x} - dCt_{AVG\ control}$. ddCt values were potentiated to the base of two, i.e., 2^{ddCt} values (because Ct values were normalized to their respective efficiencies, we can use optimal efficiencies of 2 as a basis). 2^{ddCt} values represent the fold change, or the relative gene expression ratio (relative to the control treatment) whereas ddCt values represent the $\log_2$-fold change. This procedure bears the advantage over the classical ddCt-method by Livak and Schmittgen (2001) in that Ct-values are corrected for differences in efficiencies. Although the Pfaffl-method (Pfaffl, 2001) also allows for correction of efficiencies, the values calculated according to Pfaffl should not be used for parametric statistical analyses, because Ct-values are in the exponent and normal distribution cannot be assumed. Taking $\log_2$ of Pfaffl-values would not consider the different efficiencies of GOI and REF.

Graphs present the data on two axes, $\log_2$-fold change on a linear scale (ddCt values) and fold change on a $\log_2$ scale (2^{ddCt} values, i.e., the relative gene expression ratio) to visualize that a fold change of 0.5 is an equal change to a fold change of 2. Effect quantification (fold-increase or decrease) is calculated from the 2^{ddCt}-values.

2.5.6 Histological analysis

Gonads were transferred to phosphate-buffered formaldehyde solution (ROTI®Histofix, 4%, Carl Roth) during sampling and after 48 h rinsed three times with 70% ethanol. Transverse slices from the middle of the gonads (ca. 5 mm) were dehydrated in an increasing series of ethanol (75%, 90%, 95%, 100%), rinsed in xylol (Carl Roth), and transferred into Paraplast® (Leica) with a tissue processor (Shandon Excelsior ES Tissue Processor, Thermo Fisher Scientific). 5 µm sections were cut in a half-automatic microtome (Leica Jung Supercut 2065). Sections on glass slides were stained using standard hematoxylin/eosin solution (0.1%, Carl Roth). Histological staging was performed according to the criteria in Table 2.6 using a light microscope (Nikon Eclipse Ni-U with Nikon DS-Fi3 camera).

Table 2.6
Criteria for histological classification of gonads.

Sex	State of gametogenesis	Histological classification criteria
females	Previtellogenesis	with multiple nucleoli
	early vitellogenesis	vitellogenin globules, zona radiata not divided
	mid vitellogenesis	vitellogenin globules partially merged in the middle, zona radiata not divided
males	mid spermatogenesis	spermatocysts I and II, spermatids and sperm ratio 1:1
	late spermatogenesis	spermatocysts I and II, spermatids and many sperm

2.5.7 Statistical analysis

All statistical calculations were performed in R version 4.03 (R Core R Core Team, 2020) and R Studio version 1.4.1103.

2.5.7.1 Validation of rpL8 as reference gene

Ct values of *rpL8* were compared across sexes and treatments to validate comparisons across sexes and treatments. These comparisons were made by a Kruskal-Wallis test for the climate chamber experiment and by a Mann-Whitney U-tests for the field experiment. Sex effects were tested across all treatments, and treatment effects were tested for each sex separately.

2.5.7.2 Linear (mixed) models for the climate chamber experiment

For the males of the climate chamber experiment, we used a linear mixed modelling (LMM) approach. The preferable LMM analysis including random effects was not possible for females due to low numbers of females in the experiment. Therefore, linear models (LM) neglecting dependencies of individuals from the same aquarium or run were calculated (i.e., without the random term). The explanatory variables “ALAN” and “Body mass” and their interactions were chosen as fixed terms and for males “Aquaria nested in Runs” was added as a random term. For each response variable (*fshβ*, *lhβ*, and ratio of *fshβ*-change/*lhβ*-change, 11 KT), an individual model was fitted. The interaction term was removed from the models during model selection as it did not improve the models. Model selection for the fixed terms and interaction between them was done by nested model comparison with the likelihood ratio test (LM and LMM) (Pinheiro *et al.*, 2018, Zuur *et al.*, 2009), however, the factor ALAN was always kept in the model since it was the central variable of our experiments and hypotheses. For male LMMs, the random effect term was always kept in the models independent of significance to account for the data structure. A weight term was added in LMMs for males to account for variation across aquaria. In case of a significant ALAN effect, we applied post-hoc tests using Tukey’s correction for multiple testing to compare every treatment against each other (Lenth, 2019). Regression coefficients for the LMs for the explanatory variables as well as multiple R^2 values are reported in Table 2.1. Regression coefficients for the LMMs for the fixed and random effects

as well as marginal and conditional R^2 values (Barton, 2018) each LMM of males are reported in Table 2.2. Full model specifications for each dependent variable as well as full results of the post-hoc tests are given in the supplementary material.

2.5.7.3 Statistical approaches for the field experiment

At the start of the field experiment, we planned to apply LMMs to the ILES data with ALAN treatment and body mass as fixed term and the enclosure and tagging method as random term. However, due to low re-capture rates a non-parametric test neglecting random effects was chosen as a frequentist's perspective.

The Mann-Whitney U-test reports a probability that a value from one population is larger or smaller than a value from a second, independent population. Mann-Whitney U-tests result in a *p*-value indicating whether a significant treatment effect can be assumed. However, it does not provide estimates for model parameters and associated confidence intervals and its applicability to very small sample sizes is under debate. Results from the Mann-Whitney U-test are reported for *fshβ*, *lhβ* and the ratio of *fshβ*-change/*lhβ*-change (Table 2.3).

In contrast to the frequentist's approach, the Bayesian approach has a more direct and neutral interpretation. It derives a posterior probability distribution for the model parameters based on the available data, and uncertainty due to small sample sizes is naturally incorporated on the shape of this distribution. The posterior probability distribution can then be informatively summarized in terms of credibility intervals, which report two parameter values bordering a specific probability mass, intervals of defined boundaries, or point estimates (McElreath, 2020b). Here we report as credibility intervals so called percentile intervals (PI), i.e., the two values between which the central probability mass is bounded. We chose the middle 89% interval of probability mass between the 5.5% and 94.5% percentile; 89% were chosen to dissociate from classical thresholds for *p*-values and confidence intervals (McElreath, 2020b). As intervals of defined boundaries we report the probability mass for no group difference or group difference with the opposite sign, i.e., $Pr(\beta \leq 0)$ for *lhβ*, $Pr(\beta \geq 0)$ for *fshβ*, and $Pr(\beta \geq 0)$ for the ratio of *fshβ*-change/*lhβ*-change. As point estimates we report the mode of the posterior probability distribution (maximum a posteriori; MAP), i.e., the parameter value with the highest posterior probability. In addition to the PIs, we give prediction intervals, which indicate the probability range of where to expect the next randomly drawn value of a population, i.e., prediction intervals consider the uncertainty in the parameter estimates and the stochastic nature of the response variable (σ). The results are reported in Table 2.4.

Equation 2.1 General Bayesian model definition for linear regression. *y* are the data of each response variable.

$$Pr\ (\alpha, \beta, \sigma | y) = \frac{\pi_i\, N(y_i | \alpha + \beta x_i, \sigma) f\alpha(\alpha) f\beta(\beta) f\sigma(\sigma)}{\iiint \pi i\, N(y_i | \alpha + \beta x_i, \sigma) f\alpha(\alpha) f\beta(\beta) f\sigma(\sigma) da d\beta d\sigma} \quad (2.1)$$

Due to the small sample size, we preferred to keep the model simple and the associated parameters at minimum. Quadratic approximation was used to estimate the posterior probability distribution and marginal posterior distributions of the parameters. Posterior distributions were sampled 100,000 times for summary statistics. For modelling and sampling of posterior distributions we used the

rethinking package (McElreath, 2020a). For each dependent variable x (*fshβ*, *lhβ*, or *fshβ*-change/*lhβ*-change), the model assumes $x \sim \text{Normal}(\mu_i, \sigma)$ with $\mu_i = \alpha + \beta_{sky}\, treat_i$, where α is the mean in controls as $\alpha \sim \text{Normal}$ (overall mean of x, 1) and β is the difference between skyglow treated and control animals with $\beta_{sky} \sim \text{Normal}(0, 1.5)$ and a constant residual variance of $\sigma \sim \text{Uniform}(0, 1)$. Relatively uniformed priors were chosen for every model. The priors for α assume that the most likely mean level of *fshβ*, *lhβ*, or the ratio of *fshβ*-change/*lhβ*-change in the control group is at the overall mean of the respective data, with 95% of probability between the respective $\mu_x \pm 2$. The prior for the group difference β is centered around zero, assuming no difference between groups as the most likely parameter value with 95% of probability between 0 ± 3, i.e., greatly exceeding the range between maximum and minimum values in the data of each parameter. The σ prior was also chosen very conservative, i.e., uninformative, and constrains σ to have a positive probability between zero and two, and thus exceeds the standard deviation of each data set as well as the range between minimum and maximum value in these data.

Histological data are presented as percentage of all re-captured animals from control of skyglow-treated enclosures for each vitellogenic category. Statistical analysis was not possible due to differences in the number of re-captured animals between control or skyglow treatment.

Author contributions

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• Ulrike Scharfenberger: data analysis, interpretation of results, manuscript writing
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• Franz Hölker: conceptualization, data acquisition, interpretation of results, manuscript writing

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Chapter 3

Effects of artificial light at night on thyroid hormones of Eurasian perch

Misbalance of thyroid hormones after two weeks of exposure to artificial light at night in Eurasian perch *Perca fluviatilis*

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Abstract
Artificial light at night (ALAN) can affect the physiology and behavior of animals because it alters the natural rhythm of light and darkness. Thyroid hormones (TH) are partially regulated by the light information of photoperiod and are involved in metabolic adjustments to daily and seasonal changes in the environment, such as larval and juvenile development, somatic growth and reproduction. ALAN can change photoperiodic information and might thereby lead to changes in thyroid metabolism, but so far research on this topic is scarce. Therefore, we tested in two different experiments the effects of nocturnal illumination at a wide range of light intensities on TH in plasma of Eurasian perch (*Perca fluviatilis*). Total 3,3,5-triiodo-L-thyronine (T3) was significantly affected by ALAN and reduced at the highest tested intensity of 100 lx after only two weeks of exposure. Although total L-thyroxine (T4) was not significantly affected, the ratio of T3 to T4 tended to slightly decrease at 100 lx. In a second low light experiment ALAN did not have clear effects on T3, T4 or the ratio of T3 to T4 at intensities between 0.01 lx and 1 lx. The results show first signs of endocrine disruption in thyroid metabolism after a relatively short ALAN exposure of two weeks under high-intensity streetlight conditions. Misbalanced thyroidal status can have serious implications for metabolic rates as well as developmental and reproductive processes.

Keywords: Endocrine disruption, fish, freshwater, light pollution, thyroxine, triiodothyronine

3.1 Introduction

Artificial light at night (ALAN) is an unprecedentedly increasing disturbance of natural nocturnal darkness (Gaston *et al.*, 2015b, Hölker *et al.*, 2010b). It mostly derives from centers of human activities, which are typically located in the vicinity of freshwater systems (Kummu *et al.*, 2011). Therefore, freshwater fish frequently experience nocturnal illumination, which can disturb the precise timing and optimization of their biological rhythms, for example daily and seasonal rhythms of hormones, such as melatonin (Brüning *et al.*, 2018a, Brüning *et al.*, 2015, Grubisic *et al.*, 2019, Kupprat *et al.*, 2020) or reproductive hormones (Brüning *et al.*, 2016, Brüning *et al.*, 2018b), but also food consumption or migratory behavior (Bergman, 1988, Riley *et al.*, 2012, Riley *et al.*, 2013). Light intensities of ALAN on the water surface of urban and suburban lakes or rivers normally range from 0.007 to 0.55 lx (indirect illumination by skyglow) up to 10 to 100 lx (direct exposure to a strong light source) (Hänel *et al.*, 2018, Hölker *et al.*, 2018). Hence, fish populations in such areas likely experience ALAN intensities < 1 lx close to the water surface and can occasionally be exposed to even higher intensities ranging from 1 lx up to 100 lx (e.g., swimming directly beneath a strong light source). Exposure of fish depends on turbidity and swimming depth, but specific exposure scenarios have not been quantified, yet. For comparison, daylight reaches a maximum of about 120,000 lx and ca. 800 lx at sunset (Brown, 1952, Gaston *et al.*, 2014, Grubisic *et al.*, 2019).

There are several reports of circadian or ultradian rhythms of thyroid hormones (TH) in blood plasma of teleost fish, for example in rainbow trout (*Oncorhynchus mykiss*) (Boujard and Leatherland, 1992, Cook and Eales, 1987, Gomez *et al.*, 1997, Laidley and Leatherland, 1988, Osborn *et al.*, 1978, Reddy and Leatherland, 2003), red drum (*Sciaenops ocellatus*) (Leiner and MacKenzie, 2001) or zebrafish (*Danio rerio*) (Jung *et al.*, 2016b) and some studies report lunar rhythms of TH in smolting coho salmon (*Oncorhynchus kisutch*) (Farbridge and Leatherland, 1987, Grau *et al.*, 1981). Plasma TH further exhibit seasonal rhythms, which are positively correlated to sexual steroids during the annual reproductive cycle in many seasonally spawning teleosts (Cyr and Eales, 1996). Furthermore, seasonal profiles of plasma TH are available for a number of other teleost species (Bau and Parent, 2000, Dickhoff *et al.*, 1982, Eales and Fletcher, 1982, Osborn and Simpson, 1978, Özeren *et al.*, 2019), but no general pattern can be drawn from the available data. In Eurasian perch (*Perca fluviatilis*), for example, seasonal profiles of plasma TH revealed highest concentrations in summer and low levels during winter and spring and pikeperch (*Sander lucioperca*) had highest concentrations in late spring and fall and lowest concentrations in summer (Bau and Parent, 2000). Although a clear mechanistic understanding is still needed, light perception of photoperiod or moon phases has been proposed as one of the regulatory mechanisms for daily, lunar and seasonal TH rhythms (Grau, 1988, Laidley and Leatherland, 1988, Leatherland, 1994). Therefore, ALAN is likely to disturb these rhythms. Melatonin, which has a well described direct neuroendocrine response to light and ALAN in teleosts (Grubisic *et al.*, 2019), might be an important regulatory component for rhythms of thyroid hormones; however, we know very little on this relationship for fish. In rodents, melatonin reduces thyroid function (Baschieri *et al.*, 1963, Vriend *et al.*, 1979, Wittkowski *et al.*, 1988) similar to amphibians in which

melatonin is considered to be an antagonist of thyroid function (Wright, 2002). For rodents and birds, alterations in thyroid metabolism during long-day photoperiods, associated to low melatonin production, have been suggested as long-term timers in reproductive processes (Dardente *et al.*, 2014, Ouyang *et al.*, 2018, Wood and Loudon, 2014, Yoshimura, 2010). For example, in Siberian hamsters (*Phodopus sungorus*) short days reduced the gene expression of thyroid-stimulating hormone (TSH) receptor, which is associated with reduced gonadal development. Dim light at night (5 lx), however, increased expression of TSH receptors in short-day scenarios along with increased expression of reproductive hormones (Ikeno *et al.*, 2014).

In fish, TH are mainly known for regulation of developmental (differentiation and growth) and reproductive processes (Blanton and Specker, 2007, Campinho, 2019, Carr and Patiño, 2011, Power *et al.*, 2001). The role of TH in fish is best studied in the smoltification of Atlantic salmon (*Salmo salar*) and in the metamorphosis of flatfishes, in which thyroid disruption can inhibit or delay the onset and rate of metamorphosis and lead to serious malformations, for example in olive flounder (*Paralichthys olivaceus*) (Miwa and Inui, 1987). Research on TH metabolism under ALAN exposure is hence urgently needed to estimate the potential threat that animals in light polluted areas might be exposed to.

Structurally, TH are iodothyronines of which the biologically active forms are 3,3',5-triiodo-L-thyronine (T3) and L-thyroxine (T4), the latter one being less biologically active. TH induce specific response mechanisms in various organs by binding to thyroid receptors and directly regulating gene expression in target cells. Thereby, especially T3 homeostasis in target cells is important for normal metabolic function (Brown *et al.*, 2004). However, T3 is not only directly produced by thyroid follicles because they mainly synthesize and secrete T4 rather than T3 upon stimulation by TSH. TSH is produced by the hypophyseal *pars distalis* representing the classical endocrine regulated hypothalamus-pituitary-thyroid-axis (Kloas *et al.*, 2009) but also by the photoreceptive *saccus vasculosus* in the hypothalamus of fish (Nakane *et al.*, 2013, Nakane and Yoshimura, 2014, O'Brien *et al.*, 2012). T4 is mostly catalyzed to T3 by deiodination in the outer ring of the T4 molecule and is often considered as a prohormone for T3.

Deiodination can be catalyzed by different isoforms of iodothyronine deiodinases (DIO). Type 1 deiodinase (DIO1) is thought to be rather unspecific as it catalyzes both outer-(ORD) and inner ring deiodination (IRD) with low activity levels (Orozco and Valverde-R, 2005). Type 2 deiodinase (DIO2) mainly catalyzes ORD and is thereby an "activating enzyme" as it catalyzes ORD from the biologically less active T4 into the more active T3. Type 3 deiodinase (DIO3) instead is an inactivating enzyme by catalyzing IRD from the less active T4 to the inactive 3,3',5'-trioiodo-L-thyronine (reverse T3) or from the active T3 to the inactive 3,3'-diiodothyronine (T2).

Overall, thyroid metabolism is likely to be regulated directly by endocrine as well as by daily and seasonal light information and such photic regulation might also be linked indirectly to melatonin. Exposure to ALAN can lead to modifications of both, light information and melatonin levels, but little is known about the impacts of ALAN on TH of fish. Therefore, we ran two different experiments in which *P. fluviatilis*

were exposed to a wide range of ALAN intensities. In the first experiment fish were exposed to higher intensities of 1 lx, 10 lx and 100 lx and in the second one to lower intensities of 0.01 lx, 0.1 lx and 1 lx. Total T3 and total T4 were measured in plasma after two weeks in both experiments.

3.2. Material and Methods

3.2.1. Ethical statement

The care and use of experimental animals complied with German animal welfare laws, guidelines and policies as approved by the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16).

3.2.2. Experimental fish

Eurasian perch (*P. fluviatilis*) from Lake Müggelsee (Berlin, Germany) were kept in 600-L indoor tanks with natural photoperiod (sunlight through windows and dark nights) prior to acclimation in the experimental setup (pre-acclimation, see below for details). According to the "new world atlas of artificial night sky brightness" the surface of Lake Müggelsee experiences an illumination of ca. 0.003 lx in moonless clear nights (Falchi *et al.*, 2016), which lies in the lower range of suburban skyglow (Hänel *et al.*, 2018).

3.2.3. Experimental setup

Individuals of *P. fluviatilis* (pubertal to young adults) were exposed to ALAN treatments in 80-L aquaria (length, 80 cm; width, 35 cm; height, 40 cm), which were covered with black foil to ensure independent light treatments. The lids of all aquaria were equipped with three fluorescent tubes to realize daylight intensities that reached up to 7000 lx at the water surface and were around 2900 lx averaged over 25 equally distributed points on the water surface. An additional fluorescent tube was installed for night-time illumination. Control levels were below detection limit of our luxmeter (ILT1700, Peabody, MA, USA), i.e., < 0.00167 lx and are referred to as "0 lx" in the following. Photoperiod was controlled by an automatic time switch system (Hager, Blieskastel, Germany). The experimental setup has been described before by Franke *et al.* (2013), providing quantitative and qualitative comparison between the experimental and natural conditions as well as details on the spectral composition of the light source (Biolux fluorescent tubes, Osram, Germany), which can be used to convert lux values into other illumination units (1 lx ≈ 3.7 mW m^{-2}). It covers a large part of the spectral sensitivity of *P. fluviatilis* although their spectral sensitivity, compared to humans, is slightly more red-shifted (Cameron, 1982). Light intensity was adjusted by partial cover of the light source or using neutral density filter foil (Lee Colour Filter 299 1.2 N.D.) to maintain the spectral composition of the light. Since these methods do not change the spectral composition of the light source, lux can be used as the unit of illuminance for comparison across different light intensities.

3.2.4. Exposure to high intensities of ALAN ("high ALAN experiment")

Fertilized egg ribbons of *P. fluviatilis* were collected from Lake Müggelsee (Berlin, Germany) in March 2015 to raise parasite-free fish as described by Vivas Muñoz *et al.* (2019). Pre-acclimation in 600-L indoor tanks lasted two weeks. The experiment was run twice in December 2016 and in January 2017, with each treatment in duplicate during the first run and in triplicate during the second run (i.e., n = 5 for each treatment). The experimental setup consisted of 12 identical 80-L aquaria with a tap water flow-through of ca. 10 L h^{-1} and water temperature of ca. 16°C. Each aquarium was stocked with six fish which were allowed to acclimate for two weeks without illumination during the night (0 lx) followed by two weeks of experimental conditions with the respective nocturnal light intensity or controls without illumination according to Brüning *et al.* (2015). The fish weighed 69.0 ± 18.4 g with a standard length of 15.3 ± 1.3 cm (mean ± standard deviation, n = 120). Fish were fed with commercially available food (Aller Silver, 3 mm, Emsland-Aller, Golßen, Germany) twice a day at a rate of 0.5% of their body mass until feeding stopped 24 h prior to sampling to minimize effects of feeding. Full daylight was realized from 09:00 to 15:00 with a simulated dawn or dusk period by dimming over 3 h each starting at 06:00 or 15:00, respectively. This mimics December conditions of the natural photoperiod in Lake Müggelsee (52° 26' N, 13° 39' E). Nocturnal illumination of 1 lx, 10 lx or 100 lx at the water surface was from 18:00 till 06:00.

3.2.5. Exposure to low intensities of ALAN ("low ALAN experiment")

Perca fluviatilis from Lake Müggelsee were caught by electro fishing and fish traps between July and September 2017 and fed twice a day with frozen blood worms. Preacclimation lasted 2 to 9 weeks. Thirty fish with an individual mass of 16.8 ± 4.1 g and standard length of 10.6 ± 0.9 cm (mean ± standard deviation, n = 720) were transferred to each 80-L aquarium. Fish were fed twice a day during acclimation (two weeks) and the water flow-through was adjusted to 10 L h^{-1}. The temperature during acclimation and experimental exposure to ALAN was ca. 16°C. Photoperiod was adjusted to October conditions with full daylight from 09:30 to 18:30 with a 3 h dawn or dusk period starting at 06:30 or 18:30, respectively. After acclimation, nocturnal illumination of 0.01 lx, 0.1 lx or 1 lx average intensity on the water surface was switched on from 21:30 until 06:30; controls were not illuminated during night (0 lx). During the two weeks of experimental illumination the water flow-through was reduced to 4 L h^{-1} in order to allow water-based melatonin measurements, which are described by Kupprat *et al.* (2020). To maintain good water quality, animals were not fed during the low flow-through in the two weeks of experimental illumination. The same experiment was performed twice – in October and November 2017 – with each treatment in triplicates for both runs (i.e., n = 6 for each treatment).

3.2.6. Sampling

Fish were randomly sampled on two consecutive mornings between 09:00 and 12:00 in the high ALAN experiment and in two consecutive nights between 22:00

and 04:00 in the low ALAN experiment. In the high ALAN experiment, all fish were sampled and blood sampling of one aquarium was completed within 15 min. In the low ALAN experiment, body mass and length were measured of all fish, blood was only taken from the first 15 fish and blood sampling of one aquarium was completed within 35 min. Five to six plasma samples from each aquarium, i.e., n = 35 – 36 for each experimental group, were analyzed. Males (m) and females (f) were distinguished by visual inspection of the gonads. In premature fish (nd) gonads could not be differentiated. In the low ALAN experiment sex was not determined for all fish and thus this information is not available for three blood samples (na). Blood (500 – 1000 µL) was taken from the caudal vein with heparinized syringes and transferred to a clean tube containing 1 – 2 mg Na2EDTA (EDTA). Blood and EDTA were mixed by shaking and centrifuged at 7500 × *g* for 5 min at 4°C. The plasma was transferred to a new tube and immediately frozen in liquid nitrogen and then stored at −80°C until analyzed.

3.2.7. Extraction

Extraction was performed at room temperature. Blood plasma was thawed on ice and after vortexing, 70 µL of plasma were mixed with 17.5 µL protection solution containing dithiothreitol, ascorbic acid, citric acid, each at 25 g L^{-1}, following previous protocols by Noyes *et al.* (2014) and Wang and Stapleton (2010). Then 140 µL of acetonitrile were added to the tube and vortexed for 1 min. 420 µL of ethyl acetate were added to the mixture followed by vortexing again for 1 min. After centrifugation at 4500 × *g* for 10 min, the upper phase was transferred to a new tube and the extracts were dried under vacuum at 45°C (Concentrator plus, Eppendorf, Hamburg, Germany). The ethyl acetate extraction was repeated once in the first tube and the upper phase was added to the first extract and further dried. The dried extracts were resolved in 70 µL methanol and shaken for 1 h on a 3D shaker (Polymax 1040, Heidolph Instruments, Schwabach Germany). Samples were centrifuged for 1 min at 10000 × *g* and 60 µL were transferred to an insert (250 µL) in an HPLC vial and stored at −20°C until further use.

3.2.8. LC–MS/MS measurement

We measured total T3 and total T4 from extracted plasma in a triple quadrupole tandem mass spectrometer (LC–MS/MS; 1290 Infinity II UHPLC, 6470 Triple Quadrupole Jetstream, Agilent, Santa Clara, CA, USA) with a C18 separation column (ZORBAX Eclipse XDB C18, Agilent). The injection volume was 2 µL and the flow rate was 0.4 mL min^{-1}. For LC separation, 0.1% formic acid was used as the aqueous mobile phase (A) and acetonitrile with 0.1% formic acid was used as the organic mobile phase (B). The gradient was realized as follows: 0 min 90% A, 4 min 40% A, 5 min 40% A, 7 min 90% A, 8 min 90% A, 9 min 20% A, 12 min 20% A, 13 min 90% A, 15 min 90% A. MS/MS responses of target analytes were evaluated by electrospray ionization (ESI) in positive ion mode using multiple reaction monitoring (MRM) transitions. The transitions of T3 were at 651.8 – 605.8 or 651.8 – 353.0 with collision energy of 19 eV or 40 eV for quantifier ions or qualifier ions, respectively, and fragmentor voltage of 158 V. The T4 transitions were at 777.7 – 731.7 or 777.7 – 633.7 with collision energy of 23 eV or 35 eV, respectively and fragmentor voltage of 165 V. T3 was quantified at the lower and T4 at the higher transition.

A calibration curve between 0.5 and 25 ng mL^{-1} was prepared in methanol. The limit of quantification (LOQ) was 0.5 ng mL^{-1} for both, T3 and T4. However, after measuring the high ALAN samples there was a power outage and afterwards for most of the low ALAN samples the LOQ of T4 was 1 ng mL^{-1}.

3.2.9. Validation of methodology

Prior to measuring the samples, we did a small spike and recovery experiment of three randomly chosen plasma samples (one high ALAN and two low ALAN samples) and three pools of plasma from *P. fluviatilis* of the same stock as the experimental animals of the low ALAN experiment. Subsets of each of the three plasma pools were spiked before extraction with either a high (20 ng mL^{-1}) or low (5 ng mL^{-1}) end concentration of synthetic T3 or T4 in the spiked plasma samples. Recovery of post-extraction spikes is expressed as the percentage of the T3 or T4 from the non-spiked plasma samples subtracted from the T3 or T4 in the spiked plasma sample relative to the theoretical amount of spiked T3 or T4. Extraction efficiency was calculated likewise from pre-extraction spikes. Extraction efficiency determined by pre-extraction spikes was 68.0 ± 5.1% for T3 and 68.2 ± 3.0% for T4. Recovery from post-extraction spikes was increased by 5.6 ± 4.1% for T3 and 22.3 ± 7.0% for T4. Variation in recovery in post-extraction spikes was larger in T4 than in T3. We added antioxidants to the extraction solution, but enzymatic and non-enzymatic conversion from T4 to T3 cannot be fully excluded.

Solvents were obtained in ultrapure LC–MS grade from Carl Roth GmbH (Karlsruhe, Germany). All other chemicals were mainly obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

3.2.10. Data handling

Measurements were evaluated with Agilent MassHunter Workstation software (Version B.09.00).

In the high ALAN experiment, two T4 measurements were below the LOQ of 0.5 ng mL^{-1}. In the low ALAN experiment, two samples were below 0.5 ng mL^{-1} T3. Those measurements were excluded from the data analysis. For T4, 27% of the low ALAN samples were below the LOQ and as these missing values were homogenously distributed over all treatment groups (0 lx, 25.7%; 0.01 lx: 30.6%; 0.1 lx: 22.2%; 1 lx: 34.3%), we decided to analyze available cases only. In the respective graph (Figure 3.2, middle panel) a dotted line depicts an alternative median, which takes into account the samples below LOQ. For this, all non-quantified samples were attributed a fixed constant of the respective LOQ (0.5 or 1 ng mL^{-1}) divided by two.

3.2.11. Statistical analysis

Data were log-transformed to meet the assumptions of statistical testing, i.e., normal distribution and homogeneity of residual variance. Each parameter (log(T3), log(T4) and log(ratio T3/T4) for high and low ALAN, respectively) was individually modeled in a linear mixed model (LMM) with treatment, body mass and sex as fixed effects and “aquarium nested in run” as random effects. Models were selected by dropping one factor and comparing the two models. Models were

selected according to lowest AIC and based on the LLRs and *p*-values of the analysis of variance (ANOVA) comparing two models (significance at $p < 0.05$) (Zuur *et al.*, 2009). If addition of treatment and body mass or sex did not explain significantly less variance than an interaction, additive fixed effects were modeled. If removal of body mass or sex did not significantly worsen the model, the factor was not included in the model. Treatment was kept as fixed effect in every model as it was the hypothesized effect we aimed to test for. Random effects were also kept in all LMMs to account for the data structure. In case of significant treatment, body mass or sex effects, post-hoc tests using Tukey's correction compared every treatment or sex to every other within one experiment (Lenth, 2019). Treatment, sex, body mass and random effects as well as marginal and conditional R^2 values (Barton, 2018) for each LMM are specified in Table 3.1. Full model specifications for each parameter as well as full results of the post-hoc tests are given in the supplementary material.

3.2.12. Note on the differences between the two experiments

Since several experimental variables (size, life history, feeding regimes, sampling time of day, seasonal timing of experiments, water flow-through) differed across the high and the low ALAN experiment, it is important to compare treatments only with the respective control and avoid comparisons across experiments. Originally, we planned the high ALAN experiment with a wild population from Lake Müggelsee like in the low ALAN experiment and in earlier studies (Brüning *et al.*, 2016, Brüning *et al.*, 2015, Brüning *et al.*, 2018b). Yet, in fall 2016 we did not catch enough wild *P. fluviatilis* for the experiment and worked with lab-raised fish instead, which were pre-conditioned to dry feed. In contrast, the wild *P. fluviatilis* were fed with frozen blood worms because they could not be conditioned to dry feed. Experimental fish were not fed during the low ALAN experiment to maintain water quality under the low water flow-through which was necessary to measure melatonin from the tank water (Kupprat *et al.*, 2020). It was not necessary to reduce the water flow-through and starve the experimental fish in the high ALAN experiment because changes of nocturnal melatonin were known from a previous study (Brüning *et al.*, 2015). In the high ALAN experiment, samples were taken in the mornings after fish were exposed to ALAN for the entire night. However, as there was only one effect at 100 lx in the high ALAN experiment, samples were taken at night at direct application of nocturnal illumination in the low ALAN experiment.

3.3. Results

3.3.1. ALAN effects

In the high ALAN experiment, mean T3 was significantly lowered by 28% at 100 lx illumination as compared to the dark control treatment (LMM ALAN effect: LLR = 11.94, $p = 0.008$; Tukey's post-hoc: 0 lx vs. 100 lx: $p = 0.01$, Figure 3.1). Differences of 1 lx or 10 lx compared to 0 lx were not significant (Tukey's post-hoc: 0 lx vs. 1 lx: $p = 0.69$, 0 lx vs. 10 lx: $p = 0.80$), neither were differences between 100 lx and 1 lx or 10 lx (Tukey's post-hoc: 1 lx vs. 100 lx: $p = 0.08$, 10 lx vs. 100 lx: $p = 0.06$). Mean concentrations of T4 slightly increased by 21% at 100 lx and the mean ratio of T3/T4 decreased by 41% at 100 lx compared to controls without

ALAN, although both without statistically significant treatment effects (LMM ALAN effects: T4 – LLR = 1.96, p = 0.58; T3/T4 – LLR = 6.38, p = 0.09, Table 3.1).

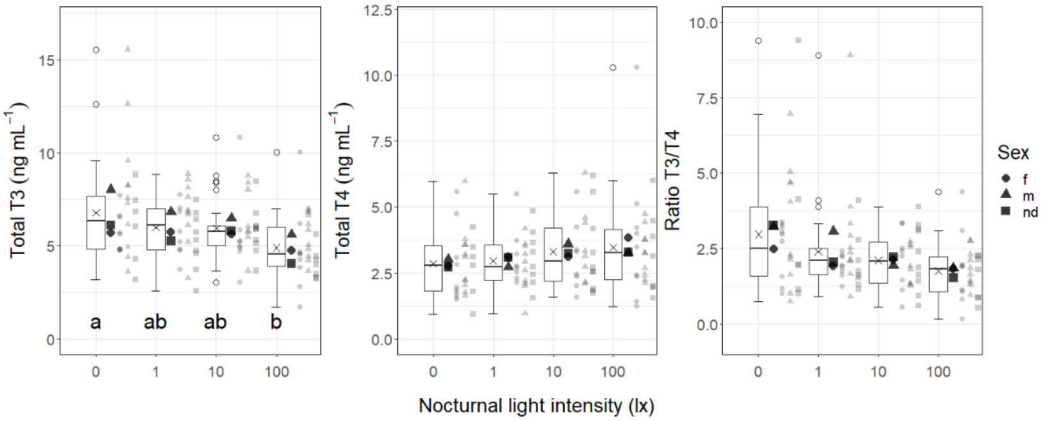

Figure 3.1 Total triiodothyronine (T3) and thyroxine (T4) in blood plasma of *P. fluviatilis* under different light pollution scenarios ("high ALAN experiment"). Samples were all taken throughout the morning (09:00–12:00). Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (T3: n = 26 for 0 lx and 10 lx, n = 27 for 1 lx and 100 lx; T4 and ratio T3/T4: n = 26 for all treatments). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5x interquartile ranges (IQR); outliers > 1.5x IQR are indicated by circles. Different shapes of big points indicate the mean of each sex (m—males, f—females, nd—not differentiated (premature fish)), whereas small points represent individuals (T3 (T4 and ratio T3/T4): n = 34 (34) for f, n = 40 (39) for m, n = 32 (31) for nd). Different letters indicate significant differences between treatments (Tukey's post-hoc, p < 0.05).

In the low ALAN experiment, no ALAN effects were detected for T3 (LMM ALAN effects: LLR = 1.05, p = 0.79, Table 3.1, Figure 3.2). An interaction of ALAN and body mass significantly explained variation of T4 and T3/T4 (LMM ALAN∗Body mass effects: T4 – LLR = 14.70, p = 0.002; T3/T4 – LLR = 9.333, p = 0.03). T4 decreased with increasing body mass at 0 lx and 1 lx but increased with increasing body mass at 0.01 lx and 0.1 lx. Overall, T3/T4 overall increased with increasing body mass, with the weakest increase at 0.1 lx and the steepest increase at 1 lx and a slight decrease at 0.01 lx (for details, see supplementary material).

3.3.2. Body mass effects

In the high ALAN experiment, body mass significantly explained variance of T3 (LMM body mass effect: LLR = 12.47, p = 0.0004) but the slope was very flat. Body mass was not kept as fixed effect in the model selection of the LMMs of T4 or T3/T4 in the high ALAN experiment as it did not significantly explain more variance.

In the low ALAN experiment, body mass also significantly explained variance of T3 (LMM body mass effect: LLR = 22.07, p < 0.0001) and T4 and T3/T4 had the above-described interaction with ALAN effects.

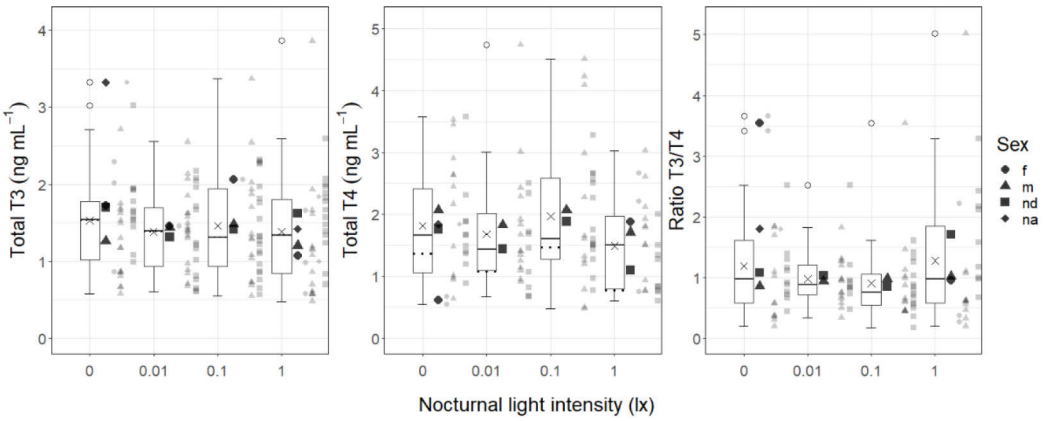

Figure 3.2 Total triiodothyronine (T3) and thyroxine (T4) in blood plasma of *P. fluviatilis* under different light pollution scenarios ("low ALAN experiment"). Samples were all taken throughout the night (22:00 – 04:00). Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (T3: n = 34 for 0 lx, n = 36 for 0.01 lx and 0.1 lx, n = 35 for 1 lx; T4 and ratio T3/T4: n = 26 for 0 lx and 0.1 lx, n = 25 for 0.01 lx, n = 23 for 1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5x interquartile ranges (IQR); outliers > 1.5x IQR are indicated by circles. Different shapes of big points indicate the mean of each sex (m—males, f—females, nd—not differentiated (premature fish), na—not available (sex not determined)), whereas small points represent individuals (T3 (T4 and ratio T3/T4): n = 8 (5) for f, n = 66 (52) for m, n = 63 (44) for nd, n = 3 (1) for na). There were no significant differences across treatments in any parameter. Dotted horizontal lines in the middle panel (total T4) display medians which take into account measurements below the limit of quantification (n = 35 for 0 lx and 1 lx, n = 36 for 0.01 lx and 0.1 lx).

Table 3.1
Treatment effects, sex effects, body mass effects and random effects and the marginal and conditional R^2 values for linear mixed models of log-transformed total triiodothyronine (log(T3)), total thyroxine (log(T4)) and the ratio of T3/T4 (log(T3/T4) in blood plasma of *P. fluviatilis* exposed to different intensities of ALAN in two different experiments ("high ALAN experiment" or "low ALAN experiment"). An asterisk marks an interaction of two fixed factors

Parameter	Fixed variable	Fixed effects		Random effects		Goodness of fit	
		LLR†	*p*-value	LLR	*p*-value	$R^2_{marginal}$	$R^2_{conditional}$
High ALAN experiment							
Log(T3)	ALAN	11.94	0.008	2.08	0.35	0.2894	0.3548
	Sex	24.34	<0.0001				
	Body mass	12.47	0.0004				
Log(T4)	ALAN	1.96	0.58	2.83	0.24	0.0246	0.1648
Log(T3/T4)	ALAN	6.58	0.09	2.16	0.34	0.0845	0.1957
Low ALAN experiment							
Log(T3)	ALAN	1.05	0.79	15.52	0.0004	0.1546	0.4138
	Body mass	22.07	<0.0001				
Log(T4)	ALAN * Body mass	14.70	0.002	0.10	0.95	0.1633	0.1873
Log(T3/T4)	ALAN * Body mass	9.33	0.03	5.19	0.07	0.1306	0.3217

† Log-likelihood ratio

3.3.3. Sex effects

In the high ALAN experiment, the sex ratios of the experimental fish for which TH were determined were 32%:38%:30% (females (f):males (m):not differentiated (nd)) and in the low ALAN experiment there were only few females resulting in sex ratios of 6%:47%:45%:2% (f:m:nd:na (not available)) for T3 or 5%:51%:43%:1% for T4, respectively (detailed number for each sex listed per treatment in the supplementary material). Interactions between sex and ALAN did not improve the models and were, thus, not included to keep the model as simple as possible. Sex effects were only significantly improving the model for T3 of the high ALAN experiment (LMM sex effect: LLR = 24.34, $p < 0.0001$) where males had significantly higher levels of T3 than females or not differentiated fish (Tukey's post-hoc: f vs. m: $p = 0.005$; nd vs. m: $p = 0.006$).

3.3.4. Random effects

Random effects did not significantly explain TH variance in the high ALAN experiment or variances of T4 or T3/T4 in the low ALAN experiment. For T3 in the low ALAN experiment, random effects significantly explained variance (LMM random effects: LLR = 15.52, $p = 0.0004$). Ca. 31% of the unexplained variance (the variance that was not explained by the fixed effects) was explained by variance across the two runs (October and November); repeatability for aquarium effects was negligibly low.

3.4. Discussion

Exposure to a high intensity of nocturnal illumination (100 lx) caused a significant reduction of T3 in the blood plasma of *P. fluviatilis* as compared to controls without illumination after two weeks. For T4 there was no significant effect of the light treatments, but a trend for a decrease of the T3/T4 ratio could be observed. In a second experiment with lower intensities of ALAN there were no clear effects on TH.

3.4.1. ALAN effect

The significant decrease in T3 at 100 lx with stable T4 levels could be explained by differences in rates of TH synthesis, secretion, conversion and excretion. One mechanistic explanation could be a reduced extrathyroidal conversion of T4 into T3 by ORD. The DIO2/DIO3 ratio could be affected or just one of the deiodinases. The trend of decreasing T3/T4-ratio further implicates alterations in deiodination activities. In contrast, in rodents short photoperiods and melatonin rather down-regulate *dio2* expression and long-photoperiods lead to high expression of *dio2* (Dardente *et al.*, 2014). Additionally, long photoperiods correlate with increased levels of TSH and T3 in birds and mammals (Nakao *et al.*, 2008, Yoshimura, 2010). Accordingly, DIO2 activity and T3 should increase under ALAN (Ouyang *et al.*, 2018), but our results rather suggest the opposite, indicating mechanistic differences between mammals or birds and teleost fish in the light-dependent regulation of thyroid metabolism. Overall, a TH-antagonistic role of melatonin, like it is described for rodents and amphibians, cannot be confirmed for *P. fluviatilis* as melatonin was reduced by ALAN (Brüning *et al.*, 2015, Kupprat *et al.*, 2020) and TH were reduced or unchanged and did not increase. The observed changes in

plasma TH are probably not a result of changed thyroid stimulation by TSH because this would likely result in an overall decrease or increase in both, T3 and T4. In future experiments, a full profile of thyroid endpoints, including plasma and peripheral tissue levels of all TH and activities of deiodinases as well as gene expression of *tsh* or deiodinases, may help to identify processes leading to the observed change.

It is interesting that total T3 is affected by ALAN already after two weeks, but the effect was significant only at 100 lx, which is at the upper end of realistic light pollution scenarios (Grubisic *et al.*, 2019, Hänel *et al.*, 2018) and *P. fluviatilis* will most likely only occasionally experience these intensities. It is in principle possible that ALAN effects are accumulating over time, i.e., an effect like the one at 100 lx could be detected at lower and more typical ALAN intensities after longer exposure (e.g., several months). Moreover, shorter exposure times to ALAN of only a few hours or days could be interesting to test for a time-specific dose–response relationship.

In the low ALAN experiment, ALAN effects were interacting with the effect of body mass on T4 and T3/T4, but there was no clear pattern along the gradient of ALAN intensities. Thus, the current data do not seem to allow a clear interpretation of the relationship between TH, body mass and ALAN. However, our data indicate that consideration of body mass can improve TH analyses.

Seasonal effects of ALAN on TH may be considered in future studies to identify if TH dynamics of *P. fluviatilis* can be also characterized by a photo-labile period, where fish are particularly susceptible to additional light at night (Falcón *et al.*, 2010), as suggested for suppression of sexual hormones in *P. fluviatilis* (Brüning *et al.*, 2016, Brüning *et al.*, 2018b). In *S. salar*, experiments on the timing of photoperiod manipulation of TH have identified a photo-labile period (McCormick *et al.*, 1987). Thus, ALAN may have particularly strong effects on TH of *P. fluviatilis* during a sensitive time window.

For a critical interpretation of our data, possible daily rhythms of plasma TH in *P. fluviatilis* need to be considered. Although to our knowledge no data on daily TH rhythms in *P. fluviatilis* is available, circadian rhythms were reported for *O. mykiss* (Osborn *et al.*, 1978) or *D. rerio* (Jung *et al.*, 2016b), and also ultradian rhythms were reported for *O. mykiss* (Gomez *et al.*, 1997, Laidley and Leatherland, 1988). Potential phase shifts of such rhythms can affect experimental results (Leatherland, 1994), for example if absolute production of TH was not affected but only a shift in the production peaks occurred under ALAN exposure. In this context, it must be mentioned that the daily peaks of plasma T3 can occur at different times than the peaks of T4, e.g., reported in *O. mykiss* (Boujard and Leatherland, 1992, Cook and Eales, 1987, Reddy and Leatherland, 2003), and *S. ocellatus* (Leiner and MacKenzie, 2001). Furthermore, peaks of plasma T3 and T4 can depend on a combination of light information and feeding times (Boeuf and Le Bail, 1999, Cook and Eales, 1987) and TH levels were lowered in Arctic charr (*Salvelinus alpinus*) (Eales and Shostak, 1985) and *O. mykiss* (Cook and Eales, 1987) when fish were starved. Since fish were not fed in the low ALAN experiment (due to water-based measurements of melatonin (Kupprat *et al.*, 2020)), starvation may have kept TH levels low enough to diminish effects besides the low levels of ALAN

and long sampling times. In comparison, circadian rhythmicity of melatonin was still measured under starvation conditions for *P. fluviatilis* with lowered amplitudes at 0.01 lx and 0.1 lx (without phase shifts) and at 1 lx first indications of a phase shift were reported (Kupprat *et al.*, 2020). At 10 lx and 100 lx, rhythms were completely depleted (Brüning *et al.*, 2015). A strong regulatory role of melatonin on TH production in *P. fluviatilis* seems unlikely, because if melatonin was a main regulator of TH production, ALAN effects on TH would have to be expected at all tested intensities, especially > 1 lx at which not only the absolute amount of melatonin was affected, but also its circadian pattern.

3.4.2 Ecophysiological implications of misbalanced thyroid metabolism

The reduction of T3 at 100 lx can have critical effects on the metabolic rate, and developmental or reproductive processes of *P. fluviatilis*. Body mass was a significant fixed effect in the LMM analysis of T3, but there were no significant ALAN-related changes of body mass within the two weeks of the experiment. A reduction of T3 would result in reduced occupancy of thyroid receptors in target cells and this would eventually lead to a reduced gene expression of TH-regulated proteins. The identification and classification of these proteins remains subject to future research but if thyroid metabolism is impaired over longer times, developmental and growth processes may change drastically (Power *et al.*, 2001). ALAN-induced changes in thyroid metabolism could be particularly harmful for fish species that undergo a dramatic metamorphosis, in which TH play an important role, e.g., salmonids (Laudet, 2011, Lorgen *et al.*, 2015) or flatfishes (Inui and Miwa, 1985, Schreiber, 2006). In aquacultural production of *S. salar* and masu salmon (*Oncorhynchus masou*), photoperiod manipulations are used as a tool to induce smoltification, which is necessary to allow physiological adjustment for the transfer to seawater and optimize growth (Boeuf and Gaignon, 1989, McCormick and Saunders, 1990, McCormick *et al.*, 1987, Okumoto *et al.*, 1989). However, TH likely also regulate less spectacular larval-juvenile transformations and juvenile-adult developments of most other teleost species (Laudet, 2011). For instance, in *D. rerio*, hypothyroid conditions (comparable to reduced T3 at 100 lx in our experiments) in eggs caused altered pace of development, changes in pigmentation and malformations in lower jaws (Carr and Patiño, 2011, Mukhi and Patiño, 2007, Walpita *et al.*, 2009, Walpita *et al.*, 2007) and paired fin development in larvae is also TH-dependent (Brown, 1997). In amphibian metamorphosis, where TH are key in regulating tail shrinking, limb growing and changes in cranial structure, an impairment of the thyroid axis can have drastic consequences, for example in the rate of metamorphosis (Fort *et al.*, 2000, Kloas, 2002, Opitz *et al.*, 2005). In the American toad (*Anaxyrus americanus*), ALAN exposure of 3 – 15 lx reduced metamorphic duration and reduced post-metamorphic growth (Dananay and Benard, 2018), but a link to changes in TH (or melatonin) by ALAN has not been considered. Whether an ALAN-induced misbalance of TH would impair growth, larval-juvenile transformations, or reproductive processes in *P. fluviatilis*, and other fish is an interesting objective for future research.

Changes in the thyroid cascade are often used as a sensitive biomarker of exposure to chemical pollutants (Brown *et al.*, 2004). Comparable effects with

lower T3 and stable or increasing T4 in teleost plasma were observed after exposure to low pH (Brown *et al.*, 1989, Brown *et al.*, 1984, Brown *et al.*, 1990), polychlorinated biphenyls (Adams *et al.*, 2000, Leatherland and Sonstegard, 1978) chemical effluents (Carletta *et al.*, 2002, Zhou *et al.*, 2000), kerosene (Peter *et al.*, 2007) as well as cortisol (Brown *et al.*, 1991) and estradiol (Cyr and Eales, 1990, Cyr *et al.*, 1988b, Leatherland, 1985). As these substances are known as endocrine disruptors, these similarities to our results underline the potential of ALAN to act as an endocrine disruptor. Currently, the public and political acknowledgement of light pollution as an environmental threat is limited and regulatory measures are, if at all, recommendations and not legally binding law (Kyba *et al.*, 2014, Schroer *et al.*, 2020). From the data presented here, TH in fish does not seem to be an exceptionally sensitive endpoint for light pollution, because an effect was only detectable at a relatively high level of illumination (100 lx). Pre-metamorphic amphibians might be more sensitive animal models to investigate effects of ALAN on TH (e.g., OECD, 2009). In fish, other hormonal endpoints appear to be more relevant with regard to ALAN, for example sex steroid blood concentration, gene expression of gonadotropins and especially nocturnal melatonin production, which are affected at low ALAN intensities within the range of typical skyglow illumination (< 2 lx) (Brüning *et al.*, 2016, Brüning *et al.*, 2018b, Grubisic *et al.*, 2019, Kupprat *et al.*, 2020). Thus, future regulatory light pollution measures need to consider the effects on the entire biodiversity in and along freshwater systems (Reid *et al.*, 2019) because light pollution has already been evidenced to affect other aquatic organisms such as microbes, algae, aquatic insects, amphibians and land-water interactions, with a potential for ecosystem-level changes through bottom-up and top-down processes (e.g., Grubisic *et al.*, 2017, Hölker *et al.*, 2015, Manfrin *et al.*, 2017, Touzot *et al.*, 2020).

3.4.3. Ratio of T3/T4

Besides the decreasing trend of the T3/T4-ratio with increasing ALAN intensity, we aimed to put the ratio levels of the control treatments into context. Our experiments revealed similar or higher levels of T3 compared to T4 in the plasma of *P. fluviatilis* and the ratio of T3/T4 lies roughly at 2.5:1 in the high ALAN experiment and in the low ALAN experiment at 1:1, although the real ratio in the low ALAN experiment is likely to be higher as ca. 27% of T4 (but not T3) measurements were below the limit of quantification. Another study, which measured plasma T3 and T4 from *P. fluviatilis* throughout one year *in situ*, reported mean ratios of 1:1 in winter up to 1:12 in early summer (Bau and Parent, 2000). Since there is no reference value available for the ratio of T3/T4 in teleost fish, we have reviewed relevant literature to better place our results in perspective. Most studies on fish TH report T3 and T4 values with a T3/T4 ratio of about 1:1 to 1:5 (Adams *et al.*, 2000, Bau and Parent, 2000, Boujard and Leatherland, 1992, Cook and Eales, 1987, Cyr *et al.*, 1988a, Eales and Fletcher, 1982, Eales and Shostak, 1985, Farbridge and Leatherland, 1987, Gomez *et al.*, 1997, Hoseini *et al.*, 2016, Laidley and Leatherland, 1988, McCormick and Saunders, 1990, McCormick *et al.*, 1987, Osborn and Simpson, 1978), but also extreme ratios from 16:1 (Cyr *et al.*, 1988a) to 1:10 (Bau and Parent, 2000, Jung *et al.*, 2016b, Zhao *et al.*, 2016) or up to 1:20 (Arkoosh *et al.*, 2017, Boujard and Leatherland, 1992, Chen *et al.*, 2017a) have been published. Still, higher T3 than T4 levels are not unusual and have been reported frequently

in teleost plasma (Bau and Parent, 2000, Cook and Eales, 1987, Cyr *et al.*, 1988a, Eales and Fletcher, 1982, Eales and Shostak, 1985, Leiner and MacKenzie, 2001, Osborn and Simpson, 1978, Osborn *et al.*, 1978, Reddy and Leatherland, 2003), as well as in fertilized eggs of teleost fish (Power *et al.*, 2001, Weber *et al.*, 1992). Overall, the ratio seems to depend on several factors, such as species, time of day or year, photoperiod, but also body mass and feeding regime. Hence, the high T3/T4 ratio in the high ALAN experiment is not unusual for teleost fish but is not in line with the measurements from an earlier study (Bau and Parent, 2000). The 1:1 ratio in the low ALAN experiment (in October conditions) matches the results of Bau and Parent (2000) und is also the most commonly reported T3/T4 ratio for teleost fish.

3.4.4. Sex effects

Males had significantly higher levels of T3 than females and non-differentiated fish in the high ALAN experiment, but T4 was not significantly affected by sex. These results might be related to suppression of T3 (but not T4) by estradiol in females, as reported for *O. mykiss* (Cyr and Eales, 1990, 1996, Cyr *et al.*, 1988b, Leatherland, 1985). In the low ALAN experiment, however, these sex effects were not confirmed, probably due to low number of females (5%).

3.5. Conclusion

Our results are among the first steps towards understanding the impacts of ALAN on thyroid metabolism in fish. High intensities of ALAN at 100 lx led to an increasing misbalance of thyroid metabolism in *P. fluviatilis* after only two weeks with decreased plasma T3 and stable plasma T4. However, ALAN intensities of 10 lx and 1 lx did not show a significant decrease. In a second experiment with even lower intensities, representing realistic skyglow exposure, there were no clear effects of ALAN on TH. Still, it is possible that a longer exposure to lower intensities causes a similar reduction of T3 as measured after two weeks at a high intensity. If the misbalance of thyroid metabolism as measured at 100 lx persisted in the long run, metabolic mismatches could lead to impaired developmental (larval-juvenile development) and reproductive processes. Teleost species undergoing a distinctive metamorphosis, such as flatfishes or salmonids could be particularly vulnerable to thyroid-related ALAN effects.

Conflict of interest
None declared.

Author contributions
Conceptualization: F.H., F.K., W.K.; Funding acquisition: F.H., W.K.; Experiments: F.K.; Lab analyses: A.K., C.S., F.K.; Provision of material and equipment: A.K., F.H., W.K.; Data analysis: F.K.; Initial draft preparation: F.K.; Manuscript editing and reviewing: all authors.

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Chapter 4

Effects of artificial light at night on the immune system, antioxidative system and body indices of Eurasian perch

Innate immunity, oxidative stress and body indices of Eurasian perch *Perca fluviatilis* after two weeks of exposure to artificial light at night

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Additional supporting information may be found online in the Supporting Information section (https://doi.org/10.1111/jfb.14703) and is also attached as Appendix E to this dissertation.

Abstract
Artificial light at night (ALAN) can disrupt biological rhythms of fish and other vertebrates by changing the light information of the nocturnal environment. Disrupted biorhythms can impair the immune system of vertebrates as it has been shown for conditions with continuous illumination or long-day photoperiod in many vertebrates, including fish. Nonetheless, this has not been shown so far for typical ALAN scenarios with high light intensities during day and low light intensities at night. Therefore, in this study, proxies for the innate immune system and oxidative stress as well as body indices of Eurasian perch *Perca fluviatilis* were measured under a wide range of intensities of nocturnal illumination. The authors found no changes in parameters of the innate immune system and no significant changes in proxies for oxidative stress after 2-week exposures to nocturnal illuminance ranging from 0.01 lx to 1 lx in one experiment or from 1 lx to 100 lx in a second experiment. A decrease in the hepatosomatic index at the highest tested light intensity of 100 lx compared to the dark control was the only significant difference in all parameters among treatments. After 2 weeks of exposure, ALAN does not seem to seriously challenge the innate immune system and seems to cause less oxidative stress than expected. The results of this study contradict the findings from other studies applying continuous illumination or long-day photoperiod and highlight the importance of further research in this field. Because ALAN represents a sustained modulation of the environment that may have cumulative effects over time, long-term studies are required for a better understanding of how ALAN modulates the health of fish.

Keywords: ALAN, fish, freshwater, light pollution, non-specific immune system, skyglow

4.1. Introduction

The health of fish is protected by physiological defense mechanisms against environmental or biological hazards, which can be critical for survival and reproductive success. The numerous components of the immune system and the antioxidative defense system of fish can display daily and seasonal rhythms as they are shaped by environmental factors, such as photoperiod and temperature (Bowden, 2008, Fortes-Silva *et al.*, 2019, Hidalgo *et al.*, 2017). Artificial light at night (ALAN) is an unprecedentedly increasing environmental change, which is introduced by humans to the nocturnal environment (Hölker *et al.*, 2010b) and changes the information on nocturnal illumination and photoperiod, which may result in suboptimal functioning of the immune system of fish and other vertebrates (Bowden, 2008, Navara and Nelson, 2007). Artificial light that is scattered in the atmosphere creates a dim glow of the night sky over large areas even many kilometers away from the original light source (Jechow *et al.*, 2020, Kyba *et al.*, 2011). This phenomenon, referred to as skyglow, illuminates the nocturnal environment at low intensities, and because of scattering, it affects not only cities but also suburban areas including the surrounding waters (Hänel *et al.*, 2018). Thereby, skyglow can extend periods of twilight and can even blur rhythms of lunar illumination, especially in cloudy nights (Jechow *et al.*, 2020, Puschnig *et al.*, 2014b).

An impairment of the immune system of fish by ALAN has mainly been studied in terms of 24 h illumination (LL) with nocturnal light intensities equal to experimental daylight intensities, which has been reviewed for fish (Bowden, 2008) and for mammals and birds (Navara and Nelson, 2007). Effects of LL on the immune system of fish include increased lysozyme activity (Burgos *et al.*, 2004), reduced peripheral leucocyte numbers (Valenzuela *et al.*, 2007) or increased antibody levels (Melingen and Wergeland, 2002) in salmonids. Furthermore, a prolonged exposure to light by a photoperiod with 14 h light and 10 h darkness (14 L:10 D) decreased the respiratory burst of blood leucocytes in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) after 1 week exposure compared to a photoperiod with 12 L:12 D (Burgos *et al.*, 2004). In this context, effects of melatonin on the immune system are typically discussed as well because the nocturnally produced hormone transduces the light/dark information to immune cells. Fish leucocytes possess melatonin receptors, indicating a mediating effect of melatonin on leucocyte numbers and functioning as it has been shown in carp *Cyprinus carpio* L. 1758 (Kepka *et al.*, 2015).

On top of the modulation of leucocyte activity, melatonin also acts as an antioxidant and modulator for antioxidative enzymes (Carrillo-Vico *et al.*, 2013, Reiter *et al.*, 2000). The antioxidative potential of melatonin in fish has been reviewed recently by Esteban *et al.* (2013). ALAN potently reduced nocturnal melatonin secretion in Eurasian perch *Perca fluviatilis* L. 1758 at nocturnal intensities between 0.01 lx and 100 lx (Brüning *et al.*, 2015, Kupprat *et al.*, 2020) and in many teleost species melatonin was likewise reduced even at low intensities of nocturnal illumination below 1 lx (e.g., Brüning *et al.*, 2018a, Nikaido *et al.*, 2009). Indeed, in most vertebrate taxa this dose-dependent suppression of melatonin by ALAN can be observed (Grubisic *et al.*, 2019). Because of the lack

of the antioxidative potential of melatonin, an increase in oxidative stress is likely at LL or exposure to ALAN (Navara and Nelson, 2007). Increased activities of superoxide dismutase (SOD) and catalase (CAT) among other antioxidative enzymes were measured in fish exposed to LL as compared to a 12 L:12 D photoperiod in several studies (Corona-Herrera *et al.*, 2018, Sreejith *et al.*, 2007) indicating an increase in oxidative stress. Some of these effects could be reversed by the administration of melatonin *in vivo* or *in vitro* (Shin *et al.*, 2011, Sreejith *et al.*, 2007). Furthermore, melatonin had immuno-enhancing effects on leucocytes of sea bass *Dicentrarchus labrax* (L. 1758) and sea bream *Sparus aurata* L. 1758 (Cuesta *et al.*, 2008) as well as *C. carpio* (Kepka *et al.*, 2015). These studies support the idea that melatonin is a main modulator of the innate immune system of fish.

The above-mentioned studies revealed effects on the immune system under LL or long-day photoperiod, with daylight intensities of hundreds to thousands of lux. This study is the first to investigate the effects of typical ALAN scenarios (bright daylight and dimly lit nights) on innate immunity and indicators for oxidative stress in fish. The authors of this study took an explorative approach by measuring several parameters associated with the health of *P. fluviatilis* in response to ALAN. Generally, the innate or non-specific immune system is the first line of defense against pathogens. Thus, the authors measured the lysozyme activity in the blood plasma and the respiratory burst activity of head kidney leucocytes. Furthermore, they assessed thiobarbituric acid reactive substances (TBARS) as a measure for lipid peroxidation, and activities of SOD as well as CAT from liver tissue as indirect proxies for oxidative stress. In addition, the condition factor (K), the splenosomatic index (I_S) and the hepatosomatic index (I_H) served as measures for the overall condition of the fish. The exposure to ALAN lasted 2 weeks (according to Brüning *et al.*, 2015), and a large range of nocturnal light intensities were tested because there is little information on the sensitivity of the immune system of fish to ALAN. Intensities of nocturnal illumination ranged from very low typical skyglow intensities of 0.01 lx to 1 lx in one experiment or, in a separate experiment, from 1 lx up to very extreme ALAN intensities of 100 lx that would occur only locally close to a strong streetlight (Hänel *et al.*, 2018).

The hypotheses of this study are based on the findings described above in which exposure to continuous illumination or long-day photoperiod led to a modulated immune status and an increase in oxidative stress in different fish species. Moreover, the hypotheses are indirectly based on immuno-enhancing and antioxidative effects of melatonin, which is reduced under ALAN, as it has previously been shown in *P. fluviatilis* with a similar set-up as in the present study (Brüning *et al.*, 2015, Kupprat *et al.*, 2020). The authors expected a reduction in respiratory burst activity of head kidney leucocytes and increased lysozyme activity as well as an increased lipid peroxidation and an increase in SOD and CAT activities in the liver as indirect measures for an increase in oxidative stress.

4.2. Materials and methods

4.2.1. Ethical statement

The care and use of experimental animals complied with German animal welfare laws, guidelines and policies as approved by the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16).

4.2.2. Experimental fish

The authors studied *P. fluviatilis* (pubertal and young adults) from Lake Müggelsee (Berlin, Germany). All experimental fish were kept in 600-L indoor tanks at 16°C for at least 2 weeks before the animals were transferred to the experimental set-up. During this time, fish experienced natural photoperiod (sunlight through windows) with natural dark nights and were fed twice a day with the food source of the respective experiment (see below). According to the "new world atlas of artificial night sky brightness," the surface of Lake Müggelsee experiences an illumination of ca. 0.003 lx in moonless clear nights (Falchi *et al.*, 2016), which lies in the lower range of suburban skyglow (Hänel *et al.*, 2018).

4.2.3. Experimental set-up

The experimental set-up has been described in detail by Franke *et al.* (2013). Fish were exposed to ALAN treatments in 80-L aquaria, which were covered with black foil to ensure independence of the light treatments. The lids of all aquaria were equipped with three fluorescent tubes to realize daylight intensities that reached up to 7000 lx at the brightest spot on the water surface and around 2900 lx averaged over 25 equally distributed points on the water surface. An additional fluorescent tube was installed for night-time illumination. Control levels were below the detection limit of the luxmeter (ILT1700, Peabody, MA, USA) used in this study, i.e., < 0.00167 lx, and are referred to as "0 lx" in the following. Photoperiod was controlled by an automatic time switch system (Hager, Blieskastel, Germany). The spectral composition of the light source (Biolux fluorescent tubes, Osram, Germany) has been reported by Franke *et al.* (2013) and can be used to convert lux values into other illumination units (e.g., 1 lx ≈ 3.7 mW m^{-2}). It covers a large part of the spectral sensitivity of *P. fluviatilis* although their spectral sensitivity is slightly more red-shifted (Cameron, 1982). Light intensity was adjusted by partial cover of the light source or using neutral density filter foil (Lee Colour Filter 299 1.2 N.D.). Because these methods do not change the spectral composition of the light source, lux can be used as the unit of illuminance for comparison across different light intensities.

4.2.3.1. Exposure to high intensities of ALAN ("high ALAN experiment")

Parasite-free *P. fluviatilis* were raised from fertilized egg ribbons collected from Lake Müggelsee (Berlin, Germany) in March 2015 as described by Vivas Muñoz *et al.* (2019). The experimental set-up consisted of 12 identical 80-L aquaria with a tap water flow-through of 10 L h^{-1} and a water temperature of ca. 16°C. Six fish were placed into each aquarium and allowed to acclimate for 2 weeks without illumination during night (0 lx) followed by 2 weeks of experimental conditions with the respective nocturnal light intensity or controls without illumination according to

Brüning *et al.* (2015). Average mass of the fish was 69.0 ± 18.4 g with an average standard length of 15.3 ± 1.3 cm (mean ± S.D., n = 120). Fish were fed with commercially available food (Aller Silver 3 mm, Emsland-Aller Aqua, Golßen, Germany) twice a day at a rate of 0.5% of their body mass. Feeding stopped 24 h before sampling. Full daylight was realized from 09:00 to 15:00 hours with a simulated dawn or dusk period over 3 h each starting at 06:00 or 15:00 hours, respectively. Nocturnal illumination with 1 lx, 10 lx or 100 lx on the water surface was from 18:00 till 06:00 hours. The experiment was run twice in December 2016 and January 2017, with each treatment in duplicate during the first run and in triplicate during the second run (i.e., n = 5 for each treatment).

4.2.3.2. Exposure to low intensities of ALAN ("low ALAN experiment")

P. fluviatilis from Lake Müggelsee were caught between July and September 2017 and fed twice a day with frozen blood worms. Thirty fish with a mass of 16.8 ± 4.1 g and standard length of 10.6 ± 0.9 cm (mean ± S.D., n = 720) were transferred to each 80-L aquarium. During 2 weeks of acclimation, fish were fed twice a day and the water flowthrough was adjusted to 10 L h^{-1}. The temperature during acclimation and experimental exposure to ALAN was kept around 16°C. Photoperiod was adjusted to October conditions with full daylight from 09:30 to 18:30 hours with a 3 h dawn or dusk period starting at 06:30 and 18:30 hours, respectively. After acclimation, the nocturnal illumination from 21:30 to 6:30 hours was switched on with 0.01 lx, 0.1 lx or 1 lx average intensity on the water surface for 2 weeks according to Brüning *et al.* (2015). Controls were not illuminated during night (0 lx). During experimental illumination, the water flow-through was reduced to 4 L h^{-1} to allow water-based melatonin measurements, which are described by Kupprat *et al.* (2020). To maintain good water quality, animals were not fed during the 2 weeks of exposure. The same experiment was performed twice – in October and November 2017 – with each treatment in triplicates for both runs (i.e., n = 6 for each treatment).

4.2.4. Sampling

Fish were randomly sampled on two consecutive mornings between 09:00 and 12:00 hours in the high ALAN experiment and in two consecutive nights between 22:00 and 04:00 hours in the low ALAN experiment. In the high ALAN experiment, all parameters were measured from all fish. In the low ALAN experiment, body mass and length were measured of all fish, but blood was only taken from the first 15 fish of each aquarium, and the first 10 fish were killed for sampling of livers as well as spleens. Males (m) and females (f) were distinguished by a visual inspection of the gonads. In premature fish (nd) gonads could not be differentiated. Sex ratios were 34%:37%:29% (f:m:nd) in the high ALAN experiment and 5%:57%:38% (f:m:nd) in the low ALAN experiment. In the low ALAN experiment, sex could be determined only for fish that were killed, and thus this information is not available for some blood samples (na).

Blood (500 – 1000 µL) was taken from the caudal vein with heparinized syringes and transferred to a tube containing ca. 1 – 2 mg Na_2EDTA (EDTA). Blood and EDTA were mixed by shaking and centrifuged at 7500 × *g* for 5 min at 4°C. The plasma was transferred to a new tube and immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Fish were stunned by a blow to the head and killed by cutting the neck. Wet body mass was measured to the nearest 0.1 g and standard length with an accuracy of 1 mm. Fish were then cut open dorsally, and the liver and spleen were excised and weighed to an accuracy of 1 mg. The liver was immediately frozen in liquid nitrogen and stored at −80°C until further analysis. In the low ALAN experiment, 68% of the dissected fish had plerocercoids of *Triaenophorus nodulosus* (Pallas 1781) in the liver, which were excised before freezing. Only noninfected livers were used for calculations of the hepatosomatic index.

Finally, the head kidneys were excised and squeezed through a 70 µm cell sieve (EASYstrainer™, Greiner Bio One International, Kremsmünster, Austria) by adding ice-cold washing medium (RPMI 1640 medium with phenol red with 25 mM HEPES, 100 U mL^{-1} penicillin–streptomycin, 2.05 mM L-glutamine, Biowest, Nuaillé, France and 20 IU mL^{-1} heparin) and stored on ice until the end of sampling. If not indicated otherwise, chemicals were obtained from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

4.2.5. Respiratory burst activity

The respiratory burst activity assay was based on the method originally described by Secombes (1990) and performed following the protocol by Liu *et al.* (2017) with some modifications. Cell cultures were only prepared in the high ALAN experiment. After sampling, cells were centrifuged at 500 × *g* for 15 min at 4°C, and the volume of the suspension was adjusted to 5 mL. Density gradient centrifugation was used to enrich macrophages and to separate them from erythrocytes: The cell suspension was layered on a discontinuous gradient consisting of 51% and 34% Percoll (GE Healthcare, Chicago, IL, USA) and centrifuged at 500 × *g* for 40 min at 4°C. Cells collected from the 34 – 51% interphase were washed once in washing medium and once with cell culture medium (RPMI 1640 with 25 mM HEPES, 100 U mL^{-1} penicillin–streptomycin, 2.05 mM L-glutamine, Biowest) by centrifugation at 500 × *g* for 15 min at 4°C and re-suspended in cell culture medium. The cell concentration was adjusted to 107 cells mL^{-1} and 100 µL of this suspension was added to eight wells of a cell culture-treated 96-well plate (Nunclon® Surface, Thermo Scientific, Waltham, MA, USA). Cells were allowed to adhere for 1 h at room temperature. Non-adherent cells were then removed by carefully rinsing with 150 µL cell culture medium. Subsequently, cells were incubated for 1 h at 25°C in culture medium containing 1 mg mL^{-1} nitroblue tetrazolium chloride, and half of the cells were stimulated with 1 µg mL^{-1} phorbol 12-myristate 13-acetate (PMA). Cells without stimulation served as control. Finally, cell layers were washed again with 150 µL of cell culture medium and fixed in 100 µL methanol, and then washed twice with 100 µL of 70% methanol and air-dried. The intracellularly produced formazan was dissolved with 100 µL of 2 M KOH and 100 µL of DMSO and mixed thoroughly. Absorption was measured at 620 nm.

4.2.6. Lysozyme activity

Lysozyme activity was measured according to Ellis (1990) adapted to a 96-well plate and optimized for plasma of *P. fluviatilis*. Plasma samples were diluted 1:2 with 0.025 M potassium sodium phosphate buffer pH 6.2, and 25 µL of diluted

sample was added to 175 µL *Micrococcus lysodeikticus* suspension (0.2 mg lyophilized bacteria in 1 mL of the same buffer). After the sample was shaken for 5 min at 21°C on an orbital shaker at 300 rpm, the optical density was measured at 530 nm every minute over 15 min at 25°C. A linear regression was calculated for each sample for the time interval between 7 and 12 min, at which the decline was linear in all samples. According to Ellis (1990), a decline of 0.001 min^{-1} was defined as one unit of lysozyme activity.

4.2.7. Liver extracts

As an indirect measure for oxidative stress, two antioxidative enzymes and one indicator for lipid peroxidation were measured from liver tissue. In the high ALAN experiments all liver samples were analyzed. For the low ALAN experiment six liver samples were randomly chosen from the 10 fish sampled from each aquarium. Livers were homogenized manually with a glass homogenizer by adding sodium phosphate buffer pH 7.0 with 0.5 M EDTA at 1 mL per 0.1 g liver mass. A 300 µL aliquot of this homogenate was taken for analysis of TBARS and stored at −80°C until assayed. Another milliliter of the homogenate was centrifuged at 10,000 × g, and the supernatant was aliquoted for analyses of protein, SOD and CAT and stored at −80°C until assayed.

4.2.7.1. Thiobarbituric acid reactive substances

Lipid peroxidation was measured with the TBARS assay based on the procedure described by Uchiyama and Mihara (1978) with a standard curve of tetraethoxypropane ranging from 1 to 50 nmol mL^{-1}. To lyse fatty acids from the liver homogenate, 250 µL of samples and standards were mixed with 250 µL of 7% SDS solution and incubated for 5 min at room temperature. After putting samples on ice and adding 500 µL of 12.5% TCA in 0.8 M HCl, 500 µL of 1% thiobarbituric acid (TBA) was added and the mixture was heated to 95°C for 45 min. Under these conditions, TBA and malondialdehyde from samples or standards reacted to produce a pink-coloured dye, which was extracted with 1500 µL of 1-butanol by vortexing for 1 min followed by centrifugation at 4500 × *g* for 10 min at 4°C. The absorbance of the supernatants was measured in triplicates at 535 nm.

4.2.7.2. Superoxide dismutase

The activity of SOD was measured with a commercially available kit (Item 706002, Cayman Chemicals, Ann Arbor, MI, USA). Liver extracts were diluted 1:200 to 1:400 with “assay sample buffer” from the kit and measured according to the manufacturer's protocol. Before measuring all samples, the authors measured three samples at dilutions of 1:100, 1:200 and 1:400, and the calculated, original concentrations did not differ more than 20% from each other. Activities were quantified by a standard curve of bovine SOD covering the range from 0.005 to 0.05 U mL^{-1}.

4.2.7.3. Catalase

The activity of CAT was measured according to the protocol of Aebi (1984) adapted to a 96-well format. The ratio of the reaction media was adjusted for this study's samples. Liver extracts were diluted 1:50 with 50 mM sodium phosphate buffer pH 7.0. First, 180 µL of buffer was added to a 96-well plate (UV-star, Greiner Bio One International, Kremsmünster, Austria), and 15 µL of 150 mM H_2O_2 solution

(prepared in the same buffer) was added. Then, 5 µL of diluted sample was added to the mixture, the plate was shaken for 5 s at 500 rpm at 25°C and then the absorption was measured at 240 nm every 30 s for 10 min at 25°C. The linear regression of decreasing absorption had $R^2 > 0.8$ in all analyzed samples. CAT activity is expressed as units (U), which equals 1 µmol of H_2O_2 consumed per minute with an extinction coefficient for H_2O_2 of 43.6 M^{-1} cm^{-1} and a path length of 0.5 cm.

4.2.7.4. Liver protein content

To normalize SOD and CAT activities, the total protein concentration in the liver extracts was measured with the Biuret reaction using a commercially available kit (RotiQuant® Universal, Roth, Karlsruhe, Germany). Quantification was made by a standard curve of bovine serum albumin.

4.2.8. Body indices: condition factor, hepatosomatic index and splenosomatic index

To assess rough measures for the overall health of the fish, three body indices were calculated. The condition factor was calculated by $K = 100\ M_W\ L_S^{-3}$, where M_W (g) is the wet mass and L_S (cm) is the standard length. The hepatosomatic index was calculated by $I_H = 100\ M_L\ M_W^{-1}$, where M_L (g) is the liver mass and the splenosomatic index was calculated likewise by $I_S = 100\ M_S\ M_W^{-1}$, where M_S (g) is the spleen mass.

4.2.9. Statistical analysis

A linear mixed modelling (LMM) approach was chosen to account for the data structure with treatment and sex as fixed factors and individuals nested within aquaria nested within runs as random factors (R Core Team, 2020, Zuur *et al.*, 2009). For the respiratory burst activity, stimulation was added as a fixed factor, and interaction with treatment was tested. Statistical significance was assumed with $p < 0.05$. In case of significant treatment or sex effects, post hoc tests using Bonferroni's correction compared every treatment to every other treatment within one experiment. Treatment, sex and random effects as well as marginal and conditional R^2 values for each LMM are specified in Table 4.1. Full model specifications for each parameter and each experiment as well as full results of the post hoc tests are given in Supporting Information. In addition, effects of liver parasitization were tested by comparing infected to non-infected individuals by Mann–Whitney U-tests. R packages used for statistical analysis and data visualization are listed in Supporting Information.

4.2.10. Note on the differences between the two experiments

Because of the differences in size and life history of the fish as well as differences in feeding regimes and different sampling times among the two experiments, the authors only compare the measured parameters within one experiment and not across the low and high ALAN experiments. Originally, the authors aimed to do the high ALAN experiment with a wild population from Lake Müggelsee as it was later realized in the low ALAN experiment and has been realized in an earlier study (Brüning *et al.*, 2015). Nonetheless, in fall 2016, the authors were unable to catch sufficient wild *P. fluviatilis* for the experiment and therefore decided to work with

lab-raised fish, which were conditioned to dry feed. Contrarily, the wild *P. fluviatilis* in the low ALAN experiment could not be conditioned to dry feed and were thus fed with frozen blood worms. As mentioned earlier in Section 4.2.3.2, fish in the low ALAN experiment were not fed during the experimental time because of low water flow-through to measure melatonin from the aquarium water (Kupprat *et al.*, 2020). This was not necessary in the high ALAN experiment because changes in nocturnal melatonin were known from a previous study (Brüning *et al.*, 2015). In the high ALAN experiment, sampling occurred in the morning after fish were exposed to ALAN for the whole night. When the authors measured only few differences between the treatments in the high ALAN experiment, they supposed that effects at even lower intensities at the same time of day would be unlikely, and therefore, they took the samples throughout the night in the low ALAN experiment.

4.3. Results

4.3.1. Respiratory burst activity

The respiratory burst was strongly stimulated by PMA in all cultures of head kidney leucocytes in the high ALAN experiment (LMM: fixed factor stimulation: $p < 0.0001$; Figure 4.1). Random effects explained a significant proportion of the variance that was not explained by the fixed effect ($p < 0.0001$, Table 4.1), mainly because of individual variation (high ALAN: $\sigma^2_{ind} > \sigma^2_{aqu} > \sigma^2_{run}$). There were no significant differences between sexes in respiratory burst activity.

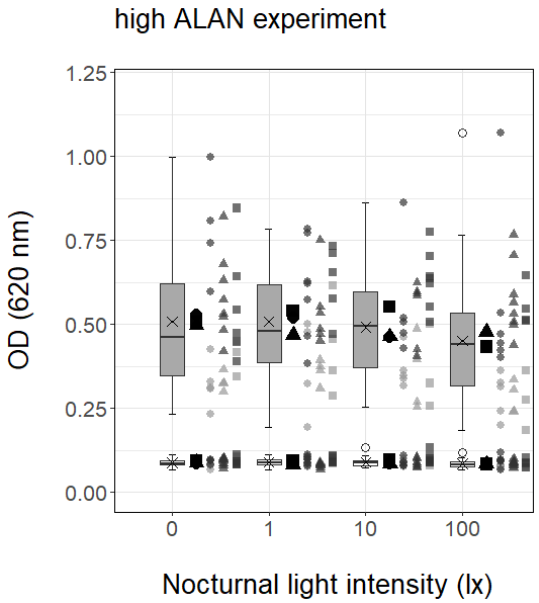

Figure 4.1 Respiratory burst activity in head kidney leucocytes of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks. Boxplots display non-stimulated cells (white boxplots) and cells stimulated with phorbol 12-myristate 13-acetate (PMA; grey boxplots) for each treatment (high ALAN experiment: n = 30). Optical density (OD) was determined at 620 nm. Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers > 1.5 × IQR are indicated by circles and "X"s inside the boxes indicate the mean. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)]. Small points represent individual cell cultures, whereas different shades of grey indicate different runs. There were no significant differences across treatments [linear mixed model, ALAN effect: loglikelihood ratio (LLR) = 2.05, $p = 0.56$] (□) no stimulation, (■) PMA stimulation, (○) December, (●) January, (●) f, (▲) m, (■) nd

Table 4.1
Effects of artificial light at night (ALAN) and sex as well as random effects and the marginal and conditional R^2 values for the linear mixed models of different immune parameters, proxies of oxidative stress and body indices in two different experiments exposing *Perca fluviatilis* to different nocturnal light intensities for 2 weeks

	ALAN effect		Sex effect		Random effects		Goodness of fit	
High ALAN experiment	**LLR†**	***p*-value**	**LLR**	***p*-value**	**LLR**	***p*-value**	**$R^2_{marginal}$**	**$R^2_{conditional}$**
Respiratory burst activity	2.05	0.56	5.96	0.051	400.03	<0.0001	0.9955	0.9988
Lysozyme activity	3.07	0.38	6.15	0.046‡	621.02	<0.0001	0.0684	0.9511
Thiobarbituric acid reactive substances	7.61	0.055	59.77	<0.0001	1237.5	<0.0001	0.4172	0.9990
Liver protein	1.61	0.66	14.22	0.0008	217.71	<0.0001	0.1154	0.9355
Superoxide dismutase activity	1.61	0.63	5.27	0.07	138.38	<0.0001	0.0584	0.9798
Catalase activity	1.73	0.63	15.06	0.0005	260.88	<0.0001	0.1071	0.9308
Condition factor	3.64	0.30	6.01	0.0495‡	0.05	0.97	0.0929	0.1142
Splenosomatic index	3.21	0.36	11.39	0.003	1.62	0.44	0.0409	0.0751
Hepatosomatic index	8.93	0.03	20.49	<0.0001	9.61	0.008	0.1943	0.3762
Low ALAN experiment	**LLR**	***p*-value**	**LLR**	***p*-value**	**LLR**	***p*-value**	**$R^2_{marginal}$**	**$R^2_{conditional}$**
Lysozyme activity	1.66	0.64	6.44	0.09	275.87	<0.0001	0.0308	0.9290
Thiobarbituric acid reactive substances	0.38	0.94	29.42	<0.0001	1003.6	<0.0001	0.1710	0.9914
Liver protein	1.21	0.75	1.49	0.48	432.32	<0.0001	0.0120	0.9923
Superoxide dismutase activity	1.68	0.64	9.68	0.008	108.01	<0.0001	0.0788	0.9017
Catalase activity	0.55	0.91	0.61	0.73	304.48	<0.0001	0.0067	0.7895
Condition factor	1.40	0.71	45.05	<0.0001	209.44	<0.0001	0.0366	0.4522
Splenosomatic index	2.36	0.50	19.89	0.0001	6.32	0.04	0.1384	0.2815
Hepatosomatic index	2.25	0.52	25.92	<0.0001	6.1^{-9}	1	0.1422	0.1422

† Log-likelihood ratio
‡ significant effect in model selection, but no significant differences in post-hoc testing

4.3.2. Lysozyme activity

Antibacterial activity of lysozyme in the blood plasma was measured as a humoral factor of the innate immune system and did not differ across treatments in both experiments (Figure 4.2, Table 4.1). There were no significant random effects in either experiment as well as no significant differences between sexes in lysozyme activity ($p > 0.05$, Table 4.1). Although in the model selection a sex effect for the high ALAN experiment was suggested ($p = 0.046$, Table 4.1), post hoc testing did not reveal significant differences ($p > 0.05$). Parasitization in the liver did not affect lysozyme activity in the low ALAN experiment (Mann–Whitney U-test, $p = 0.51$, Supporting Information).

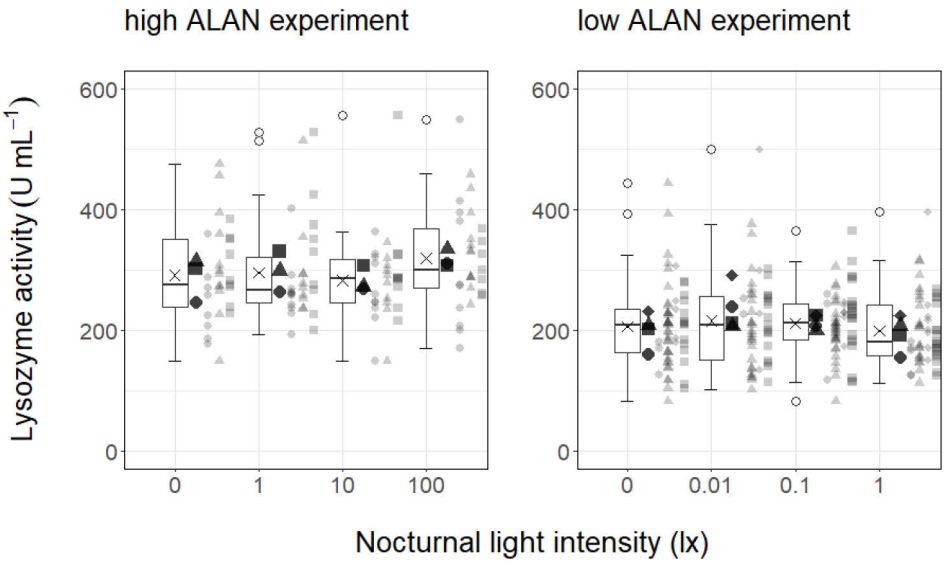

Figure 4.2 Lysozyme activity in the blood plasma of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Activity is expressed as units (U) per milliliter. Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (high ALAN experiment: n = 29 for 0 lx and 100 lx, n = 30 for 1 lx and 10 lx; low ALAN experiment: n = 57 for 0 lx, n = 49 for 0.01 lx, n = 59 for 0.1 lx, n = 54 for 1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers > 1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish), na – not available (sex not determined)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effect in high ALAN experiment: log-likelihood ratio (LLR) = 3.07, p = 0.38; ALAN effect in low ALAN experiment: LLR = 1.66, p = 0. 64] (●) f, (▲) m, (■) nd, (◆) na

4.3.3. Oxidative stress in the liver: TBARS, SOD, CAT and liver protein

The indirect measures for oxidative stress, measured by the TBARS assay from liver homogenate (Figure 4.3) and by SOD and CAT activities in liver extracts (Figure 4.4), did not differ significantly across treatments of different nocturnal illumination in both experiments (p > 0.05, Table 4.1). The protein content in the liver was used to standardize enzyme activities and did not differ significantly across treatments in both experiments, either (p > 0.05, Figure 4.4, Table 4.1). The graphs for the liver protein are attached in Supporting Information. All LMMs for the liver parameters revealed significant random effects with highest variance of individuals, which explained the biggest portion of the data variance ($\sigma^2_{ind} > \sigma^2_{run}$ or σ^2_{aqu}).

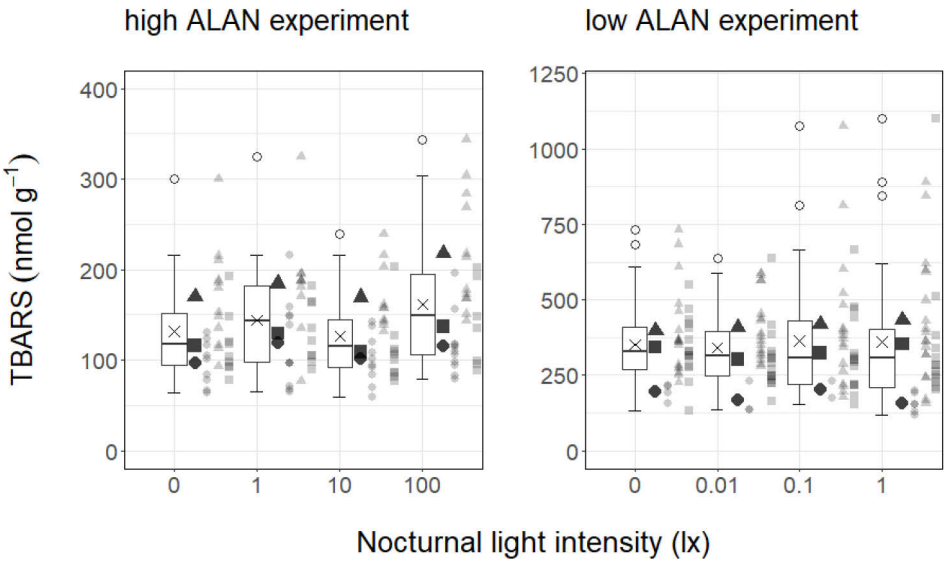

Figure 4.3 Thiobarbituric acid reactive substances (TBARS) of liver homogenate of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. The measurements are expressed as nanomoles malondialdehyde equivalent per gram liver mass. Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (high ALAN experiment: n = 30; low ALAN experiment: n = 36 for 0 lx and 0.01 lx, n = 34 for 0.1 lx, n = 37 for 1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers > 1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effect in high ALAN experiment: loglikelihood ratio (LLR) = 7.61, $p = 0.055$; ALAN effect in low ALAN experiment: LLR = 0.38, $p = 0.94$] (●) f, (▲) m, (■) nd

All liver parameters had sex effects in at least one experiment (Table 4.1). Males had higher TBARS levels than females (both experiments: $p < 0.0001$) and premature (nd) fish (high ALAN exp.: $p < 0.0001$, low ALAN exp.: $p = 0.003$). In addition, in the low ALAN experiment premature fish had higher levels of TBARS in the liver than females ($p = 0.005$). The protein content in the liver was higher in premature fish than in males or females in the high ALAN experiment ($p < 0.0001$), and in the low ALAN experiment there were no significant sex effects ($p = 0.48$, Table 4.1). The activity of SOD showed no sex effects in the high ALAN experiment ($p = 0.07$), but in the low ALAN experiment the premature fish had higher activities than males or females (nd vs. m: $p = 0.03$, nd vs. f: $p = 0.047$). The activity of CAT was higher in males of the high ALAN experiment than in females or premature fish (m vs. f: $p = 0.0005$, m vs. nd: $p = 0.047$), but had no sex effect in the low ALAN experiment ($p = 0.74$, Table 4.1). Parasitization in the liver did not affect any of the hepatic parameters in the low ALAN experiment (Mann–Whitney U-test, $p > 0.05$, Supporting Information).

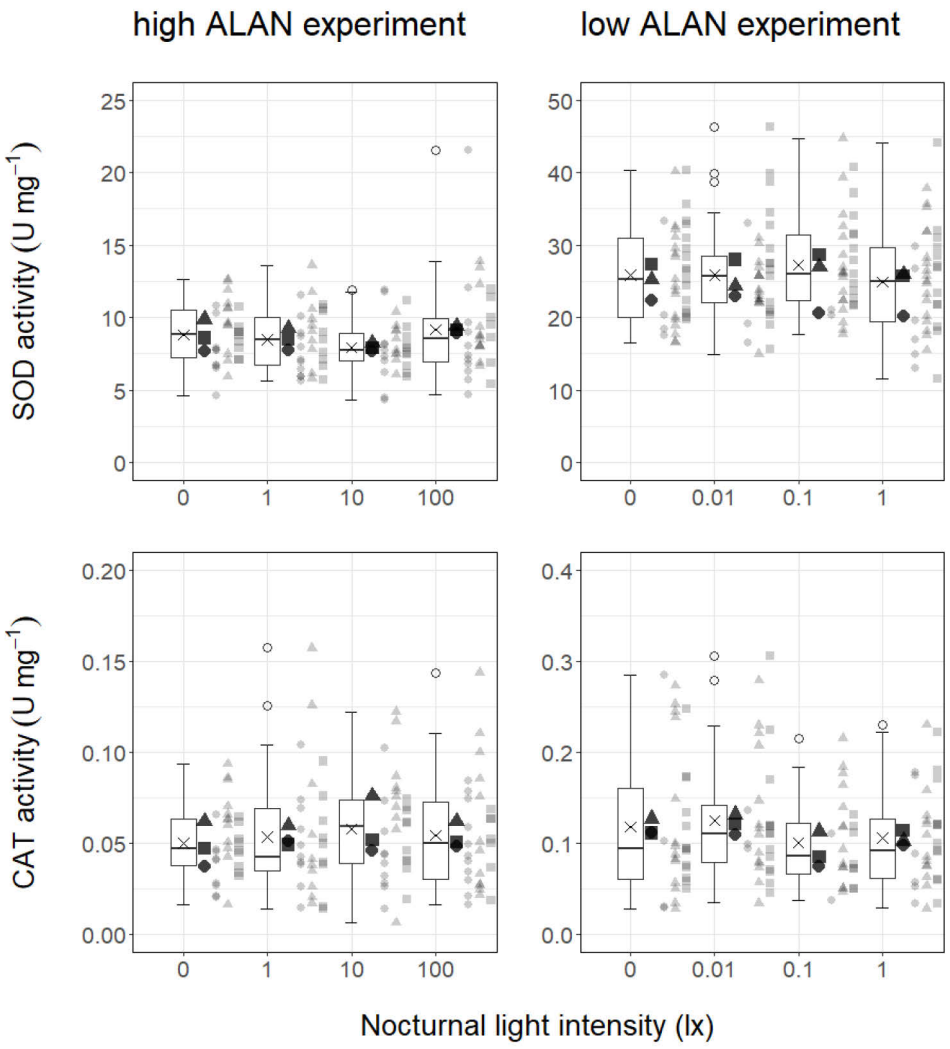

Figure 4.4 Activities of the antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT) measured from liver extracts of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Specific activities of both enzymes are expressed as units (U) per milligram total liver protein. Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (high ALAN experiment – SOD: n = 30, − CAT: n = 30 for 0 lx, 10 lx and 100 lx, n = 29 for 1 lx; low ALAN experiment – SOD: n = 37 for 0 lx and 1 lx, n = 35 for 0.01 lx, n = 33 for 0.1 lx, − CAT: n = 31 for 0 lx, 0.01 lx and 1 lx, n = 24 for 0.1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers > 1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effects in high ALAN experiment: SOD – log-likelihood ratio (LLR) = 1.61, p = 0.63, CAT – LLR = 1.73, p = 0.63; ALAN effect in low ALAN experiment: SOD – LLR = 1.68, p = 0.64, CAT – LLR = 0.55, p = 0.91] (●) f, (▲) m, (■) nd

4.3.4. Body indices

There were no significant effects across the different nocturnal illuminations both in K and I_S of either experiment or in the I_H of the low ALAN experiment ($p > 0.05$, Table 4.1, Figure 4.5). The graphs for K are attached in Supporting Information. At 100 lx, I_H was significantly lowered by 15% compared to 0 lx in the high ALAN experiment (LMM, treatment as fixed effect: $p = 0.0303$, Tukey's post hoc: $p = 0.046$).

The random effects were significant for I_H in the high ALAN experiment in which run had a higher variance than aquarium ($\sigma^2_{run} > \sigma^2_{aqu}$) and for K and I_S of the low ALAN experiment in which run or aquarium had a higher variance, respectively (K: $\sigma^2_{run} > \sigma^2_{aqu}$; I_S: $\sigma^2_{aqu} > \sigma^2_{run}$).

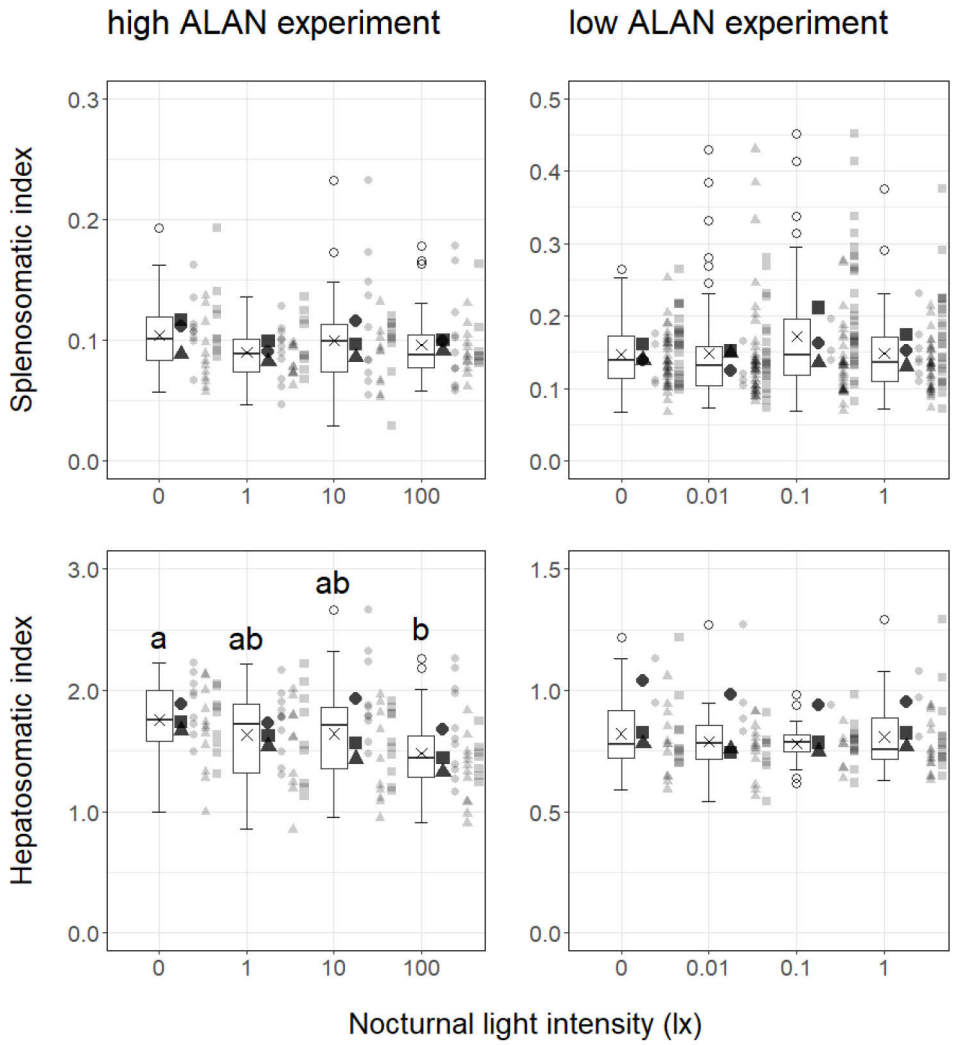

Figure 4.5 Splenosomatic index (I_S) and hepatosomatic index (I_H) of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Boxplots display data for each treatment, and "X"s inside the boxes indicate the mean (high ALAN experiment: n = 30; low ALAN experiment – I_S: n = 63 for 0 lx, n = 64 for 0.01 lx, n = 65 for 0.1 lx and 1 lx, − I_H: n = 24 for 0 lx, n = 25 for 0.01 lx, n = 22 for 0.1 lx, n = 31 for 1 lx). Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. Letters indicate a significant decrease at 100 lx as compared to 0 lx (Tukey's post hoc, $p = 0.0457$) for the I_H in the high ALAN experiment. There were no significant differences across treatments in the I_S in either experiment [linear mixed models, ALAN effect in high ALAN experiment: I_S – log-likelihood ratio (LLR) = 3.21, $p = 0.36$, I_H – LLR = 8.93, $p = 0.03$; ALAN effect in low ALAN experiment: I_S – LLR = 2.36, $p = 0.50$, I_H – LLR = 2.25, $p = 0.52$] (●) f, (▲) m, (■) nd

Sex had a significant effect on all body indices in both experiments ($p < 0.05$, Table 4.1). Nonetheless, post hoc tests revealed no significant differences between sexes in K of the high ALAN experiment. In the low ALAN experiment,

premature fish had a lower *K* than males or females (nd vs. f: $p = 0.0156$; nd vs. m: $p < 0.0001$). I_S was higher in premature fish than in males in both experiments (high ALAN exp.: $p = 0.0088$; low ALAN exp.: $p < 0.0001$). I_H was higher in females than in premature or male fish in both experiments (f vs. m: $p < 0.0001$; f vs. nd (high ALAN exp.): $p < 0.0117$; f vs. nd (low ALAN exp.): $p < 0.0001$). The I_S was significantly lower (ca. 6%) in fish infected with liver parasites compared to fish without infected livers (Mann–Whitney U-test, $p = 0.01$, Supporting Information).

4.4. Discussion

This study aimed to assess the effects of ALAN at illuminances between 0.01 lx and 100 lx on the innate immune system, indicators for oxidative stress and body condition of *P. fluviatilis* after 2 weeks. In contrast to most of the hypotheses of this study, the measured parameters remained largely unchanged at all tested levels of ALAN. Nonetheless, there was a significant decrease in the hepatosomatic index (I_H) at the highest ALAN level of 100 lx compared to 0 lx.

4.4.1. Respiratory burst activity

The respiratory burst activity – a parameter for innate cellular immunity – showed no significant changes in response to 1 lx, 10 lx or 100 lx of nocturnal illumination. This lack of effects of high ALAN intensities on respiratory burst activity contrasts results from studies with continuous illumination or long-day photoperiod in *O. mykiss* or *C. carpio* (Burgos *et al.*, 2004, Kepka *et al.*, 2015). The natural variation in respiratory burst activity among individuals might have been too broad to detect effects of ALAN in *in vivo* experiments after only 2 weeks. Significant changes in respiratory burst activity in *in vivo* experiments were measured in experiments, which lasted 4–8 weeks and had harmful or stimulating additives in the fish food as a treatment (e.g., Adel *et al.*, 2016, Pietsch *et al.*, 2014), which might modulate the immune system more effectively than ALAN. Furthermore, ALAN or indirectly the reduced melatonin might come into effect only in combination with other factors. Accordingly, in leucocytes of *C. carpio* melatonin treatment only changed respiratory burst activity *in vivo* in a zymosan-induced peritonitis but did not show enhancing effects at *in vitro* treatments with different melatonin concentrations (Kepka *et al.*, 2015).

4.4.2. Lysozyme activity in blood plasma

Opposing the hypothesis of this study, lysozyme activity was not elevated by any ALAN treatment in the experiments of this study. This contradicts previous findings in *O. mykiss* at continuous illumination or long-day photoperiod with elevated lysozyme content after 1 or 4 weeks, respectively (Burgos *et al.*, 2004). It is possible that typical ALAN levels have no effect on lysozyme activity of *P. fluviatilis* or an exposure to ALAN may take longer than 2 weeks for lysozyme activity to respond. For example, it was only recently shown that the overall bacterial-killing activity in blood plasma of king quail *Excalfactoria chinensis* (L. 1766) was increased only after 4 or 6 weeks exposure to weak ALAN (0.3 lx) in developing females and males (Saini *et al.*, 2019).

4.4.3. Oxidative stress in the liver

The TBARS and the activities of SOD and CAT in liver extracts did not significantly change after 2-week exposures to ALAN, suggesting that there were no substantial changes in oxidative stress in this tissue. Similar to the results of this study, the oxidative status in wild songbirds was not affected by ALAN at an illuminance level of 3 lx, although body mass changed after 2 days of exposure (Raap *et al.*, 2016). Yet, some antioxidant capacity was likely missing in the experimental fish of this study, as it was shown in earlier publications that melatonin concentrations decreased in *P. fluviatilis* under ALAN in a dose–response manner (Brüning *et al.*, 2015, Kupprat *et al.*, 2020). Probably, other antioxidative mechanisms compensated for the lacking melatonin without a measurable increase in activity of SOD and CAT. For further testing of the hypothesis that a lack of melatonin can lead to an increase in oxidative stress, the combination of ALAN with another stressor, e.g., increased temperature, might be a promising approach to evaluate how the antioxidative system of fish responds to ALAN under realistic oxidative stress. Furthermore, *in vitro* experiments exposing isolated cells or tissues to oxidative stress with simultaneous melatonin treatment might also help understanding the mechanisms of melatonin and its contribution to the antioxidative capacity in fish tissues. A similar approach was used by Sreejith *et al.* (2007) in climbing perch *Anabas testudineus* (Bloch 1792) in which oxidative stress was induced by 6-propylthiouracil (PTU) treatment or continuous illumination. In cultivated liver tissue of *A. testudineus*, melatonin reduced the lipid peroxidation caused by continuous illumination and reduced the increased activities of, e.g., SOD and CAT in combination with 6-PTU treatment even below control levels, whereas 6-PTU alone increased activities of SOD and CAT. The results therefore suggest a strong contribution of melatonin to the antioxidative capacity in fish.

4.4.4. Body indices: I_S and I_H

The I_S as an indicator for hematopoietic and immunological activity did not change in the experiments of this study. In contrast, as in small rodents short-day photoperiods increased splenic mass (e.g., Vaughan *et al.*, 1987), decreased splenic mass might be expected at long-day photoperiods or continuous light. The results of this study, however, showed that the splenic mass of *P. fluviatilis* is not affected by ALAN between 0.01 and 100 lx within 2 weeks. Irrespective of the ALAN treatment, infections with the liver parasite *T. nodulosus* slightly lowered relative splenic mass as compared to non-infected *P. fluviatilis*. *T. nodulosus* is a common parasite of the Northern pike *Esox lucius* L. 1758 and frequently infects *P. fluviatilis* as an intermediate host in which most worms encapsulate in the liver. Although most other parameters reported here were not affected by the liver parasite, the lowered I_S may indicate a weak suppression of hematopoietic and immune function of the spleen.

The significant decrease in I_H at 100 lx can be interpreted as an indicator of decreased energy storage, such as glycogen reserves (Chellappa *et al.*, 1995). Interestingly, there seems to be an inverse trend of I_H and TBARS at 100 lx with decreased I_H and increased TBARS, despite a *p*-value closely above the threshold of statistical significance for the latter one (Table 4.1). A confirmation of this inverse

relationship at high intensities of ALAN is necessary before further conclusions about the effects of ALAN on the liver metabolism of fish can be drawn. In contrast, other studies rather indicate a positive relationship between TBARS and I_H in fish (e.g., Chien and Hwang, 2001). Further experiments with a longer exposure time and subsequent analysis of the caloric composition of protein, lipid and carbohydrate in the liver could reveal further insights into the effects of ALAN on liver metabolism. Such experiments should take sex-dependent differences into account because of differences in I_H and TBARS across sexes in the results of this study.

If the decrease in I_H results from a decrease in energy storage such as glycogen, future studies should also address the processes leading to an increased energy demand. For example, increased locomotor activity and concomitant increased oxygen consumption could lead to a higher energy demand in general or to increased oxidative stress. For example, farmed Atlantic salmon *Salmo salar* L. 1758 showed continuous swimming activity at night under continuous light as compared to reduced activity in dark nights of natural photoperiod (Oppedal *et al.*, 2001). It is not possible to estimate these complex processes from the results of this study, but the liver metabolism is probably of interest in future ALAN experiments.

4.4.5. General discussion of hypotheses

Most of the initial hypotheses were not verified by the experiments of this study. Therefore, based on the current results of this study, new hypotheses and follow-up experiments are needed to further investigate the effects of ALAN on the immune system and antioxidative responses. In the following, the authors discuss explanations for why the hypotheses were not verified and formulate research questions for future research.

Firstly, the direct conclusion would be that ALAN has no effect on the measured parameters of the immune system and indicators for oxidative stress in *P. fluviatilis*. Negative effects of ALAN could be compensated by means that were not measured in this study. *P. fluviatilis* is an euryoecious species that is known for its high potential for adaptation to a wide range of environmental conditions, which may be one of the reasons of its wide distribution range, and a robust immune system might be a key factor in this ability to adapt to a wide range of environmental conditions. There is hardly any information about adaptation potential of animals to ALAN. Because the origin of the experimental animals is only slightly light polluted (Lake Müggelsee experiences horizontal illuminance levels because of skyglow of probably ca. 0.005 lx, Jechow *et al.*, 2020), the authors do not assume that a lack of effects is because of adaptation of the experimental animals to the low intensities of skyglow. Nonetheless, if the immune system and the oxidative status of *P. fluviatilis* withstand ALAN effects, other fish species might be less flexible in adjusting to ALAN. For example, species-specific effects of continuous illumination were shown in early life stages of four different freshwater species (Brüning *et al.*, 2010). ALAN scenarios, however, might not be as comparable with scenarios of continuous illumination or long-day photoperiods as assumed. Even at extremely light polluted locations, days are still orders of magnitudes brighter than nights and therefore physiological rhythms may be maintained under ALAN

but not under continuous illumination depending on the intensity of nocturnal illumination. For example, rhythmicity of melatonin secretion in *P. fluviatilis* was only depleted at nocturnal intensities above 10 lx (Brüning *et al.*, 2015), whereas lower intensities of ALAN below 10 lx led to strongly reduced levels of nocturnal melatonin but rhythmicity was maintained (Brüning *et al.*, 2015, Kupprat *et al.*, 2020). In studies with long-day photoperiod, animals still experience dark nights but with a shortened scotophase. Thus, the hypothesized effects based on reduced melatonin may not come into effect because an intact rhythmicity of melatonin is still present despite changes in the amplitude.

Secondly, the duration of exposure may be critical for the consideration of responses to ALAN. To better predict the physiological implications of ALAN, it would be critical to also include long-term processes because ALAN may elicit rather slowly increasing responses such as physiological and behavioural compensatory mechanisms (Gaston *et al.*, 2015b). Similar to negative implications of lacking vitamin C in humans (e.g., Pohanka *et al.*, 2012), it is possible that changes because of reduced melatonin will come into effect only after longer exposure times, i.e., several months. Nonetheless, short-term effects after several days with compensatory acclimation processes after 2 weeks are also possible, but continuous monitoring of immune, antioxidative and conditional parameters is necessary to estimate the time scales of these processes.

Lastly, another option to further test the hypotheses of this study even in short-term experiments could be a combination of ALAN with another environmental stressor. Possible factors for multi-factorial experimental designs are increased temperature, reduced pH, reduced food availability or food quality, a combination with typical ecotoxicological stressors (e.g., exposure to heavy metals) as well as increased predation pressure or outbreaks of diseases, whereas the latter two are experimentally more sophisticated.

4.4.6. Sex effects

The LMMs of lysozyme activity and *K* from the high ALAN experiment suggested significant sex effects but did not have significant differences in the post hoc testing. For lysozyme activity and *K* in the high ALAN experiment, *p*-values of the sex effects are closely below the threshold for significance, which might explain why no effects in the post hoc test could be resolved.

In general, the results of this study show that there are differences among sexes in some physiological parameters of *P. fluviatilis*, particularly in TBARS, in the activities of antioxidative enzymes and in I_S and I_H, but without sex effects on the innate immune parameters. These results suggest that oxidative stress, hematopoietic and immunological activity of the spleen as well as hepatic energy storage are different across sexes. This underlines the importance of considering sex-dependent differences in future research investigating oxidative stress or body indices in fish.

4.5. Conclusion

In contrast to the initial hypotheses of this study based on previous research on the effects of continuous illumination or long-day photoperiod, the results of this

study do not indicate that ALAN affects the innate immune system or oxidative stress in *P. fluviatilis* after 2 weeks of exposure. The response parameters did not differ from controls with dark nights at various levels of nocturnal illumination between 0.01 lx and 100 lx in two separate experiments. Still, long-term studies or a combination with other factors such as elevated temperature as well as studies on other fish species should be the subject for future research on fish health with respect to light pollution and circadian rhythms. Because the authors found a significant decrease in the hepatosomatic index at 100 lx, the hepatic metabolism might be of interest for such studies. Further, both short-term and long-term studies are required for a better mechanistic understanding of how ALAN-disrupted behavioral and physiological biorhythms might modulate the immune system and oxidative stress of fish. This will lead to a deeper overall understanding of the potential threats of light pollution to fish.

Author contributions

F.H., F.K. and W.K. conceptualized the experiments. F.K., K.K. and T.P. planned and optimized the lab analyses. F.K. and T.P. ran the experiments, samplings and laboratory analyses. F.K. did the statistical analysis of the data and wrote the initial draft of the manuscript. All authors contributed to reviewing and editing the manuscript.

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Discussion

Revisiting the research objectives

At the beginning of my doctoral research, it was known that ALAN at intensities ≥ 1 lx suppresses nocturnal melatonin, and that ALAN reduces reproductive hormones during certain time windows in Eurasian perch and roach. The overall aim of this thesis was to find out if the low ALAN intensities of skyglow would already induce these effects. The underlying central motivation for ecophysiological ALAN research is the identification of effect threshold intensities to define appropriate regulatory measures for reducing light pollution in the future. For effects on melatonin (Chapter 1) and reproductive hormones (Chapter 2) a range for thresholds has already been known and thus, primarily lower light intensities were tested. For thyroid metabolism (Chapter 3) and for the immune system, antioxidative defense as well as for gross physiological condition (relative organ and body masses) (Chapter 4), only little was known about the effects of ALAN on fish. Hence, in Chapters 3 and 4, a wide range of light intensities was studied to see whether ALAN has an effect at all and if so, to determine respective thresholds.

Major findings

The low ALAN intensities of skyglow (0.01 – 1 lx) induced clear effects on melatonin and some effects on reproductive hormones (overview in Figure 6).

Low intensities of 0.1 lx and 0.01 lx suppressed absolute melatonin levels but did not impair rhythmicity. A threshold for absolute suppression could not be determined as 0.01 lx still evoked suppression of nocturnal melatonin in the water-based measurements (Chapter 1). At 1 lx, melatonin is further suppressed and the threshold for disturbance of rhythmicity appears to be around 1 lx (Chapter 1, Brüning *et al.*, 2015). Taking into account the results of Brüning *et al.* (2015), the dose-response relationship between ALAN intensity and suppression of nocturnal melatonin levels can be described (Figure 7), yet for full modelling, determination of a NOEL would be advantageous.

Effects on reproductive hormones (plasma 11 KT, pituitary expression of gonadotropins) induced by low skyglow-like ALAN intensities showed a rather complex picture (Chapter 2). In females, gene expressions of the *β*-subunits of gonadotropins (*fshβ* and *lhβ*) showed a differential response in both, a controlled and a field experiment, with a tendency for reduced *fshβ* expression but to lesser extent for *lhβ* in the controlled climate chamber experiment and significantly reduced *lhβ* but not *fshβ* expression in the field experiment. Male reproductive hormones were not affected by low skyglow-like ALAN intensities in our experiments, but the time of the year may be a factor influencing the sensitivity towards ALAN exposure (photo-labile period, see discussion in Chapter 2). Overall, skyglow-like low intensities of ALAN (< 1 lx) might not lead to the strong

effects on reproductive hormones observed in earlier studies with higher intensities representing direct exposure to ALAN (1 – 100 lx).

The thyroid hormones and different health parameters were mainly unaffected by ALAN (Chapter 3 and Chapter 4, overview in Figure 6). Few effects were statistically significant at the highest tested intensity of 100 lx, but at intensities ≤ 1 lx (skyglow), no effects were measured.

Triiodothyronine (T3) was significantly reduced at the highest tested ALAN intensity of 100 lx. Thyroxine (T4) and T3/T4 ratios were not significantly affected by ALAN but the mean T3/T4-ratio tended to be lower at ALAN intensities between 1 lx and 100 lx, indicating endocrine disruption (Chapter 3).

The hepatosomatic index (I_H; liver mass relative to body mass) was significantly lowered at the highest tested intensity of 100 lx. The splenosomatic index (I_S; spleen mass relative to body mass) and the condition factor (K; body mass relative to standard length) were not affected (Chapter 4). There were no effects of ALAN on respiratory burst activity of head kidney leucocytes or plasma lysozyme activity or antioxidative enzymes at any tested intensity (0.01 – 1 lx or 1 – 100 lx). Mean thiobarbituric acid reactive substances (TBARS) in liver homogenates were notably increased at 100 lx but not statistically significant. This may be an indicator for increased hepatic liver peroxidation. Despite indications in the literature that ALAN may lead to changes in these parameters directly or indirectly via reduced melatonin, the results do not suggest an effect of ALAN on the innate immune system or antioxidative defense system. However, long-term effects remain to be investigated.

Physiological implications of the observed ALAN effects

Melatonin as a sensitive proxy for physiological effects?

Melatonin appears to be the most sensitive of the investigated parameters (Figure 6) and elicits a clear response to ALAN (Figure 7A; Chapter 1). In a recent review, it was summarized that this sensitive response parameter appears to be consistent across different fish species from marine or freshwater ecosystems from different latitudes and temperatures (Grubisic *et al.*, 2019). In the relevant studies considered in this review, white light of 1 lx reduced plasma melatonin below 70 – 90% of the dark control levels in ten different teleost species (Grubisic *et al.*, 2019). The significant melatonin suppression at 0.01 lx in Chapter 1 is one of the lowest observed effect levels for melatonin suppression in teleost fish. Still, a NOEL could not be defined.

Not only the suppression of absolute melatonin levels is an important indicator of physiological ALAN effects, but also the impairment of melatonin rhythmicity by ALAN needs to be considered. The distinct pattern of daily plasma melatonin with low levels during the day and higher levels throughout the night (i.e., rhythmicity), might be equally or even more important as the total amount of circulating melatonin. The two melatonin parameters might be linked to other physiological parameters investigated in Chapter 2 – 4. The correlation of melatonin with other

physiological parameters requires further investigation and modes of action need to be established. Melatonin and melatonin rhythmicity may then serve as a fast and sensitive proxy for ALAN effects on the physiology of vertebrates. Ultimately, the aim would be *ex vivo* systems, (e.g., cultured pineal glands) to screen sensitivities towards ALAN exposure for many species.

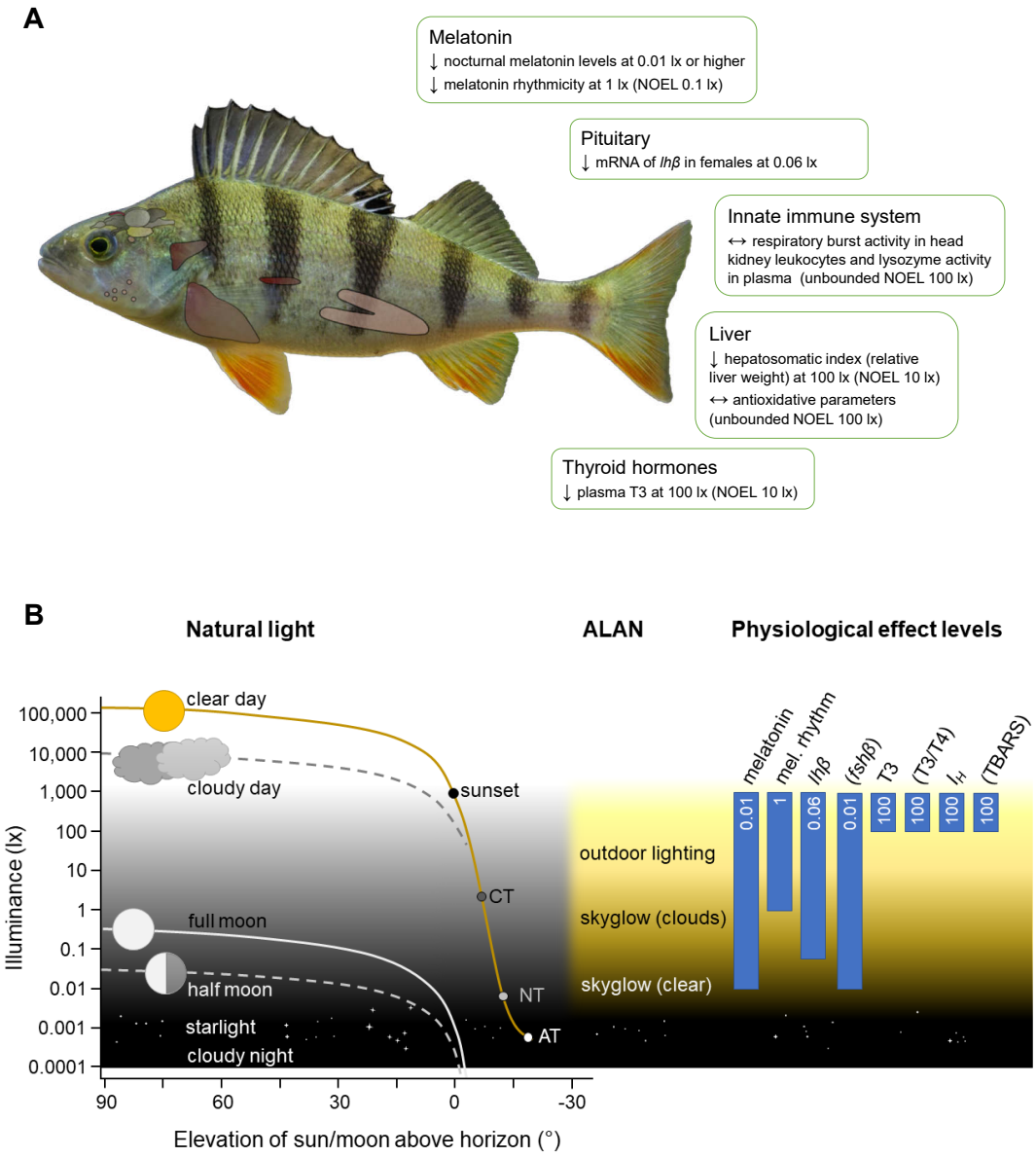

Figure 6 A) The physiological effects of artificial light at night (ALAN) in different organs of Eurasian perch (*Perca fluviatilis*). Lowest-observed-effect levels (LOEL, $p < 0.05$) or no-observed-effect level (NOEL) as determined after exposure to surface nocturnal illuminations of 0.01 lx up to 100 lx for two weeks, or of 0.06 lx for 1 – 2 months (*lhβ*). Photo courtesy of Andreas Hartl. **B)** LOELs of ALAN effects in Eurasian perch (blue boxes on the right) compared to natural illuminance on the left-hand side. On the right-hand side, above the blue boxes, effects in brackets had considerably different means but were not statistically significant ($p > 0.05$) due to low sample size. On the left-hand side, natural illuminance is displayed as a function of sun (solid yellow line: clear day; dashed yellow line: cloudy day) or moon (solid gray line: full moon illuminance; dashed gray line: illuminance by first-/last-quarter moon, "half-moon") elevation. CT: lower boundary of civil twilight, NT: lower boundary of nautical twilight; AT: lower boundary of astronomical twilight. In the middle, ALAN sources refer to a range of illuminances. Modified from Grubisic *et al.* (2019) and Gaston *et al.* (2014)

The dose-response relationships of nocturnal melatonin levels and melatonin rhythmicity and ALAN intensities are represented in Figure 7. For this figure, mean melatonin concentrations were calculated as relative change from the controls without nocturnal light ("0 lx") from Chapter 1 (Kupprat *et al.*, 2020) and a previous, methodologically similar study (Brüning *et al.*, 2015). The combined relative-change means are presented in Figure 7A. For melatonin levels, the response looks rather linear at lower ALAN intensities. An effect plateau at intensities below the NOEL (as it is typically modelled in dose-response relationships, e.g., by the Hill equation) might not be overly pronounced for melatonin due to the direct release of melatonin from the pineal gland to the cerebrospinal fluid and the direct control of AANAT2 protein amount by light (hyperpolarization of pineal photoreceptor cells and subsequent degradation of AANAT2) (Falcón, 1999, Falcón *et al.*, 2010). This would mean, that even with natural nocturnal illumination of starlight and moonlight, nocturnal melatonin values would vary naturally, depending on moon phases and cloud cover. Skyglow illuminates the sky persistently at low levels of starlight and moonlight intensities, and can cover up lunar illuminance rhythms (Puschnig *et al.*, 2014a). Therefore, the question arises, which role lunar rhythms play in the physiology of Eurasian perch and which consequences can be expected if these rhythms were lost due to light pollution (see discussion in Chapter 1).

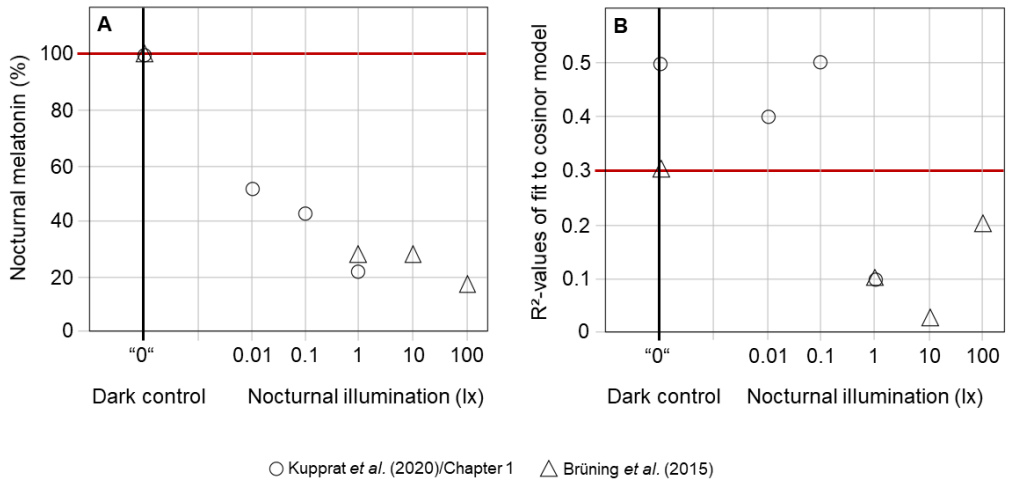

Figure 7 A) Relative melatonin content in the aquarium water of Eurasian perch (*Perca fluviatilis*) exposed to different intensities of artificial light at night (ALAN, 0.01, 0.1, 1 lx or 1, 10, 100 lx) compared to control levels without nocturnal illumination (< 0.00167 lx, "0 lx"). Thirty Eurasian perch in 80-L aquaria were exposed to ALAN for 10 – 11 days with a low water flow-through of 4 L h^{-1}. The red line depicts the reference at 100% (controls). Values represent the mean change of measured melatonin concentrations at night (maximum values at 5 a.m.) relative to the controls. All data were significantly different from controls ($p < 0.05$). **B)** Melatonin rhythmicity in Eurasian perch at different intensities of ALAN. R^2 values of the fits to the cosinor model serve as a measure for rhythmicity of the data from Kupprat *et al.* (2020)/Chapter 1 (circles) and Brüning *et al.* (2015) (triangles). The red horizontal line depicts the threshold of rhythmicity (lowest control value) as defined by Brüning *et al.* (2015).

Moreover, the fit of the cosinor model served as a proxy for rhythmicity (Chapter 1) and R^2 values from two studies are presented together. The threshold for rhythmicity ($R^2 = 0.3$) is defined by the controls from the experiment by Brüning *et al.* (2015) (Figure 7B). The threshold for impaired melatonin rhythmicity lies in the range between 0.1 lx and 1 lx. This range of ALAN intensities covers the maximum

natural nocturnal illumination by the full moon, which does not exceed 0.4 lx (Kyba *et al.*, 2017b). Hence, melatonin rhythmicity is maintained at ALAN illuminance in the range of naturally varying nocturnal illuminance of the moon phases, but rhythmicity is disturbed when illuminance exceeds natural levels. Although still below the 0.3, it is not fully clear why the R^2 value at 100 lx is larger than at 10 lx and 1 lx. This could be incidental, but it is also conceivable that there is a certain threshold where the light is so bright that internal rhythms outweigh melatonin regulation, like a “safety-switch” to maintain some rhythmicity.

Linkage of suppressed melatonin to other physiological effects

We have a good understanding of the action of ALAN on plasma melatonin at various intensities in teleost fish (Grubisic *et al.*, 2019) and of the underlying mechanisms, e.g., depolarization of photoreceptor cells in the pineal gland (Falcón *et al.*, 2010), but less is known about the mode of action of melatonin on other physiological processes, i.e., secondary effects of ALAN. Results from my thesis give first insights into which parameters might be correlated to melatonin levels or the more complex parameter of melatonin rhythmicity. A statistical analysis of correlations between melatonin and other physiological effects was not feasible because melatonin values were from indirect water-based measurements. The causality of correlations will have to be established on a molecular level with mode of action studies including description of the molecular clock systems and identification of melatonin receptors in the affected tissues of Eurasian perch. The presence and expression pattern of melatonin receptors can be a valuable hint on the action of melatonin and thereby also indicate secondary effects of reduced melatonin (see introduction on melatonin receptors). Understanding the mode of action of melatonin or rather reduced melatonin on various physiological functions, will be important to extrapolate from this sensitive endpoint to other physiological effects.

The absolute nocturnal production of melatonin is impaired at low intensities of 0.01 lx. Reproductive processes can be impaired at these low intensities, which might be linked to the reduction of nocturnal melatonin levels. Melatonin can act on the pituitary gland and thereby influence gonadotropin gene expression (Falcón *et al.*, 2010, Isorna *et al.*, 2017, Maitra and Hasan, 2016) (also see Introduction on melatonin receptors). For example, in European seabass, decreased plasma melatonin levels induced by long photoperiod were associated to a decrease of nocturnal LH (Bayarri *et al.*, 2004). The other way around, additional melatonin administration led to an increase in reproductive parameters such as the gonadosomatic index (I_G) or sexual hormones in masu salmon (*Oncorhynchus masou* Brevoort, 1856) (Amano *et al.*, 2000), zebrafish (Carnevali *et al.*, 2011), or Atlantic croaker (*Micropogonias undulatus* L., 1766) (Khan and Thomas, 1996). Opposingly, other studies suggested the opposite, i.e., melatonin administration led to decreased reproductive performance, e.g., in European seabass (Alvarado *et al.*, 2015), European eel (*Anguilla anguilla* L. 1758) (Sébert *et al.*, 2008), or Gangetic catfish (*Mystus cavasius* Hamilton, 1822) (Badruzzaman *et al.*, 2020). Such controversial results suggest that the mode of action of melatonin on the HPG axis might depend on species, sex, maturation status, time of the year, and also time of the day of melatonin administration (Renuka and Joshi, 2010). This

species- and time-specific reaction to light and melatonin might be explained by adaptation to the respective ecological niche of the species. Since nocturnal melatonin levels vary naturally throughout seasons in temperate regions, due to changing daylengths (and temperature, also see Figure 3), the ALAN-dependent suppression of melatonin and its role in ALAN-dependent reduction of reproductive hormones would at best have to be considered over the full annual cycle to estimate effects of ALAN on reproduction. As shown in Chapter 2, responses to low intensities of ALAN can be differential and weak. The natural role of lunar rhythms on reproductive processes in temperate fish species has hardly been considered in the literature (except for smoltification of salmon), but since lunar rhythms can apparently be sensed (possibly via melatonin) there might be an underestimated fine-tuning by the moon involved in reproductive and developmental processes. A melatonin-mediated sensing of lunar phases in order to synchronize lunar or semi-lunar spawning has been suggested for tropical coral reef fish species (Takemura *et al.*, 2010). Growth rates of early life stages of coral reef fish were recently reported to be dependent on the lunar phase, but melatonin was not measured in this study (Shima *et al.*, 2021).

Correlations of ALAN, melatonin levels or melatonin rhythmicity on thyroid metabolism are discussed in Chapter 3 and require further investigations before conclusions can be drawn. Interestingly, plasma T3 tended to gradually decrease between 1 lx and 100 lx (statistical significance reached at the highest tested intensity of 100 lx) which coincides with impaired melatonin rhythmicity at intensities of ≥ 1 lx. Other species might be affected at even lower levels like migrating salmonids who showed significant correlation of T4 surge at dates of the new moon and the full moon light might be involved in preventing the T4 surge to ensure safe seaward migration of smolts in the darkest nights (Grau *et al.*, 1981). To date, no data are available for melatonin receptors in thyroid follicles of fish but TSH production in the pituitary gland might be regulated by melatonin via melatonin receptors (see Introduction on melatonin receptors). In zebrafish, melatonin synthesis starts as early as 1 day post-fertilization, which indicates that melatonin may play crucial role in early development at which TH are key (Elbaz *et al.*, 2013).

The ALAN effects of reduced plasma T3 and hepatosomatic index might each have a causal association to melatonin rhythmicity, but less likely not to absolute nocturnal melatonin levels, as these two parameters were not impaired at lower intensities at which melatonin was still reduced.

The hypothesis of increased oxidative stress due to the lacking antioxidative potential of melatonin under ALAN (Chapter 4) was built upon the absolute melatonin levels and to a lesser extent on rhythmicity. However, no indication of increased oxidative stress could be measured in Eurasian perch, which means that within two weeks, decreased melatonin is not seriously affecting the antioxidative defense system. Although melatonin is often discussed as an important antioxidant, reduced or lacking melatonin may only lead to measurable changes in antioxidative defense proxies at higher intensities or longer exposure.

Melatonin and parameters of the innate immune system did not correlate as expected (Chapter 4), but there are hints in the literature from other species that there might be effects of ALAN on the immune system, but perhaps only at very

high intensities and/or only after very long exposures (also see discussion in Chapter 4). Baekelandt *et al.* (2019) reported that an increase of melatonin in fall boosts immune parameters in pikeperch (*Sander lucioperca* L., 1758). In carp, melatonin receptors were evidenced in peripheral blood leucocytes, head kidney leucocytes and lymphatic tissues (Kepka *et al.*, 2015). Moreover, melatonin administration increased the respiratory burst activity of monocytes *in vitro* but not *in vivo* (Kepka *et al.*, 2015). However, ALAN cannot lead to direct effects of additional melatonin, as it reduces natural levels of melatonin. It is conceivable that a "buffering zone" with "physiologically acceptable" melatonin reduction and time frame of reduced melatonin; this physiological acceptance may cover the extent of natural melatonin fluctuations by varying moon phases and cloud cover. If this concept proves correct, an ALAN exposure duration of several months would be necessary to clearly precede natural moon light variations and exceed "physiologically acceptable" melatonin reduction.

As partially discussed in Chapter 1, melatonin might also be linked to behavioral patterns, which could partially explain ALAN effects on different behaviors of fish. For example, ALAN increased foraging activity (Bergman, 1988, Flik *et al.*, 1997, Ohlberger *et al.*, 2008, Townsend and Risebow, 1982), swimming activity (Oppedal *et al.*, 2001), daytime risk taking (Kurvers *et al.*, 2018), and parental care behavior (Foster *et al.*, 2016). Although none of the behavioral studies measured melatonin, all these behaviors could be associated to reduced plasma melatonin as they are consistently reduced by ALAN in different fish species (Grubisic *et al.*, 2019). Moreover, reduced melatonin is associated to sleep deprivation of diurnal vertebrates, for fish mainly investigated in zebrafish (Elbaz *et al.*, 2013, Gandhi *et al.*, 2015), which is in line with increased locomotor activity (Foster *et al.*, 2016, Oppedal *et al.*, 2001), but could also be associated with increased risk-taking during daytime (Kurvers *et al.*, 2018). Overall, there is a large data gap in sleep research and the functional meaning of sleep for the physiology of fishes other than zebrafish. In nocturnal fish species, the link of melatonin to behavior might be different. In European eel, for instance, migratory behavior is reduced when exposed to ALAN (Lowe, 1952, Vowles and Kemp, 2021, Walker *et al.*, 2014), but again melatonin was not subject to investigation in these studies. If activity of Eurasian perch was constantly increased under skyglow illuminance, this would require more energy, which may partially be compensated for by increased feeding (Bergman, 1988, Flik *et al.*, 1997), but could also lead to increased use of energy storage and energetic mismatches.

The liver as a central organ of physiological ALAN effects?

One direct effect on the liver and two liver-related effects were observed after two-week exposures to ALAN of 100 lx (Chapter 3 and 4). Firstly, the I_H was reduced indicating reduced energy reserves, and secondly, the TBARS in liver homogenates were considerably increased but without statistical significance indicating increased peroxidation of liver lipids (Chapter 4). Thirdly, the reduced plasma T3 and trend of reduced T3/T4 ratio might be explained by a decreased activity of deiodinase 2 (DIO2, Chapter 3). The decreased plasma T3 in Eurasian perch might originate from reduced DIO2 activity in the liver, assuming similar liver-derived plasma T3 levels as reported in smolting Atlantic salmon (Morin *et al.*,

1993). Concurringly, outer-ring deiodination was greater in liver than in other investigated organs in American plaice (*Hippoglossoides platessoides* Fabricius, 1780) (Adams *et al.*, 2000). Thyroid metabolism has generally been suggested to be associated with hepatic metabolism in fish (Peter, 2011). It remains entirely open whether the different liver parameters (directly or loosely related to liver metabolism) are related to each other, because the liver is responsible for a large variety of metabolic functions and causality needs to be investigated in more specific mode of action studies. Nevertheless, these are the first results that indicate that the liver can be a very interesting organ to look at in future ALAN research.

Can observed ALAN effects become adverse effects on the long run?

The International Programme on Chemical Safety defined physiological effects as adverse when an effect is a "*change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences*" (IPCS, 2004). Adverse effects can include for example, increased mortality, irreversible developmental malformations or impaired reproductive success. If an environmental stressor results in adverse effects, it can strengthen the argumentation for regulatory conservation measures. The results presented in this thesis are not considered adverse *per se* but show some indications that adversity may be observed after longer, continuous exposures due to ALAN being present every night and not just occasionally.

Adverse effects related to reproduction (Chapter 2)

Reduction of gonadotropin expression is not an adverse effect *per se*. However, if it is linked to reduced fertility and less offspring later in the reproductive cycle, it can be used as a proxy for adverse outcomes. Long-term studies which link reduced gonadotropin expression early in the reproductive cycle to adverse outcomes with respect to ALAN exposure, still need to be carried out.

In Eurasian perch, gonadal development was completely suppressed in males and females exposed to continuous illumination of 500 lx over a 10-month period (Migaud *et al.*, 2006). In the same experiment, long photoperiod (16 L:8 D) applied for 10 months reduced I_G, delayed spawning compared to natural photoperiod, and 65% of the maturing females did not spawn at all. Moreover, prolonged photoperiod resulted in lower fecundities, reduced egg quality and lower fertilization rates (8% compared to 57% under natural photoperiod). Hatching rates were null compared to 54% under natural photoperiod and parental mortality doubled under longer photoperiod (Migaud *et al.*, 2006). Reproductive hormones were not addressed in this study, but this study clearly shows the potential of artificial light and photoperiod manipulations for adverse reproductive outcomes after longer exposure. Still, realistic ALAN exposure is not as extreme as the applied experimental conditions.

With respect to adverse reproductive outcomes, fertilization, hatching and larval survival are key parameters, which have been investigated under ALAN exposure in different fish species. For clownfish, hatching was entirely suppressed by ALAN of 25 lx (parents and eggs exposed, controls with 86% hatching rates), but no relations to hormones were made in this study (Fobert *et al.*, 2019). Hatching of larvae (time to 50% hatch) was shown to be delayed by experimental application of continuous illumination of 3500 lx in chub and bleak whereas the duration of the hatching period was prolonged in Eurasian perch and roach (Brüning *et al.*, 2010). Dispersal of Atlantic salmon fry was prolonged at exposure to ALAN of 12 lx (Riley *et al.*, 2013). Such delay in hatching or larval dispersal can lead to increased predation and finally to reduced numbers of surviving offspring, as also discussed by Brüning *et al.* (2010). In amphibians, fertilization rates were affected corresponding to delayed reproductive behavior by ALAN of 0.1 lx and 5 lx in the common toad, but testosterone was not affected (Touzot *et al.*, 2020). A related study further found reduced activity and changed energy allocation after exposure of male breeding common toads to ALAN of 5 lx or 20 lx (Touzot *et al.*, 2019) which may be related to the reduced reproductive success.

As proposed in Chapter 2, one- or two-generational experiments are needed to investigate the effects of ALAN on the full reproductive cycle and understand the mechanism leading to the outcome (e.g., OECD, 2015).

Adverse effects related to thyroid hormones (Chapter 3)

Reduced T3, as observed in Eurasian perch (Chapter 3), can lead to lower metabolism and impaired development and malformations (also see discussion Chapter 3). With respect to this, there might also be a link to the observed effects of ALAN on hatching and development as described above (Brüning *et al.*, 2010, Fobert *et al.*, 2019). Given the precise timing of spawning to guarantee optimal use of the temporal ecological niche for each species, offspring might run into fundamental trouble if this timing is disturbed by delayed development or growth. The fry dispersal of Atlantic salmon was delayed by ALAN of 12 lx (Riley *et al.*, 2013) but it is not clear whether this behavior of salmon is mediated by melatonin or thyroid hormones. In a coral reef fish, convict surgeonfish, whole-body T3 was significantly reduced on day 2 and T4 increased without statistical significance on day 5. After the study period, larval mortality rates were significantly increased under ALAN of 20 – 25 lx (O'Connor *et al.*, 2019). Similarly, underwater illuminance of approximately 4 lx reduced survival and growth of juvenile Orangefin anemonefish (*Amphiprion chrysopterus* Cuvier, 1830), but TH were not measured in this study (Schligler *et al.*, 2021). Studies on hatching and larval survival (Brüning *et al.*, 2010, O'Connor *et al.*, 2019, Riley *et al.*, 2013) were also conducted at rather high light intensities (15 – 3500 lx continuous illumination) and do not resemble typical skyglow intensities. Therefore, the question whether skyglow is already bright enough to induce the listed adverse developmental effects should be subject for future research. In amphibians, metamorphosis was impaired in the American toad at ALAN exposure of 3 – 15 lx, but TH were not assessed in this study (Dananay and Benard, 2018).

Irrespective of the major concern of developmental effects, T3 also has some regulatory role of metabolism in adults which might be measured by reduced

oxygen consumption potentially related to minor changes in liver metabolism (see discussion above), but this is likely not adverse. Reduced TH in adults may become adverse if they reduce fertility or impair other reproductive processes but understanding of the involvement of TH in reproductive cycle is little understood in teleosts.

Unrelated to ALAN, reduced T3 can lead to a variety of reproductive and developmental adverse effects, which has so far mainly been reported for zebrafish. For instance, reduced TH in parental female zebrafish (experimentally evoked by exposure to perchlorate) was associated to malformed lowered jaws in the offspring (Mukhi and Patiño, 2007). Reduced T3 and increased T4 in zebrafish larvae (after experimental exposure to butylated hydroxyanisole) were related to increased rates of malformation and decreased calcification of vertebrae (Zhao *et al.*, 2020). Swim bladders did not inflate in zebrafish larvae along with reduced TH after exposure of embryos to perfluoropolyether carboxylic acids (Wang *et al.*, 2020). More studies with direct association to ALAN are necessary, but in a worst-case scenario, similar effects might occur after continuous reduction of T3 caused by ALAN exposure as observed in Eurasian perch (Chapter 3).

Adverse effects related to reduced I_H (Chapter 4)

Reduced I_H can be interpreted as reduced energy storage in the liver, which can become disadvantageous for a fish on the long run.

Reduced T3 and reduced I_H were observed only at 100 lx which is in the range of direct illumination, and it is not expected that Eurasian perch or any other fish are exposed to such levels for a long time. Therefore, an establishment of long-term effects of realistic low light ALAN conditions on T3 or I_H would be needed first before conclusions or extrapolations to adverse effects can be drawn. For now, it can only be assumed that effects at high ALAN intensities after two weeks, would persist or even worsen over longer exposure of several months and years. Some examples from the literature support this assumption. For example, clear increases of lysozyme concentrations in rainbow trout after photoperiod manipulations were only evident after 142 d, whereas earlier measurements (30, 60, or 90 d) did not show clear increase (Burgos *et al.*, 2004). Likewise, total immature erythrocytes of rainbow trout increased only significantly after 60 d of long photoperiod, and results were ambiguous at day 7 and 30 (Valenzuela *et al.*, 2007). In the same study, changes in leucocytes, lymphocytes, and thrombocytes were only significant after 60 d of treatment with 14 L:10 D and 90 d recovery in 12 L:12 D (Valenzuela *et al.*, 2007). Reduction of I_H was only significant after 20 and 30 days, but not after 10 days of arsenic treatment in striped dwarf catfish (*Mystus vittatus* Bloch, 1794) (Verma and Praksh, 2019). Still, the cumulative nature of ALAN effects remains speculation until more elaborated long-term experiments are conducted. It is also conceivable that the effects of realistic ALAN exposure (in low intensities similar to skyglow, i.e., < 1 lx), can be compensated each day which would limit the overall impact of ALAN on the long run. In general, it can be assumed that effects, which are directly induced by light/ALAN, would intensify over longer exposures, whereas for indirect effects (which again depend on several parameters) intensification of ALAN effects cannot be taken for granted since they might be compensated for on a regular basis. A non-experimental, correlational approach to analyze long-term

effects of ALAN on physiological parameters would require sampling of fish from many different lakes with a variety of turbidities and skyglow exposure levels. If such a sampling would occur regularly throughout the year, not only melatonin effects could be correlated but also reproductive parameters.

Light pollution in comparison to other pollutants or environmental stressors

For a better classification of ALAN as an environmental stressor on fish physiology, the following summarizes effects from exposure to known pollutants (thermal or chemical pollution) that are comparable to the ALAN effects in this thesis.

Melatonin

Rhythmic interchange of light and darkness appears to be the main regulator of melatonin rhythms. To date, no other pollutants or environmental stressors are known that ALAN could be compared to with respect to the observed effects of reduced nocturnal melatonin and shortening of rhythmic phase. As fish are ectothermic animals, temperature is a natural co-variant to light modulation of melatonin rhythms. Porter *et al.* (2001) measured melatonin profiles in juvenile Atlantic salmon under various nocturnal illuminations and at two different temperatures (4 and 12°C). The reduced temperature (difference of 8°C) slows the metabolism of ectothermic animals and reduced the amplitude of nocturnal melatonin by 50% of the amplitude at 12°C. Hence, reduced temperature had comparable effects as 400 lx of ALAN in this study. However, shortening of the phase of the melatonin rhythm were not reported for ALAN nor for reduced temperature (Porter *et al.*, 2001). No effects of other pollutants comparable to the observed phase shift of melatonin rhythms have been reported in fish (Chapter1). The only vaguely comparable effect in the literature is a phase shift of pineal melatonin rhythms in rats under timed food restriction diet (50% of ad libitum food intake only for 2 h in the morning) compared to ad libitum feeding rats (Challet *et al.*, 1992). Overall, with respect to the observed effects on melatonin, it is rather difficult to compare ALAN to other environmental stressors or pollutants.

Reproduction

Reduced expression of gonadotropins, as partially observed after ALAN exposure, have been reported for several fish species after exposure to different chemical or pharmaceutical pollutants. For example, diclofenac exposure significantly reduced *lhβ* expression from pituitaries of juvenile Nile tilapia (80 days post hatching) with no significant *fshβ* reduction (Gröner *et al.*, 2017). Another example is the significant suppression of *fshβ* in the pituitary gland of juvenile zebrafish by bisphenol A exposure of 20 d (with no significant change in *lhβ* expression) (Chen *et al.*, 2017b). Expression of both *fshβ* and *lhβ* were reduced in adult female zebrafish after 96 h embryonic exposure to benzo(a)pyrene (Gao *et al.*, 2018) or in female medaka after exposure to phenanthrene for 80 d (Sun *et al.*, 2015). These are just a few examples for well-studied pollutants that result in similar effects like ALAN in the experiments described in Chapter 2 and justify the term light pollution for ALAN. Recently, it was demonstrated in Arctic charr that photoperiod manipulations can be more effective in preventing sexual maturation than food deprivation in winter (Liu and Duston, 2019), which may be beneficial in

aquaculture appliances but also underlines the power of ALAN to disrupt reproductive processes in fish.

Thyroid hormones

As discussed in Chapter 3, reduced T3 and stable or slightly increased T4 observed after ALAN exposure are comparable to effects of low pH (Brown *et al.*, 1989, Brown *et al.*, 1984, Brown *et al.*, 1990), and typical endocrine disruptors such as polychlorinated biphenyls (Adams *et al.*, 2000, Leatherland and Sonstegard, 1978), kerosene (Peter *et al.*, 2007), or estradiol (Cyr and Eales, 1990, Cyr *et al.*, 1988b, Leatherland, 1985).

Liver weight

The observed ALAN-effect size of reduced I_H is comparable to effects of increased temperature for 20 d (+4°C) or fasting (15 d fasting, 5 days re-feeding) in juveniles of a freshwater carnivorous catfish (*Lophiosilurus alexandri* Steindachner, 1876) (Favero *et al.*, 2019). Further, 30 d exposure of striped dwarf catfish to arsenic (Verma and Praksh, 2019) reduced I_H to a comparable extent as observed in this thesis (Chapter 4). Exposure of adult female banded gourami (*Trichogaster fasciata* Bloch and Schneider, 1801) to high concentrations of manganese sulphate (2500 mg L^{-1}) reduced I_H to a stronger extent than reported in this work for ALAN (Chapter 4) (45%reduction from control) after only 90 h (Agrawal and Srivistava, 1980).

Ecological implications of the observed ALAN effects

In situ occurrence of investigated ALAN intensities

The lower ALAN intensities (≤ 1 lx) in the climate chamber experiments resembled the illumination by skyglow in transparent shallow waters (see discussion of Chapter 1 for details). The light intensities that fish actually experience and also the spectrum of light depend on the predominant light sources, the water turbidity as well as the swimming depth of the fish. Furthermore, the individual extent of skull pigmentation determines how much light reaches the pineal photoreceptor cells.

According to “the new world atlas of artificial night sky brightness” (Falchi *et al.*, 2016) roughly 14% of the worldwide population (2% of area) and 21% of the EU population (0.6% of area) are exposed to more than 3000 µcd m^{-2} (converted by $E_V = \pi * L_V$ this is roughly comparable to 0.01 lx). This may appear a small fraction of the global area but as humans settle close to water (Kummu *et al.*, 2011), exposures of water bodies, especially shores may be much brighter. Moreover, the radiance values in Falchi *et al.* (2016) were calculated from satellite data in moonless clear nights and thus, irradiance may be manyfold brighter when clouds cover the sky and intensify the scattering of ground-based light sources (Jechow *et al.*, 2016, Kyba *et al.*, 2011, Kyba *et al.*, 2015). For marine coastlines it was estimated for 2010 that roughly 22% of coastlines worldwide (excl. Antarctica), and more than 50% of European coastlines regularly experience ALAN (Davies *et al.*, 2014). It is currently not possible to calculate the percentage of freshwater areas (lakes and rivers) that is affected by certain levels of ALAN on a global scale. The

spatial resolution of remote sensing from satellite data is not enough, yet, to resolve for rivers and small lakes (Jechow and Hölker, 2019a). Estimates of the extent of light pollution on the surface of freshwaters in Berlin showed that flowing waters (rivers and canals, 1.5% of the area) are illuminated 6 times brighter than standing waters (ponds, lakes, 3.5% of the area); for comparison streets (13.6% of the area) were 511-times brighter than standing waters (Kuechly *et al.*, 2012). At best, spatial 3D models also of the underwater light propagation (like Jechow *et al.*, 2021, Tamir *et al.*, 2017) would be needed to really estimate how much ALAN fish actually experience.

Overall, skyglow-like intensities below 1 lx reduced nocturnal melatonin (Chapter 1) and reduced gonadotropin expression under specific circumstances in Eurasian perch (Chapter 2), but a threshold for these effects could not be established. Twilight-like, strong nocturnal illuminance of 100 lx acts as a physiological threshold in Eurasian perch for reduced liver weight (Chapter 4), reduced triiodothyronine (but not thyroxine, Chapter 3) as well as reduced cortisol in an earlier study (Brüning *et al.*, 2015). However, only few freshwaters are expected to experience such high illuminance by ALAN and if so, only occasionally. In general, Eurasian perch is a representative for freshwater fish in Europe, which is likely to experience light pollution over vast areas. However, this species has been reported to be overrepresented in urban freshwater ecosystems and may overall still benefit from the urban ecosystem for reasons which may not be related to light pollution, and which are not investigated in this thesis (e.g., lack of competition due to undemanding requirements for spawning grounds). For example, 90% of the sampled fish in channels in Berlin were Eurasian perch and roach (Jürgensen *et al.*, 2019).

Which other fish species might be vulnerable to ALAN exposure?

My thesis investigated ALAN effects on the physiology of Eurasian perch. At higher ALAN intensities ≥ 1 lx, physiological effects on Eurasian perch were comparable to effects in roach (Brüning, 2016). Generally, the Eurasian perch and roach are considered eurytope species with a high potential for adaptation to new environmental conditions. Fishes with more specific adaptation to daily and/or seasonal changes in light levels may have a narrower defined temporal ecological niche. Among teleosts, similar modes of action of ALAN on pineal melatonin production can be assumed as results are widely consistent across species and habitats (Grubisic *et al.*, 2019). For thyroid metabolism in teleosts, available data in the literature is less consistent with respect to photoperiod manipulations. Therefore, more experimental data is required to understand similarities and differences across species and habitats. Similarly, some contradictions have been revealed between the data on reproductive hormones (Chapter 2), thyroid hormones (Chapter 3) as well as antioxidative and immune parameters (Chapter 4) presented here and data available from the literature on effects of continuous light or long photoperiods in other species.

Specific sensitivity towards ALAN widely depends on the ability to sense light in general, i.e., it depends on the sensitivity of the photoreceptors with respect to

intensities and spectrum. Moreover, a variety of photoreceptors must be considered (pineal, ocular, deep-brain, skin; see Introduction). Naturally, fish species with the highest sensitivity towards light would be most likely to be affected by ALAN.

Many salmonid species are known to lack a clock-controlled production of melatonin and hence, melatonin production is controlled in an on/off-switch manner by light (Falcón *et al.*, 2010). This can lead to a particular sensitivity towards ALAN. Especially salmon undergoing long and exhausting migrations to spawning grounds can be particularly vulnerable (Falcón *et al.*, 2020). Timing of seaward migration of salmon smolts occurs during night (Grau *et al.*, 1981) and ALAN can disturb this timing in Atlantic salmon smolts (Riley *et al.*, 2012). Moreover, fry of Atlantic salmon disperses from their nests during darkness and streetlights can seriously delay fry dispersal into times of daylight, when fry is more easily visible to predators (Riley *et al.*, 2013). Further, smoltification is a critical step in development and involves a surge of T4 to induce changes necessary for the seaward migration. The T4 surge coincided with the new moon period in several salmonid species and populations (Grau *et al.*, 1981). Overall, salmonids appear to be particularly vulnerable towards artificial light exposure. Hence, ALAN exposure of native streams of salmonids should be avoided for protection of these economically important species.

Flatfishes also show a distinct metamorphosis triggered by thyroid hormones, which can potentially be varied by ALAN. However, flatfishes typically live close to the ground and are therefore less likely to experience light pollution.

In general, species and certain life stages that live close to the surface or in shallow waters (e.g., littoral species or life stages) are expected to experience more light (natural and artificial) and can therefore be expected to have a higher light sensitivity, which might result in stronger effects of ALAN. However, nocturnal whereabouts remain mostly unstudied for diurnal species known to live close to the surface (e.g., bleak) or diurnal littoral species (juvenile chub or dace), and therefore it is hard to predict the susceptibility of these species to ALAN. Moreover, species that show pronounced positive phototactic behavior are expected to be affected strongly by ALAN in terms of behavioral changes but also with strong physiological effects as the experienced light intensities will increase the closer the fish gets to the light source. However, although perceived illuminance will be much lower, species with a strong negative phototaxis can also be vulnerable to ALAN in terms of behavioral changes. For example, European eel avoid crossing illuminated passages during their naturally nocturnal migration (Lowe, 1952, Vowles and Kemp, 2021, Walker *et al.*, 2014). In general, species with a distinct behavioral temporal pattern (strictly diurnal or strictly nocturnal) will be more affected by ALAN than species that are temporally more flexible. For example, nocturnal prey fish need the safety of darkness throughout the night to avoid being seen by (day-active) visual predators. Nocturnal predators would require darkness to not be avoided by their (day-active) prey. Diurnal, visual predator fishes (such as Eurasian perch) may have a foraging advantage due to ALAN by expanding their temporal ecological feeding niche. It remains to be investigated further whether such an advantage outweighs the potential adverse effects of ALAN on

population level (higher visibility for other larger predators, omission of resting phases, and the physiological effects investigated in this thesis).

Ecosystem-scaled effects of ALAN

All in all, light pollution seems like a minor stressor compared to other extensively studied stressors like global warming or increasing loads of xenobiotics and nutrients through sewage water and agricultural run-off into freshwaters. However, light pollution is a rather recently recognized additional stressor and may exacerbate other effects. Since the fundamental environmental cue of photoperiod is altered, which most organisms have adapted to in terms of biological rhythms, ecosystem-scaled effects of ALAN are conceivable (e.g., shifts in the food web or effects on predator-prey interactions).

Activity of visual feeders like Eurasian perch is generally low at night (especially in dark new moon nights), but even low light intensities can already increase feeding behavior (Bergman, 1988, Czarnecka *et al.*, 2019, Flik *et al.*, 1997, Nakayama *et al.*, 2018). This might be linked to increased swimming behavior, which in turn could be associated with lower levels of melatonin in the blood (discussed in Chapter 1). The ecosystem-scaled effects of increased predation are difficult to estimate because also prey behavior needs to be considered. Especially juvenile Eurasian perch visually feed on zooplankton, for example on species of *Daphnia* (Cladocera). Eurasian perch and *Daphnia magna* are a popular predator-prey-model system as *Daphnia* species (like many other zooplankton species) display a pronounced predator-avoidance behavior called diel vertical migration (DVM): At a certain predator density, zooplankton species spend daytime in deeper and darker water layers to avoid visual predation and migrate to the surface during night, when chances of visual predation are lower. Zooplankton can sense chemical cues of predators, called kairomones, but to anticipate changes in predation pressure, DVM evolved a light-sensitive process. *D. magna* also expresses insect-like AANAT at the onset of darkness and the subsequent peak of melatonin in *D. magna* may play a role in the regulation of DVM (Bentkowski *et al.*, 2010, Schwarzenberger and Wacker, 2015). ALAN can thus suppress *Daphnia* DVM either directly by light itself or indirectly by increased predator activity (and hence, increased distribution of kairomones) or by a complex interaction of both. For example, a reduction of *Daphnia* DVM magnitude and amplitude was observed under urban light pollution in a suburban lake (Moore *et al.*, 2001) and the amplitude of *Daphnia* DVM was reduced by the light of full moon (Dodson, 1990, Gliwicz, 1986). However, in an earlier LakeLab experiment with a similar setup as the field experiment (described in Material and Methods, and in Chapter 2), similar changes could not be confirmed (Walles, 2020). In this experiment, horizontal movement due to uneven light distribution on the water surface were discussed to have affected the measurements. Additionally, for the predominant Cladocera species in this LakeLab experiment – *Ceriodaphnia sp.* – DVM was not reported, yet (Stephan, 2021, Walles, 2020). It is also possible that skyglow levels are not enough to reduce DVM of zooplankton but bright enough to increase predation at least to some extent (or vice versa). If there is a large difference between adapted light sensitivities of predators and preys, ecosystem-scaled effects might be expected. Hence, the lowest light levels to induce a) DVM and b) increased visual

predation by fish need to be established to estimate the effects of ALAN on multi-species ecological processes. In a marine study, growth rates of larval coral reef fish were recently reported to be dependent on the lunar phase (Shima *et al.*, 2021), and the authors suggested this to be an effect of increased visibility of prey items and higher feeding rates in interplay with predator and prey DVM. In addition to zooplankton-fish interactions, reduced grazing of zooplankton may lead to increases in phytoplankton blooms, especially in urban freshwaters. The physiology of plankton itself can also be impaired by low levels of ALAN (Poulin *et al.*, 2014, Stephan, 2021), but threshold intensities remain to be established also for this trophic level.

Conservational implications of the observed ALAN effects

Different types of known effects of ALAN on fish in general are summarized in Figure 8. Considering the above discussions on adversity of effects as well as the comparison to other pollutants, such an overview may allow a first hazard profile of ALAN for fish, which can be useful for conservational issues.

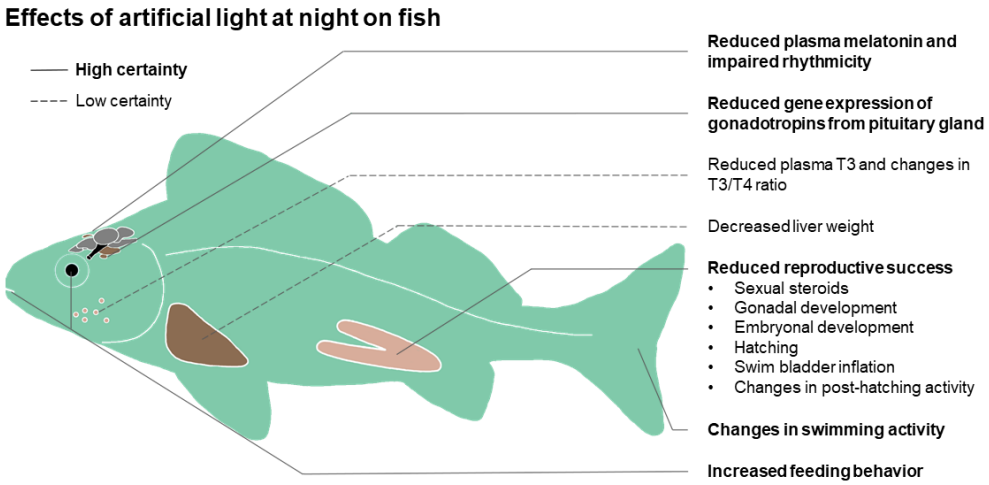

Figure 8 Effects of ALAN on fish physiology and behavior combining results from this thesis and from the discussed literature. “High certainty” refers to effects observed in multiple species at several different ALAN illuminances (solid lines). “Low certainty” refers to effects observed in only one species and/or only at high intensities of ALAN (dashed lines). Illustration by Franziska Kupprat.

Optimum nocturnal illumination of the outdoors depends on different needs of different species, including humans. For most wildlife, optimum conditions are assumed to represent pre-industrial or even pre-human conditions. For humans, aesthetic, societal and economic benefits of light, but also human health hazards need to be taken into account.

IUCN lists light pollution as a threat to species, categorized as a form of pollution under “9.6 excess energy” along with thermal and noise pollution (IUCN, 2021). To further characterize the threat of light pollution, IUCN suggests a categorization

scheme for “Threat Impact” including “Timing options” (at which time scale is the threat expected to cause an impact?), “Scope options” (how much of a population will be affected?), and “Severity options” (how likely and at which rate are population declines expected?). For timing options, light pollution would clearly be categorized as “Ongoing”. If population effects occur at skyglow intensities, which remains to be experimentally proven, scope options would range from “affects the whole population (> 90%)” to “affects the majority of the population (50 – 90%)”, depending on the exposure scenarios, e.g., skyglow intensities, or shelter option of the habitat. Severity options require understanding of multi-generation population predictions. For some species in which effects on reproductive success are evident, a characterization as “Causing or likely to cause fluctuations” may be applicable. In general, it can be assumed that the higher the light intensity threshold of an observed adverse effect, the higher the severity, but the lower the affected portion of the population.

Still, the general population does not generally perceive ALAN as a pollutant in a way that for instance chemical pollution is seen, except if they want to observe stars (Schulte-Römer *et al.*, 2018). However, the weak perception of ALAN as a pollutant does not resemble its actual threat to wildlife as manifested by the effects described in this thesis. For precautionary reasons, the general goal should be to stop all unnecessary or non-essential ALAN. The criteria to determine to which spatial and temporal extent ALAN is essential needs to be defined in a dialogue involving stakeholders from lighting engineers, ecologists, and municipalities (Hölker *et al.*, 2021, Schulte-Römer *et al.*, 2018). Safety issues in traffic would most likely be considered essential whereas the socio-economic benefits of bright advertising billboards or aesthetic landmark illumination will have to be weighed against the ecological risks (Gaston *et al.*, 2015a). At best, light pollution measures will go hand in hand with financial savings and reductions of greenhouse gas emissions. Globally, lighting accounts for 19% of energy consumption and emits 1900 million tonnes (Mt) of CO_2, making lighting one of the biggest causes of energy-related greenhouse gas emissions (IEA, 2006). Electric lighting caused CO_2 emission of 1528 Mt and stationary outdoor lighting accounts for 8% of lighting electricity (IEA, 2006). Thus, outdoor lighting contributed 122 Mt of CO_2 globally and accounted for more than 1.5 % of total energy consumption (calculated with numbers from IEA, 2006). Besides energy savings, another societal benefit of reduced light pollution would be a better visibility of the night sky, which is generally perceived as aesthetic, beautiful and worth preserving (Duriscoe, 2001, Stone, 2018).

As aquatic areas are hardly visited at night, there appears to be little need to illuminate the water surfaces of rivers, channels, and lakes, or sea coastlines at night. Lights on bridges and shorelines would have to be shielded and light intensities dimmed to the necessary purpose-fit minimum. Connected protection areas may serve as refuge areas for light-sensitive species that actively seek to avoid ALAN (e.g., IDA, Dark Sky Places). Avoidance of ALAN would also have to be implemented in areas already protected from other pollutants, as it has been pointed out for marine protected areas (Davies *et al.*, 2016). Still, for species which are trapped in light beams or for which light serves as a lure (e.g., migratory birds), dark sky parks might be less helpful.

Vulnerable ecosystems, which are currently already regressing due to other stressors might also be susceptible to light pollution. For example, global warming and ocean acidification but also chemical pollution challenge coral reef ecosystems and on top they have recently been shown to suffer from light pollution as well (Ayalon *et al.*, 2021, Levy *et al.*, 2020, Rosenberg *et al.*, 2019). Whereas rising atmospheric CO_2 leading to global warming and ocean acidification are difficult to tackle on a local basis and require a long time for measures to come into effect, light pollution can be ameliorated rather easily with relatively low socio-economic trade-offs. Moreover, physiological effects of ALAN are expected to recover quickly, because this type of pollution ends instantly when lights are turned off; unlike chemical pollution, with chemicals often remaining in the environment for a longer time even if pollution stopped immediately (Duriscoe, 2001).

In some places where light pollution has obvious and rather dramatic effects, some measures have already been implemented. For example, at beaches in Florida, so many hatching sea turtles had died due to disorientation by streetlamps that most coastal counties are now regulating ALAN close to turtle nesting grounds (Salmon, 2003). Furthermore, in Northeast Florida, the “Lights Out” campaign promotes a reduction of commercial and private ALAN emission during migratory seasons of birds (Florida Museum, Lights Out Project). This measure resulted from die-offs of migrating birds due to disorientation by light pollution in several places on the globe and has gathered societal attention (Cabrera-Cruz *et al.*, 2018, Schulte-Römer *et al.*, 2018, pp. 51 – 52, van Doren *et al.*, 2021).

Still, for more and farther-reaching regulatory measures, a more comprehensive data base is required to characterize risks of light pollution (e.g., for the above-described IUCN criteria). This thesis indicates that for freshwater fish, melatonin, endocrine disruption, and reproduction could be of particular interest for regulatory argumentation concerning aquatic vertebrates.

Conclusions

The aim of my thesis was to understand the physiological impacts of skyglow-like low intensities of ALAN on fish. Moreover, my goal was to contribute to the general understanding of physiological ALAN effects in fish and estimate threshold effect levels.

Skyglow-like low ALAN intensities induced changes in the physiology of Eurasian perch in terms of reduced absolute nocturnal melatonin levels (LOEL 0.01 lx) and disturbed melatonin rhythmicity (LOEL 1 lx). Moreover, small changes in the gene expression of gonadotropins were observed (LOEL 0.06 lx for *lhβ*), although the overall picture remains inconclusive. More than any other investigated physiological endpoint, the reduction of gonadotropins might lead to critical adverse effects on population level, if changes in gonadotropins affect fertility and ultimately the number or fitness of offspring.

For the first time, the present research demonstrated that ALAN reduces plasma T3 and reduced I_H in Eurasian perch at a high intensity of ALAN (LOEL at 100 lx). It remains subject to future investigations whether the observed ALAN effect on thyroid hormones also occur in early life stages and if it affects development and

growth. The reduction of I_H is also an ALAN effect that has not been previously reported and raises many questions about the metabolic mode of action that led to this ALAN-induced reduction in liver weight. Reduced glycogen storage by increased activity pattern or changes in lipid metabolism are just two potential explanations of this effect. In general, the liver seems to be a sensitive and interesting tissue to investigate in future ALAN studies.

Absolute nocturnal melatonin levels and melatonin rhythmicity were the most sensitive physiological parameters affected by ALAN exposure, but reduction of melatonin or disturbed melatonin rhythmicity are not considered adverse *per se*. The mode of action of melatonin itself or melatonin rhythmicity are not fully understood. From our results, absolute melatonin levels may be linked to reductions of gonadotropins, but the effects on T3 and I_H are more likely to be linked to melatonin rhythmicity as they occurred at higher intensities.

Upcoming ALAN experiments should consider longer time frames, as the exposure in the present climate chamber experiments (two weeks) was rather short. It is possible that ALAN effects elicit a slow but persistent response which could lead to latent effects over longer exposure times. Based on the findings presented in this thesis, a worst-case scenario might include adverse long-term effects such as reduced reproductive success, delayed development, or lower energy reserves on a population level. Hence, the effects of ALAN on fish might not be the most prominent in short-term experiments but can serve as supporting argument on the need for regulatory measures. Until final thresholds for ALAN intensity for short- and long-term exposure are established for a variety of species, ALAN should be considered a serious concern for physiological fitness of aquatic wildlife. Overall, there is a need to re-think public nocturnal lighting for protecting freshwater ecosystems, but a variety of socio-economic factors need to be considered when establishing new lighting concepts. For conservational issues, it seems obvious that nocturnal illumination of freshwaters should be timely restricted, as dim and as well-shielded as possible to protect wildlife.

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References

Adams BA, Cyr DG, Eales JG (2000) Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* 127: 367-378. https://doi.org/10.1016/S0742-8413(00)00164-X

Adel M, Yeganeh S, Dadar M, Sakai M, Dawood MAO (2016) Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754). *Fish Shellfish Immunol.* 56: 436-444. https://doi.org/10.1016/j.fsi.2016.08.003

Aebi H (1984) Catalase *in vitro*. *Methods Enzymol.* 105: 121-126.

Agrawal SJ, Srivistava AK (1980) Haematological responses ina fresh water fish to experimental manganese poisoning. *Toxicology* 17: 97-100. https://doi.org/10.1016/0300-483x(80)90031-1

Alvarado MV, Carrillo M, Felip A (2015) Melatonin-induced changes in *kiss/gnrh* gene expression patterns in the brain of male sea bass during spermatogenesis. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 185: 69-79. https://doi.org/10.1016/j.cbpa.2015.03.010

Amano M, Iigo M, Ikuta K, Kitamura S, Yamada H, Yamamori K (2000) Roles of melatonin in gonadal maturation of underyearling precocious male masu salmon. *Gen. Comp. Endocrinol.* 120: 190-197. https://doi.org/10.1006/gcen.2000.7547

Appelbaum L, Vallone D, Anzulovich A, Ziv L, Tom M, Foulkes NS, Gothilf Y (2006) Zebrafish arylalkylamine-N-acetyltransferase genes - targets for regulation of the circadian clock. *J. Mol. Endocrinol.* 36: 337-347. https://doi.org/10.1677/jme.1.01893

Arkoosh MR, Van Gaest AL, Strickland SA, Hutchinson GP, Krupkin AB, Dietrich JP (2017) Alteration of thyroid hormone concentrations in juvenile chinook salmon (*Oncorhynchus tshawytscha*) exposed to polybrominated diphenyl ethers, BDE-47 and BDE-99. *Chemosphere* 171: 1-8. https://doi.org/10.1016/j.chemosphere.2016.12.035

Aube M (2015) Physical behaviour of anthropogenic light propagation into the nocturnal environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370: 1667. https://doi.org/10.1098/rstb.2014.0117

Aulsebrook AE, Jones TM, Mulder RA, Lesku JA (2018) Impacts of artificial light at night on sleep: A review and prospectus. *Journal of Experimental Zoology. Part A: Ecological and Integrative Physiology* 329: 409-418. https://doi.org/10.1002/jez.2189

Ayalon I, Rosenberg Y, Benichou JIC, Campos CLD, Sayco SLG, Nada MAL, Baquiran JIP, Ligson CA, Avisar D, Conaco C, Kuechly HU, Kyba CCM, Cabaitan PC, Levy O (2021) Coral gametogenesis collapse under artificial light pollution. *Curr. Biol.* 31: 413-419.E413. https://doi.org/10.1016/j.cub.2020.10.039

Badruzzaman M, Ikegami T, Amin AKMR, Shahjahan M (2020) Melatonin inhibits reproductive activity through changes of serotonergic activity in the brain of freshwater catfish (*Mystus cavasius*). *Aquaculture* 526: 735378. https://doi.org/10.1016/j.aquaculture.2020.735378

Baekelandt S, Mandiki SNM, Schmitz M, Kestemont P (2019) Influence of the light spectrum on the daily rhythms of stress and humoral innate immune markers in pikeperch Sander lucioperca. *Aquaculture* 499: 358-363. https://doi.org/10.1016/j.aquaculture.2018.09.046

Barton K (2018) MuMIn: Multi-model inference. R package version 1.42.1. https://CRAN.R-project.org/package=MuMIn

Baschieri L, de Luca F, Cramarossa L, de Martino C, Oliverio A, Negri M (1963) Modifications of thyroid activity by melatonin. *Experientia* 19: 15-17. https://doi.org/10.1007/bf02135330

Bau F, Parent J-P (2000) Seasonal variations of thyroid hormone levels in wild fish. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie* 323: 365-372. https://doi.org/10.1016/s0764-4469(00)00137-2

Bayarri MJ, Rodríguez L, Zanuy S, Madrid JA, Sánchez-Vázquez FJ, Kagawa H, Okuzawa K, Carrillo M (2004) Effect of photoperiod manipulation on the daily rhythms of melatonin and reproductive hormones in caged European sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.* 136: 72-81. https://doi.org/10.1016/j.ygcen.2003.12.004

Bentkowski P, Markowska M, Pijanowska J (2010) Role of melatonin in the control of depth distribution of *Daphnia magna*. *Hydrobiologia* 643: 43-50. https://doi.org/10.1007/s10750-010-0134-x

Bergman E (1988) Foraging abilities and niche breadths of two percids, *Perca fluviatilis* and *Gymnocephalus cernua*, under different environmental conditions. *J. Anim. Ecol.* 57: 443-453. https://doi.org/10.2307/4916

Blanton ML, Specker JL (2007) The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Crit. Rev. Toxicol.* 37: 97-115. https://doi.org/10.1080/10408440601123529

Boeuf G, Gaignon J-L (1989) Effects of rearing conditions on growth and thyroid hormones during smolting of Atlantic salmon, *Salmo salar* L. *Aquaculture* 82: 29-38. https://doi.org/10.1016/0044-8486(89)90393-1

Boeuf G, Le Bail P-Y (1999) Does light have an influence on fish growth? *Aquaculture* 177: 129-152. https://doi.org/10.1016/S0044-8486(99)00074-5
Bolton D, Mayer-Pinto M, Clark GF, Dafforn KA, Brassil WA, Becker A, Johnston EL (2017) Coastal urban lighting has ecological consequences for multiple trophic levels under the sea. *Sci. Total Environ.* 576: 1-9. https://doi.org/10.1016/j.scitotenv.2016.10.037
Boujard T, Leatherland JF (1992) Circadian pattern of hepatosomatic index, liver glycogen and lipid content, plasma non-esterified fatty acid, glucose, T3, T4, growth hormone and cortisol concentrations in *Oncorhynchus mykiss* held under different photoperiod regimes and fed using demand-feeders. *Fish Physiol. Biochem.* 10: 111-122. https://doi.org/10.1007/BF00004522
Bowden TJ (2008) Modulation of the immune system of fish by their environment. *Fish Shellfish Immunol.* 25: 373-383. https://doi.org/10.1016/j.fsi.2008.03.017
Brown DD (1997) The role of thyroid hormone in zebrafish and axolotl development. *Proc. Natl. Acad. Sci. U.S.A.* 94: 13011-13016. https://doi.org/10.1073/pnas.94.24.13011
Brown DRE (1952) Natural illumination charts. Department of the Navy, Bureau of Ships.
Brown JA, Edwards D, Whitehead C (1989) Cortisol and thyroid hormone responses to acid stress in the brown trout, *Salmo trutta* L. *J. Fish Biol.* 35: 73-84. https://doi.org/10.1111/j.1095-8649.1989.tb03394.x
Brown SB, Adams BA, Cyr DG, Eales JG (2004) Contaminant effects on the teleost fish thyroid. *Environ. Toxicol. Chem.* 23: 1680-1701. https://doi.org/10.1897/03-242
Brown SB, Eales JG, Evans RE, Hara TJ (1984) Interrenal, thyroidal, and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification. *Can. J. Fish. Aquat. Sci.* 41: 36-45. https://doi.org/10.1139/f84-004
Brown SB, Evans RE, Majewski HS, Sangalang GB, Klaverkarnp JF (1990) Responses of plasma electrolytes, thyroid hormones, and gill histology in Atlantic salmon (*Salmo salar*) to acid and limed river waters. *Can. J. Fish. Aquat. Sci.* 47: 2431-2440. https://doi.org/10.1139/f90-271
Brown SB, MacLatchy DL, Hara TJ, Eales JG (1991) Effects of cortisol on aspects of 3,5,3'-triiodo-L-thyronine metabolism in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 81: 207-216. https://doi.org/10.1016/0016-6480(91)90005-Q
Brüning A (2016) Spotlight on fish: The biological impacts of artificial light at night. Dr. rer. nat. Monographic dissertation, Humboldt University of Berlin
Brüning A, Hölker F, Franke S, Kleiner W, Kloas W (2016) Impact of different colours of artificial light at night on melatonin rhythm and gene expression of gonadotropins in European perch. *Sci. Total Environ.* 543: 214-222. https://doi.org/10.1016/j.scitotenv.2015.11.023
Brüning A, Hölker F, Franke S, Kleiner W, Kloas W (2018a) Influence of light intensity and spectral composition of artificial light at night on melatonin rhythm and mRNA expression of gonadotropins in roach *Rutilus rutilus*. *Fish Physiol. Biochem.* 44: 1-12. https://doi.org/10.1007/s10695-017-0408-6
Brüning A, Hölker F, Franke S, Preuer T, Kloas W (2015) Spotlight on fish: light pollution affects circadian rhythms of European perch but does not cause stress. *Sci. Total Environ.* 511: 516-522. https://doi.org/10.1016/j.scitotenv.2014.12.094
Brüning A, Hölker F, Wolter C (2010) Artificial light at night: implications for early life stages development in four temperate freshwater fish species. *Aquat. Sci.* 73: 143-152. https://doi.org/10.1007/s00027-010-0167-2
Brüning A, Kloas W, Preuer T, Hölker F (2018b) Influence of artificially induced light pollution on the hormone system of two common fish species, perch and roach, in a rural habitat. *Conserv. Physiol.* 6: coy016. https://doi.org/10.1093/conphys/coy016
Burgos A, Valenzuela A, González M, Klempau A (2004) Non-specific defence mechanisms of rainbow trout (*Oncorhynchus mykiss*) during artificial photoperiod. *Bull. Eur. Assoc. Fish Pathol.* 24: 240-245. https://eafp.org
Cabrera-Cruz SA, Smolinsky JA, Buler JJ (2018) Light pollution is greatest within migration passage areas for nocturnally-migrating birds around the world. *Sci. Rep.* 8: 3261. https://doi.org/10.1038/s41598-018-21577-6
Cahill GM (2002) Clock mechanisms in zebrafish. *Cell Tissue Res.* 309: 27-34. https://doi.org/10.1007/s00441-002-0570-7
Cameron NE (1982) The photopic spectral sensitivity of a dichromatic teleost fish (*Perca fluviatilis*). *Vision Res.* 22: 1341-1348. https://doi.org/10.1016/0042-6989(82)90223-1
Campinho MA (2019) Teleost metamorphosis: the role of thyroid hormone. *Front. Endocrinol. (Lausanne)* 10: 383. https://doi.org/10.3389/fendo.2019.00383
Carazo I, Norambuena F, Oliveira C, Sánchez-Vázquez FJ, Duncan NJ (2013) The effect of night illumination, red and infrared light, on locomotor activity, behaviour and melatonin of Senegalese sole (*Solea senegalensis*) broodstock. *Physiol. Behav.* 118: 201-207. https://doi.org/10.1016/j.physbeh.2013.05.032
Carleton KL, Escobar-Camacho D, Stieb SM, Cortesi F, Marshall NJ (2020) Seeing the rainbow: mechanisms underlying spectral sensitivity in teleost fishes. *J. Exp. Biol.* 223: jeb193334. https://doi.org/10.1242/jeb.193334
Carletta MA, Weis P, Weis JS (2002) Development of thyroid abnormalities in mummichogs, *Fundulus heteroclitus*, from a polluted site. *Mar. Environ. Res.* 54: 601-604. https://doi.org/10.1016/S0141-1136(02)00133-2

Carnevali O, Gioacchini G, Maradonna F, Olivotto I, Migliarini B (2011) Melatonin induces follicle maturation in *Danio rerio*. *PLoS One* 6: e19978. https://doi.org/10.1371/journal.pone.0019978
Carr JA, Patiño R (2011) The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. *Gen. Comp. Endocrinol.* 170: 299-312. https://doi.org/10.1016/j.ygcen.2010.06.001
Carrier-Belleau C, Drolet D, McKindsey CW, Archambault P (2021) Environmental stressors, complex interactions and marine benthic communities' responses. *Sci. Rep.* 11: 4194. https://doi.org/10.1038/s41598-021-83533-1
Carrillo-Vico A, Lardone PJ, Álvarez-Sánchez N, Rodriguez-Rodriguez A, Guerrero JM (2013) Melatonin: buffering the immune system. *Int. J. Mol. Sci.* 14: 8638-8683. https://doi.org/10.3390/ijms14048638
Carrillo M, Zanuy S, Felip A, Bayarri MJ, Molés G, Gómez A (2009) Hormonal and environmental control of puberty in perciform fish - the case of sea bass. *Ann. N. Y. Acad. Sci.* 1163: 49-59. https://doi.org/10.1111/j.1749-6632.2008.03645.x
Challet E, Pévet P, Vivien-Roels B, A. M (1992) Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime. *J. Biol. Rhythms* 12: 65-79. https://doi.org/10.1177/074873049701200108
Chellappa S, Huntingford FA, Strang RHC, Thomson RY (1995) Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. *J. Fish Biol.* 47: 775-787. https://doi.org/10.1111/j.1095-8649.1995.tb06002.x
Chen R, Yuan L, Zha J, Wang Z (2017a) Developmental toxicity and thyroid hormone-disrupting effects of 2,4-dichloro-6-nitrophenol in Chinese rare minnow (*Gobiocypris rarus*). *Aquat. Toxicol.* 185: 40-47. https://doi.org/10.1016/j.aquatox.2017.02.005
Chen W, Lau SW, Fan Y, Wu RSS, Ge W (2017b) Juvenile exposure to bisphenol A promotes ovarian differentiation but suppresses its growth - Potential involvement of pituitary follicle-stimulating hormone. *Aquat. Toxicol.* 193: 111-121. https://doi.org/10.1016/j.aquatox.2017.10.008
Cheng GF, Yuen CW, Ge W (2007) Evidence for the existence of a local activin follistatin negative feedback loop in the goldfish pituitary and its regulation by activin and gonadal steroids. *J. Endocrinol.* 195: 373-384. https://doi.org/10.1677/JOE-07-0265
Chien L-T, Hwang D-F (2001) Effects of thermal stress and vitamin C on lipid peroxidation and fatty acid composition in the liver of thornfish *Terapon jarbua*. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 128: 91-97. https://doi.org/10.1016/S1096-4959(00)00299-2
Ciocca M, Wang J (2013) By the light of the silvery moon: fact and fiction. *PhyEd* 48: 360-367. https://doi.org/10.1088/0031-9120/48/3/360
Confente F, Rendón M, Besseau L, Falcón J, Muñoz-Cueto JA (2010) Melatonin receptors in a pleuronectiform species, *Solea senegalensis*: Cloning, tissue expression, day-night and seasonal variations. *Gen. Comp. Endocrinol.* 167: 202-214. https://doi.org/10.1016/j.ygcen.2010.03.006
Cook RF, Eales JG (1987) Effects of feeding and photocycle on diel changes in plasma tyhroid hormone levels in rainbow trout, *Salmo gairdneri*. *J. Exp. Zool.* 242: 161-169 https://doi.org/10.1002/jez.1402420207
Cornelissen G (2014) Cosinor-based rhythmometry. *Theor. Biol. Med. Model.* 11: 16. https://doi.org/10.1186/1742-4682-11-16
Corona-Herrera GA, Arranz SE, Martínez-Palacios CA, Navarrete-Ramírez P, Toledo-Cuevas EM, Valdez-Alarcón JJ, Martínez-Chávez CC (2018) Experimental evidence of masculinization by continuous illumination in a temperature sex determination teleost (Atherinopsidae) model: is oxidative stress involved? *J. Fish Biol.* 93: 229-237. https://doi.org/10.1111/jfb.13651
Costache M, Bucur C, Costache M, Radu D, Nicolae CG (2017) Research on the use of different hormonal substances to stimulate maturation and ovulation in perch (*Perca fluviatilis* L.). *Scientific Papers: Series D, Animal Science-The International Session of Scientific Communications of the Faculty of Animal Science* 60: 333-336.
Cowan M, Azpeleta C, Lopez-Olmeda JF (2017) Rhythms in the endocrine system of fish: a review. *J. Comp. Physiol., B* 187: 1057-1089. https://doi.org/10.1007/s00360-017-1094-5
Cuesta A, Cerezuela R, Esteban MA, Meseguer J (2008) *In vivo* actions of melatonin on the innate immune parameters in the teleost fish gilthead seabream. *J. Pineal Res.* 45: 70-78. https://doi.org/10.1111/j.1600-079X.2008.00557.x
Cyr DG, Bromage NR, Duston J, Eales JG (1988a) Seasonal patterns in serum levels of thyroid hormones and sex steroids in relation to photoperiod-induced changes in spawning time in rainbow trout, *Salmo gairdneri*. *Gen. Comp. Endocrinol.* 69: 217-225. https://doi.org/10.1016/0016-6480(88)90008-1
Cyr DG, Eales JG (1990) Influence of short-term oestradiol treatment on plasma thyroid hormone kinetic in rainbow trout, *Salmo gairdneri*. *J. Fish Biol.* 36: 391-400. https://doi.org/10.1111/j.1095-8649.1990.tb05619.x
Cyr DG, Eales JG (1996) Interrelationships between thyroidal and reproductive endocrine systems in fish. *Rev. Fish Biol. Fish.* 6: 165-200. https://doi.org/10.1007/BF00182342
Cyr DG, McLatchy DL, Eales JG (1988b) The influence of short-term 17β-estradiol treatment on plasma T_3 levels and *in vitro* hepatic T_4 5'-Monodeiodinase Activity in immature rainbow trout,

Salmo gairdneri. *Gen. Comp. Endocrinol.* 69: 431-438. https://doi.org/10.1016/0016-6480(88)90035-4
Czarnecka M, Kakareko T, Jermacz L, Pawlak R, Kobak J (2019) Combined effects of nocturnal exposure to artificial light and habitat complexity on fish foraging. *Sci. Total Environ.* 684: 14-22. https://doi.org/10.1016/j.scitotenv.2019.05.280
Dabrowski K, Ciereszko RE, Ciereszko A, Toth GP, Christ SA, El-Saidy D, Ottobre JS (1996) Reproductive physiology of yellow perch (*Perca flavescens*): environmental and endocrinological cues. *J. Appl. Ichthyol.* 12: 139-148. https://doi.org/10.1111/j.1439-0426.1996.tb00079.x
Dananay KL, Benard MF (2018) Artificial light at night decreases metamorphic duration and juvenile growth in a widespread amphibian. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 285: 20180367. https://doi.org/10.1098/rspb.2018.0367
Danilova N, Krupnik VE, Sugden D, Zhdanova IV (2004) Melatonin stimulates cell proliferation in zebrafish embryo and accelerates its development. *FASEB J.* 18: 751-753. https://doi.org/10.1096/fj.03-0544fje
Dardente H, Hazlerigg DG, Ebling FJ (2014) Thyroid hormone and seasonal rhythmicity. *Front. Endocrinol. (Lausanne)* 5: 19. https://doi.org/10.3389/fendo.2014.00019
Datta PC, King MG (1980) Melatonin: Effects on brain and behavior. *Neurosci. Biobehav. Rev.* 4: 451-458. https://doi.org/10.1016/0149-7634(80)90034-2
Davie A, Mazorra de Querob C, Bromage N, Treasurer J, Migaud H (2007) Inhibition of sexual maturation in tank reared haddock (*Melanogrammus aeglefinus*) through the use of constant light photoperiods. *Aquaculture* 270: 379-389. https://doi.org/10.1016/j.aquaculture.2007.04.052
Davies TW, Coleman M, Griffith KM, Jenkins SR (2015) Night-time lighting alters the composition of marine epifaunal communities. *Biol. Lett.* 11: 20150080. https://doi.org/10.1098/rsbl.2015.0080
Davies TW, Duffy JP, Bennie J, Gaston KJ (2014) The nature, extent, and ecological implications of marine light pollution. *Front. Ecol. Environ.* 12: 347-355. https://doi.org/10.1890/130281
Davies TW, Duffy JP, Bennie J, Gaston KJ (2016) Stemming the tide of light pollution encroaching into marine protected areas. *Conservation Letters* 9: 164-171. https://doi.org/10.1111/conl.12191
de Jong M, Jeninga L, Ouyang JQ, van Oers K, Spoelstra K, Visser ME (2016) Dose-dependent responses of avian daily rhythms to artificial light at night. *Physiol. Behav.* 155: 172-179. https://doi.org/10.1016/j.physbeh.2015.12.012
Delgado MJ, Cerda-Reverter JM, Soengas JL (2017) Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the control of food intake. *Front. Neurosci.* 11: 354. https://doi.org/10.3389/fnins.2017.00354
Diamantopoulou C, Christoforou E, Dominoni DM, Kaiserli E, Czyzewski J, Mirzai N, Spatharis S (2021) Wavelength-dependent effects of artificial light at night on phytoplankton growth and community structure. *Proc R Soc B* 288: 20210525. https://doi.org/10.1098/rspb.2021.0525
Dickhoff WW, Folmar LC, Mighell JL, Mahnken CVW (1982) Plasma thyroid hormones during smoltification of yearling and underyearling coho salmon and yearling chinook salmon and steelhead trout. *Aquaculture* 28: 39-48. https://doi.org/10.1016/0044-8486(82)90006-0
Dodson S (1990) Predicting diel vertical migration of zooplankton. *Limnol. Oceanogr.* 35: 1195-1200. https://doi.org/10.4319/lo.1990.35.5.1195
Dolomatov SI, Kubyshkin AV, Kutia SA, Zukow W (2013) Role of thyroid hormones in fishes. *J. Health Sci.* 3: 279-296. https://depot.ceon.pl/handle/123456789/2527
Duriscoe D (2001) Preserving pristine night skies in national parks and the wilderness ethic, Protecting Dark Skies, The George Wright FORUM, https://www.georgewrightsociety.org/georgewrightforum
Durrant J, Michaelides EB, Rupasinghe T, Tull D, Green MP, Jones TM (2015) Constant illumination reduces circulating melatonin and impairs immune function in the cricket *Teleogryllus commodus*. *PeerJ* 3: e1075. https://doi.org/10.7717/peerj.1075
Eales JG, Fletcher GL (1982) Circannual cycles of thyroid hormones in plasma of winter flounder (*Pseudopleuronectes americanus* Walbaum). *Can. J. Zool.* 60: 304-309. https://doi.org/10.1139/z82-040
Eales JG, Shostak S (1985) Correlations between food ration, somatic growth parameters and thyroid function in Arctic charr, *Salvelinus alpinus* L. *Comp. Biochem. Physiol. A Physiol.* 80A: 553-558. https://doi.org/10.1016/0300-9629(85)90411-6
Eilertsen M, Drivenes, Øyvind, Edvardsen RB, Bradley CA, Ebbesson LO, Helvik JV (2014) Exorhodopsin and melanopsin systems in the pineal complex and brain at early developmental stages of Atlantic halibut (*Hippoglossus hippoglossus*). *J. Comp. Neurol.* 522: 4003-4022. https://doi.org/10.1002/cne.23652
Eisenbeis G (2006) Artificial night lighting and insects: Attraction of insects to streetlamps in a rural setting in Germany. In Rich C, Longcore T eds, Ecological consequences of artificial night lighting, Washington, DC: Island Press, 1718 Connecticut Avenue, N.W., Suite 300, Washington, DC 20009, pp. 281-304,
Ekström P, Meissl H (1997) The pineal organ of teleost fishes. *Rev. Fish Biol. Fish.* 7: 199-284. https://link.springer.com/article/10.1023/A:1018483627058
Ekström P, Vaněcek J (1992) Localization of 2-[^{125}I]iodomelatonin binding sites in the brain of the Atlantic salmon, *Salmo salar* L. *Neuroendocrinology* 55: 529-537. https://doi.org/10.1159/000126166

Elbaz I, Foulkes NS, Gothilf Y, Appelbaum L (2013) Circadian clocks, rhythmic synaptic plasticity and the sleep-wake cycle in zebrafish. *Front Neural Circuits* 7: 9. https://doi.org/10.3389/fncir.2013.00009
Ellis AE (1990) Lysozyme assays. In Stolen JS, Fletcher TC, Anderson DP, Roberson BS, van Muiswinkel WB eds, Techniques in Fish Immuology Vol. 1, Fair Haven, N.J., USA: SOS Publications, pp. 101-103,
Ellis T, James JD, Scott AP (2005) Branchial release of free cortisol and melatonin by rainbow trout. *J. Fish Biol.* 67: 535-540. https://doi.org/10.1111/j.0022-1112.2005.00740.x
Esteban MA, Cuesta A, Chaves-Pozo E, Meseguer J (2013) Influence of melatonin on the immune system of fish: a review. *Int. J. Mol. Sci.* 14: 7979-7999. https://doi.org/10.3390/ijms14047979
Esteban MA, Cuesta A, Rodríguez A, Meseguer J (2006) Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system. *J. Pineal Res.* 41: 261-266. https://doi.org/10.1111/j.1600-079X.2006.00362.x
Falchi F, Cinzano P, Duriscoe D, Kyba CCM, Elvidge CD, Baugh K, Portnov BA, Rybnikova NA, Furgoni R (2016) The new world atlas of artificial night sky brightness. *Science Advances* 2: e1600377. https://doi.org/10.1126/sciadv.1600377
Falcón J (1999) Cellular circadian clocks in the pineal. *Prog. Neurobiol.* 58: 121-162. https://doi.org/10.1016/s0301-0082(98)00078-1
Falcón J, Besseau L, Fazzari D, Attia J, Gaildrat P, Beauchaud M, Boeuf G (2003) Melatonin modulates secretion of growth hormone and prolactin by trout pituitary glands and cells in culture. *Endocrinology* 144: 4648-4658. https://doi.org/10.1210/en.2003-0707
Falcón J, Besseau L, Fuentès M, Sauzet S, Magnanou E, Boeuf G (2009) Structural and functional evolution of the pineal melatonin system in vertebrates. *Ann. N. Y. Acad. Sci.* 1163: 101-111. https://doi.org/10.1111/j.1749-6632.2009.04435.x
Falcón J, Migaud H, Muñoz-Cueto JA, Carrillo M (2010) Current knowledge on the melatonin system in teleost fish. *Gen. Comp. Endocrinol.* 165: 469-482. https://doi.org/10.1016/j.ygcen.2009.04.026
Falcón J, Torriglia A, Attia D, Viénot F, Gronfier C, Behar-Cohen F, Martinsons C, Hicks D (2020) Exposure to artificial light at night and the consequences for flora, fauna, and ecosystems. *Front. Neurosci.* 14: 602796. https://doi.org/10.3389/fnins.2020.602796
Farbridge KJ, Leatherland JF (1987) Lunar cycles of coho salmon, *Oncorhynchus kisutch* - II. Scale amino acid uptake, nucleic acids, metabolic reserves and plasma thyroid hormones. *J. Exp. Biol.* 129: 179-189. https://doi.org/10.1242/jeb.129.1.179
Favero GC, Boaventura TP, Ferreira AL, Silva ACF, Porto LA, Luz RK (2019) Fasting/re-feeding and water temperature promote the mobilization of body reserves in juvenile freshwater carnivorous catfish *Lophiosilurus alexandri*. *Aquaculture* 511: https://doi.org/10.1016/j.aquaculture.2019.734223
Felip A, Zanuy S, Muriach B, Cerdá-Reverter MJ, Carrillo M (2008) Reduction of sexual maturation in male *Dicentrarchus labrax* by continuous light both before and during gametogenesis. *Aquac. Asia* 275: 347-355. https://doi.org/10.1016/j.aquaculture.2008.01.020
Firth BT, Belan I, Kennaway DJ (2006) Persistence of a plasma melatonin rhythm in constant darkness and its inhibition by constant light in the sleepy lizard, *Tiliqua rugosa*. *J. Pineal Res.* 41: 15-20. https://doi.org/10.1111/j.1600-079X.2006.00322.x
Flik BJG, Aanen DK, Ringelberg J (1997) The extent of predation by juvenile perch during diel vertical migration of *Daphnia*. *Adv. Limnol.* 49: 51-58. http://hdl.handle.net/11245/1.132009
Florida Museum (Lights Out Project) Northeast Florida 'Lights Out' project aims to reduce bird collision deaths. https://www.floridamuseum.ufl.edu/science/florida-lights-out-project-aims-to-reduce-bird-collision-deaths/
Fobert EK, Burke da Silva K, Swearer SE (2019) Artificial light at night causes reproductive failure in clownfish. *Biol. Lett.* 15: 20190272. http://doi.org/10.1098/rsbl.2019.0272
Fort DJ, Rogers RL, Morgan LA, Miller MF, Clark PA, White JA, Paul RR, Stover EL (2000) Preliminary validation of a short-term morphological assay to evaluate adverse effects of amphibian metamorphosis and thyroid function using *Xenopus laevis*. *J. Appl. Toxicol.* 20: 419–425. https://doi.org/10.1002/1099-1263(200009/10)20:5<419::AID-JAT708>3.0.CO;2-A
Fortes-Silva R, Leme FOP, Boaventura TP, Mendonça HCP, Moreira JPL, Cunha PHH, Luz RK (2019) Daily rhythms of leukocytes populations, biochemical and enzymatic blood parameters in a carnivorous freshwater catfish (*Lophiosilurus alexandri*). *Chronobiol. Int.* 36: 276-287. https://doi.org/10.1080/07420528.2018.1537284
Foster JG, Algera DA, Brownscombe JW, Zolderdo AJ, Cooke SJ (2016) Consequences of different types of littoral zone light pollution on the parental care behaviour of a freshwater teleost fish. *Water, Air, Soil Pollut.* 227: 404. https://doi.org/10.1007/s11270-016-3106-6
Frank KD (2006) Effects of artificial night lighting on moths. In Rich C, Longcore T eds, Ecological consequences of artificial night lighting, Washington, DC: Island Press, 1718 Connecticut Avenue, N.W., Suite 300, Washington, DC 20009, pp. 305-344,
Franke S, Brüning A, Hölker F, Kloas W (2013) Study of biological action of light on fish. *J. Light Vis. Environ.* 37: 194-204. https://doi.org/10.2150/jlve.IEIJ130000518
Fukunaga K, Yamashina F, Ohta N, Mizuno H, Takeuchi Y, Yamauchi C, Takemura A (2019) Involvement of melatonin in transducing moon-related signals into the reproductive network of the

female honeycomb grouper *Epinephelus merra*. *Gen. Comp. Endocrinol.* 282: 113211. https://doi.org/10.1016/j.ygcen.2019.113211

Gaildrat P, Becq F, Falcón J (2002) First cloning and functional characterization of a melatonin receptor in fish brain: a novel one? *J. Pineal Res.* 32: 74-84. https://doi.org/10.1034/j.1600-079x.2002.1817.x

Gaildrat P, Falcón J (1999) Expression of melatonin receptors and 2-[^{125}I]iodomelatonin binding sites in the pituitary of a teleost fish. *Adv. Exp. Med. Biol.* 460: 61-72. https://doi.org/10.1007/0-306-46814-x_8

Gandhi AV, Mosser EA, Oikonomou G, Prober DA (2015) Melatonin is required for the circadian regulation of sleep. *Neuron* 85: 1193-1199. https://doi.org/10.1016/j.neuron.2015.02.016

Ganger MT, Dietz GD, Ewing SJ (2017) A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. *BMC Bioinformatics* 18: 534. https://doi.org/10.1186/s12859-017-1949-5

Gao D, Lin J, Ou K, Chen Y, Li H, Dai Q, Yu Z, Zuo Z, Wang C (2018) Embryonic exposure to benzo(a)pyrene inhibits reproductive capability in adult female zebrafish and correlation with DNA methylation. *Environ. Pollut.* 240: 403-411. https://doi.org/10.1016/j.envpol.2018.04.139

García-López Á, Pascual E, Sarasquete C, Martínez-Rodríguez G (2006) Disruption of gonadal maturation in cultured Senegalese sole *Solea senegalensis* Kaup by continuous light and/or constant temperature regimes. *Aquaculture* 261: 789-798. https://doi.org/10.1016/j.aquaculture.2006.09.005

Gaston KJ, Bennie J, Davies TW, Hopkins J (2013) The ecological impacts of nighttime light pollution: a mechanistic appraisal. *Biol. Rev. Camb. Philos. Soc.* 88: 912-927. https://doi.org/10.1111/brv.12036

Gaston KJ, Duffy JP, Gaston S, Bennie J, Davies TW (2014) Human alteration of natural light cycles: causes and ecological consequences. *Oecologia* 176: 917-931. https://doi.org/10.1007/s00442-014-3088-2

Gaston KJ, Gaston S, Bennie J, Hopkins J (2015a) Benefits and costs of artificial nighttime lighting of the environment. *Environ. Rev.* 23: 14-23. https://doi.org/10.1139/er-2014-0041

Gaston KJ, Visser ME, Hölker F (2015b) The biological impacts of artificial light at night: the research challenge. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370: 20140133. https://doi.org/10.1098/rstb.2014.0133

Geven EJW, Klaren PHM (2017) The teleost head kidney: Integrating thyroid and immune signalling. *Dev. Comp. Immunol.* 66: 73-83. https://doi.org/10.1016/j.dci.2016.06.025

Giling DP, Nejstgaard JC, Berger SA, Grossart HP, Kirillin G, Penske A, Lentz M, Casper P, Sareyka J, Gessner MO (2017) Thermocline deepening boosts ecosystem metabolism: evidence from a large-scale lake enclosure experiment simulating a summer storm. *Glob. Chang. Biol.* 23: 1448-1462. https://doi.org/10.1111/gcb.13512

Gliwicz ZM (1986) A lunar rycle in zooplankton. *Ecology* 67: 883-897. https://doi.org/10.2307/1939811

Golombek DA, Pévet P, Cardinali DP (1996) Melatonin effects on behavior: Possible mediation by the central GABAergic system. *Neurosci. Biobehav. Rev.* 20: 403-412. https://doi.org/10.1016/0149-7634(95)00052-6

Gomez JM, Boujard T, Boeuf G, Solari A, Le Bail P-Y (1997) Individual diurnal plasma profiles of thyroid hormones in rainbow trout (*Oncorhynchus mykiss*) in relation to cortisol, growth hormone, and growth rate. *Gen. Comp. Endocrinol.* 107: 74–83. https://doi.org/10.1006/gcen.1997.6897

Grau EG (1988) Environmental influences on thyroid function of teleost fish. *Am. Zool.* 28: 329–335. https://doi.org/10.1093/icb/28.2.329

Grau EG, Dickhoff WW, Nishioka RS, Bern HA, Folmar LC (1981) Lunar phasing of the thyroxine surge preparatory to seaward migration salmonid fish. *Science* 211: 607-609. https://doi.org/10.1126/science.7455703

Gröner F, Höhne C, Kleiner W, Kloas W (2017) Chronic diclofenac exposure affects gill integrity and pituitary gene expression and displays estrogenic activity in nile tilapia (*Oreochromis niloticus*). *Chemosphere* 166: 473-481. https://doi.org/10.1016/j.chemosphere.2016.09.116

Grubisic M, Haim A, Bhusal P, Dominoni DM, Gabriel KMA, Jechow A, Kupprat F, Lerner A, Marchant P, Riley W, Stebelova K, van Grunsven RHA, Zeman M, Zubidat AE, Hölker F (2019) Light pollution, circadian photoreception, and melatonin in vertebrates. *Sustainability* 11: 6400. https://doi.org/10.3390/su11226400

Grubisic M, Singer G, Bruno MC, van Grunsven RHA, Manfrin A, Monaghan MT, Hölker F (2017) Artificial light at night decreases biomass and alters community composition of benthic primary producers in a sub-alpine stream. *Limnol. Oceanogr.* 62: 2799-2810. https://doi.org/10.1002/lno.10607

Halberg F, Tong YL, Johnson EA (1967) Circadian System Phase - An Aspect of Temporal Morphology; Procedures and Illustrative Examples. In von Mayersbach H ed, The Cellular Aspects of Biorhythms, Heidelberg: Springer-Verlag Berlin. https://doi.org/10.1007/978-3-642-88394-1_2

Hänel A, Posch T, Ribas SJ, Aubé M, Duriscoe D, Jechow A, Kolláth Z, Lolkema DE, Moore C, Schmidt N, Spoelstra H, Wuchterl G, Kyba CCM (2018) Measuring night sky brightness:

methods and challenges. *J. Quant. Spectros. Radiat. Transfer* 205: 278-290. https://doi.org/10.1016/j.jqsrt.2017.09.008

Hermelink B, Würtz S, Trubiroha A, Rennert B, Kloas W, Schulz C (2011) Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. *Gen. Comp. Endocrinol.* 172: 282-292. https://doi.org/10.1016/j.ygcen.2011.03.013

Herrera-Pérez P, Del Carmen Rendón M, Besseau L, Sauzet S, Falcón J, Muñoz-Cueto JA (2010) Melatonin receptors in the brain of the European sea bass: An in situ hybridization and autoradiographic study. *J. Comp. Neurol.* 518: 3495-3511. https://doi.org/10.1002/cne.22408

Hidalgo MC, Trenzado CE, Furné M, Beltrán A, Manzaneda C, García-Gallego M, Domezain A, Sanz A (2017) Tissue-specific daily variation in the oxidative status of sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*): a comparative study. *Fish Physiol. Biochem.* 43: 1105-1115. https://doi.org/10.1007/s10695-017-0356-1

Hölker F, Bolliger J, Davies TW, Giavi S, Jechow A, Kalinkat G, Longcore T, Spoelstra K, Tidau S, Visser ME, Knop E (2021) 11 Pressing research questions on how light pollution affects biodiversity. *Frontiers in Ecology and Evolution* 9: 767177. https://doi.org/10.3389/fevo.2021.767177

Hölker F, Dörner H, Schulze T, Haertel-Borer SS, Peacor SD, Mehner T (2007) Species-specific responses of planktivorous fish to the introduction of a new piscivore: implications for prey fitness. *Freshwat. Biol.* 52: 1793-1806. https://doi.org/10.1111/j.1365-2427.2007.01810.x

Hölker F, Jechow A, Schroer S, Gessner MO (2018) Nächtliches Licht und Lichtverschmutzung in und um Gewässer. *Handbuch Angewandte Limnologie* 34: 1-26. https://doi.org/10.1002/9783527678488.hbla2018003

Hölker F, Moss T, Griefahn B, Kloas W, Voigt CC, Henckel D, Hänel A, Kappeler PM, Völker S, Schwope A, Franke S, Uhrlandt D, Fischer J, Klenke R, Wolter C, Tockner K (2010a) The dark side of light: A transdisciplinary research agenda for light pollution policy. *Ecol. Soc.* 15: 13. http://www.ecologyandsociety.org/vol15/iss4/art13/

Hölker F, Wolter C, Perkin EK, Tockner K (2010b) Light pollution as a biodiversity threat. *Trends Ecol. Evol.* 25: 681-682. https://doi.org/10.1016/j.tree.2010.09.007

Hölker F, Wurzbacher C, Weißenborn C, Monaghan MT, Holzhauer SI, Premke K (2015) Microbial diversity and community respiration in freshwater sediments influenced by artificial light at night. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370: 20140130. https://doi.org/10.1098/rstb.2014.0130

Hoseini SM, Tort L, Abolhasani MH, Rajabiesterabadi H (2016) Physiological, ionoregulatory, metabolic and immune responses of Persian sturgeon, *Acipenser persicus* (Borodin, 1897) to stress. *Aquacult. Res.* 47: 3729-3739. https://doi.org/10.1111/are.12822

IDA (Dark Sky Places) International Dark Sky Places conservation program. https://www.darksky.org/our-work/conservation/idsp/

IEA (2006) Light's labour's lost: Policies for energy-efficient lighting. In Agency IE ed, Energy Efficient Policy Profiles, OECD Publishing, Paris. https://doi.org/10.1787/9789264109520-en

Iigo M, Abe T, Kambayashi S, Oikawa K, Masuda T, Mizusawa K, Kitamura S, Azuma T, Takagi Y, Aida K, Yanagisawa T (2007) Lack of circadian regulation of *in vitro* melatonin release from the pineal organ of salmonid teleosts. *Gen. Comp. Endocrinol.* 154: 91-97. https://doi.org/10.1016/j.ygcen.2007.06.013

Ikegami T, Azuma K, Nakamura M, Suzuki N, Hattori A, Ando H (2009) Diurnal expressions of four subtypes of melatonin receptor genes in the optic tectum and retina of goldfish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 152: 219-224. https://doi.org/10.1016/j.cbpa.2008.09.030

Ikegami T, Takeuchi Y, Hur SP, Takemura A (2014) Impacts of moonlight on fish reproduction. *Mar. Genomics* 14: 59-66. https://doi.org/10.1016/j.margen.2013.11.007

Ikeno T, Weil ZM, Nelson RJ (2014) Dim light at night disrupts the short-day response in Siberian hamsters. *Gen. Comp. Endocrinol.* 197: 56-64. https://doi.org/10.1016/j.ygcen.2013.12.005

Inui Y, Miwa S (1985) Thyroid hormone induces metamorphosis of flounder larvae. *Gen. Comp. Endocrinol.* 60: 450-454. https://doi.org/10.1016/0016-6480(85)90080-2

IPCC (2014) Climate change 2014: synthesis report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change. In team Cw, Pachauri RK, Meyer LA eds, Geneva, Switzerland, https://archive.ipcc.ch/report/ar5/syr/

IPCS (2004) Part 1: IPCS/OECD Key generic terms used in chemical hazard/risk assessment, IPCS risk assessment terminology, World Health Organisation, Geneva, Switzerland, https://apps.who.int/iris/bitstream/handle/10665/42908/9241562676.pdf

Isorna E, de Pedro N, Valenciano AI, Alonso-Gomez AL, Delgado MJ (2017) Interplay between the endocrine and circadian systems in fishes. *J. Endocrinol.* 232: R141-R159. https://doi.org/10.1530/JOE-16-0330

IUCN (2021) The IUCN Red List of Threatened Species. Version 2021-2. https://www.iucnredlist.org/resources/threat-classification-scheme

James JD, Ellis T, Scott AP (2004) Water-based measurement of rainbow trout melatonin. *J. Fish Biol.* 65: 1298-1304. https://doi.org/10.1111/j.0022-1112.2004.00531.x

Jechow A, Hölker F (2019a) How dark is a river? Artificial light at night in aquatic systems and the need for comprehensive night-time light measurements. *WIREs Water* 6: e1388. https://doi.org/10.1002/wat2.1388
Jechow A, Hölker F (2019b) Snowglow-The amplification of skyglow by snow and clouds can exceed full moon illuminance in suburban areas. *J Imaging* 5: 69. https://doi.org/10.3390/jimaging5080069
Jechow A, Hölker F, Kolláth Z, Gessner MO, Kyba CCM (2016) Evaluating the summer night sky brightness at a research field site on Lake Stechlin in northeastern Germany. *J. Quant. Spectros. Radiat. Transfer* 181: 24-32. http://doi.org/10.1016/j.jqsrt.2016.02.005
Jechow A, Kyba CCM, Hölker F (2019) Beyond all-sky: Assessing ecological light pollution using multi-spectral full-sphere fisheye lens imaging. *Journal of Imaging* 5: 46. https://doi.org/10.3390/jimaging5040046
Jechow A, Kyba CCM, Hölker F (2020) Mapping the brightness and color of urban to rural skyglow with all-sky photometry. *JQSRT* 250: 106988. https://doi.org/10.1016/j.jqsrt.2020.106988
Jechow A, Schreck G, Kyba CCM, Berger SA, Bistarelli LT, Bodenlos M, Gessner MO, Grossart H-P, Kupprat F, Nejstgaard JC, Pansch A, Penske A, Sachtleben M, Shatwell T, Singer GA, Stephan S, Walles TJW, Wollrab S, Zielinska-Dabkowska KM, Hölker F (2021) Design and implementation of an illumination system to mimic skyglow at ecosystem level in a large-scale lake enclosure facility. *Sci. Rep.* 11: 23478. https://doi.org/10.1038/s41598-021-02772-4
Jones TM, Durrant J, Michaelides EB, Green MP (2015) Melatonin: a possible link between the presence of artificial light at night and reductions in biological fitness. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370: 20140122. https://doi.org/10.1098/rstb.2014.0122
Jung SJ, Choi YJ, Kim NN, Choi JY, Kim BS, Choi CY (2016a) Effects of melatonin injection or green-wavelength LED light on the antioxidant system in goldfish (*Carassius auratus*) during thermal stress. *Fish Shellfish Immunol.* 52: 157-166. https://doi.org/10.1016/j.fsi.2016.03.002
Jung SJ, Kim NN, Choi YJ, Choi JY, Choi YU, Heo YS, Choi CY (2016b) Effects of melatonin and green-wavelength LED light on the physiological stress and immunity of goldfish, *Carassius auratus*, exposed to high water temperature. *Fish Physiol. Biochem.* 42: 1335-1346. https://doi.org/10.1007/s10695-016-0221-7
Jürgensen S, Puchmüller J, Wolter C, Schomaker C (2019) Fische in Berlin - Bilanz der Artenvielfalt (Allgemeiner Teil). In Senatsverwaltung für Umwelt VuKBiZmdL-IfGuB ed, Berlin: Informierter, Senatsverwaltung für Umwelt, Verkehr und Klimaschutz Berlin, Berlin, https://www.berlin.de/fischereiamt/_assets/service/pdf/broschuere_fische_a.pdf
Kelley JL, Davies WIL (2016) The biological mechanisms and behavioral functions of opsin-based light detection by the skin. *Frontiers in Ecology and Evolution* 4: 106. https://doi.org/10.3389/fevo.2016.00106
Kepka M, Szwejser E, Pijanowski L, Kemenade B, Chadzinska M (2015) A role for melatonin in maintaining the pro- and anti-inflammatory balance by influencing leukocyte migration and apoptosis in carp. *Dev. Comp. Immunol.* 53: 179-190. https://doi.org/10.1016/j.dci.2015.07.011
Khan IA, Thomas P (1996) Melatonin influences gonadotropin II secretion in the Atlantic croaker (*Micropogonias undulatus*). *Gen. Comp. Endocrinol.* 104: 231-242. https://doi.org/10.1006/gcen.1996.0166
Kissil GW, Lupatsch I, Elizur A, Zohar Y (2001) Long photoperiod delayed spawning and increased somatic growth in gilthead seabream (*Sparus aurata*). *Aquaculture* 200: 363-379. https://doi.org/10.1016/S0044-8486(01)00527-0
Kloas W (2002) Amphibians as a model for the study of endocrine disruptors. *Int. Rev. Cytol.* 216: 1-57. https://doi.org/10.1016/S0074-7696(02)16002-5
Kloas W, Urbatzka R, Opitz R, Würtz S, Behrends T, Hermelink B, Hoffmann F, Jagnytsch O, Kroupova H, Lorenz C, Neumann N, Pietsch C, Trubiroha A, Van Ballegooy C, Wiedemann C, Lutz I (2009) Endocrine disruption in aquatic vertebrates. *Ann. N. Y. Acad. Sci.* 1163: 187-200. https://doi.org/10.1111/j.1749-6632.2009.04453.x
Kojima D, Mano H, Fukada Y (2000) Vertebrate ancient-long opsin: A green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. *J. Neurosci.* 20: 2845-2851. https://doi.org/10.1523/JNEUROSCI.20-08-02845.2000
Kolkovski S, Dabrowski K (1998) Off-season spawning of yellow perch. *Prog. Fish-Cult.* 60: 133-136. https://doi.org/10.1577/1548-8640(1998)060<0133:OSSOYP>2.0.CO;2
Kottelat M, Freyhof J (2007) Handbook of European freshwater fishes, Kottelat, Cornol, Switzerland and Freyhof, Berlin, Germany.
Kuechly HU, Kyba CCM, Ruhtz T, Lindemann C, Wolter C, Fischer J, Hölker F (2012) Aerial survey and spatial analysis of sources of light pollution in Berlin, Germany. *Remote Sens. Environ.* 126: 39-50. https://doi.org/10.1016/j.rse.2012.08.008
Kulczykowska E, Kalamarz H, Warne JM, Balment RJ (2006) Day-night specific binding of 2-[^{125}I]iodomelatonin and melatonin content in gill, small intestine and kidney of three fish species. *J. Comp. Physiol. B* 176: 277-285. https://doi.org/10.1007/s00360-005-0049-4
Kulczykowska E, Kleszczyńska A, Gozdowska M, Sokołowska E (2017) The time enzyme in melatonin biosynthesis in fish: day/night expressions of three aralkylamine *N*-acetyltransferase genes in three-spined stickleback. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 208: 46-53. https://doi.org/10.1016/j.cbpa.2017.03.005

Kummu M, de Moel H, Ward PJ, Varis O (2011) How close do we live to water? A global analysis of population distance to freshwater bodies. *PLoS One* 6: e20578. https://doi.org/10.1371/journal.pone.0020578
Kupprat F, Hölker F, Kloas W (2020) Can skyglow reduce nocturnal melatonin concentrations in Eurasian perch? *Environ. Pollut.* 262: 114324. https://doi.org/10.1016/j.envpol.2020.114324
Kupprat F, Hölker F, Knopf K, Preuer T, Kloas W (2021a) Innate immunity, oxidative stress, and body indices of the Eurasian perch *Perca fluviatilis* after two-week exposures to artificial light at night. *J. Fish Biol.* 99: 118-130. https://doi.org/10.1111/jfb.14703
Kupprat F, Kloas W, Krüger A, Schmalsch C, Hölker F (2021b) Misbalance of thyroid hormones after two weeks of exposure to artificial light at night in Eurasian perch *Perca fluviatilis*. *Conserv. Physiol.* 9: coaa124. https://doi.org/10.1093/conphys/coaa124
Kurvers RHJM, Drägestein J, Hölker F, Jechow A, Krause J, Bierbach D (2018) Artificial light at night affects emergence from a refuge and space use in guppies. *Sci. Rep.* 8: 14131. https://doi.org/10.1038/s41598-018-32466-3
Kurvers RHJM, Hölker F (2014) Bright nights and social interactions: a neglected issue. *Behav. Ecol.* 26: 334-339. https://doi.org/10.1093/beheco/aru223
Kyba CCM, Hänel A, Hölker F (2014) Redefining efficiency for outdoor lighting. *Energy Environ. Sci.* 7: 1806-1809 https://doi.org/10.1039/c4ee00566j
Kyba CCM, Kuester T, Sánchez de Miguel A, Baugh K, Jechow A, Hölker F, Bennie J, Elvidge CD, Gaston KJ, Guanter L (2017a) Artificially lit surface of Earth at night increasing in radiance and extent. *Science Advances* 3: e1701528. https://doi.org/10.1126/sciadv.1701528
Kyba CCM, Mohar A, Posch T (2017b) How bright is moonlight? *A&G* 58: 31-32. https://doi.org/10.1093/astrogeo/atx025
Kyba CCM, Ruhtz T, Fischer J, Hölker F (2011) Cloud coverage acts as an amplifier for ecological light pollution in urban ecosystems. *PLoS One* 6: e17307. https://doi.org/10.1371/journal.pone.0017307
Kyba CCM, Tong KP, Bennie J, Birriel I, Birriel JJ, Cool A, Danielsen A, Davies TW, Outer PN, Edwards W, Ehlert R, Falchi F, Fischer J, Giacomelli A, Giubbilini F, Haaima M, Hesse C, Heygster G, Hölker F, Inger R, Jensen LJ, Kuechly HU, Kuehn J, Langill P, Lolkema DE, Nagy M, Nievas M, Ochi N, Popow E, Posch T, Puschnig J, Ruhtz T, Schmidt W, Schwarz R, Schwope A, Spoelstra H, Tekatch A, Trueblood M, Walker CE, Weber M, Welch DL, Zamorano J, Gaston KJ (2015) Worldwide variations in artificial skyglow. *Sci. Rep.* 5: 8409. https://doi.org/10.1038/srep08409
Laidley CW, Leatherland JF (1988) Circadian studies of plasma, cortisol, thyroid hormone, protein, glucose and ion concentration, liver glycogen concentration, and liver and spleen weigth in rainbow trout, *Salmo gairdneri* Richardson. *Comp. Biochem. Physiol.* 89: 495-502. https://doi.org/10.1016/0300-9629(88)91063-8
Lau MT, Ge W (2005) Cloning of Smad2, Smad3, Smad4, and Smad7 from the goldfish pituitary and evidence for their involvement in activin regulation of goldfish FSHβ promoter activity. *Gen. Comp. Endocrinol.* 141: 22-38. https://doi.org/10.1016/j.ygcen.2004.10.019
Laudet V (2011) The origins and evolution of vertebrate metamorphosis. *Curr. Biol.* 21: R726-R737. https://doi.org/10.1016/j.cub.2011.07.030
Leatherland JF (1985) Effect of 17β-estradiol and methyl testosterone on the activity of the thyroid gland in rainbow trout, *Salmo gairdneri* Richardson. *Gen. Comp. Endocrinol.* 60: 343-352. https://doi.org/10.1016/0016-6480(85)90067-X
Leatherland JF (1994) Reflections on the thyroidology of fishes: from molecules to humankind. Institute of Ichthyology, University of Guelph, Guelph, Ontario, Canada.
Leatherland JF, Sonstegard RA (1978) Lowering of serum thyroxine and triiodothyronine levels in Coho salmon, *Oncorhynchus kisutch*, by dietary mirex and PCBs. *Journal of the Fisheries Research Board of Canada* 35: 1285-1289. https://doi.org/10.1139/f78-202
LeGates TA, Fernandez DC, Hattar S (2014) Light as a central modulator of circadian rhythms, sleep and affect. *Nat. Rev. Neurosci.* 15: 443-454. https://doi.org/10.1038/nrn3743
Leiner KA, MacKenzie DS (2001) The effects of photoperiod on growth rate and circulating thyroid hormone levels in the red rum, *Sciaenops ocellatus*: evidence for a free-running circadian rhythm of T_4 secretion. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 130: 141-149. https://doi.org/10.1016/S1095-6433(01)00373-7
Lenth R (2019) emmeans: Estimated marginal means, aka least-squares means. R package version 1.3.3. https://CRAN.R-project.org/package=emmeans
Levy O, Fernandes de Barros Marangoni L, J ICB, Rottier C, Beraud E, Grover R, Ferrier-Pages C (2020) Artificial light at night (ALAN) alters the physiology and biochemistry of symbiotic reef building corals. *Environ. Pollut.* 266: 114987. https://doi.org/10.1016/j.envpol.2020.114987
Lin SW, Ge W (2009) Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish – an *in vitro* approach. *Gen. Comp. Endocrinol.* 160: 183-193. https://doi.org/10.1016/j.ygcen.2008.11.020
Liu D, Straus DL, Pedersen L-F, Meinelt T (2017) Pulse versus continuous peracetic acid applications: Effects on rainbow trout performance, biofilm formation and water quality. *Aquacult. Eng.* 77: 72-79. https://doi.org/10.1016/j.aquaeng.2017.03.004

Liu Q, Duston J (2019) Long photoperiod in winter is more effective than food deprivation in stopping unwanted sexual maturation in Arctic charr. *Aquaculture* 501: 213-218. https://doi.org/10.1016/j.aquaculture.2018.11.007

Liu Q, Manning AJ, Duston J (2019) Light intensity and suppression of nocturnal plasma melatonin in Arctic charr (*Salvelinus alpinus*). *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 228: 103-106. https://doi.org/10.1016/j.cbpa.2018.11.012

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-408. https://doi.org10.1006/meth.2001.1262

Lorgen M, Casadei E, Król E, Douglas A, Birnie MJ, Ebbesson LO, Nilsen TO, Jordan WC, Jørgensen EH, Dardente H, Hazlerigg DG, Martin SA (2015) Functional divergence of type 2 deiodinase paralogs in the Atlantic salmon. *Curr. Biol.* 25: 936-941. https://doi.org/10.1016/j.cub.2015.01.074

Lorne JK, Salmon M (2007) Effects of exposure to artificial lighting on orientation of hatchling sea turtles on the beach and in the ocean. *Endang. Species Res.* 3: 23-30. https://doi.org/10.3354/esr003023

Lowe RH (1952) The influence of light and pther factors on the seaward migration of the silver eel (Anguilla anguilla L.). *J. Anim. Ecol.* 21: 275-309. https://doi.org/10.2307/1963

MacQuarrie DW, Vanstone WE, Markert JR (1979) Photoperiod induced off-season spawning of pink salmon (*Oncorhynchus gorbuscha*). *Aquaculture* 18: 289-302. https://doi.org/10.1016/0044-8486(79)90033-4

Mahlow P (2019) The influence of light pollution and brownification on the behaviour of the European perch *Perca fluviatilis* (L.). Master of Science Master's thesis, Freie Universität Berlin, Berlin

Maitra SK, Hasan KN (2016) The role of melatonin as a hormone and an antioxidant in the control of fish reproduction. *Front. Endocrinol. (Lausanne)* 7: 38. https://doi.org/10.3389/fendo.2016.00038

Manfrin A, Singer G, Larsen S, Weiß N, van Grunsven RHA, Weiß N-S, Wohlfahrt S, Monaghan MT, Hölker F (2017) Artificial light at night affects organism flux across ecosystem boundaries and drives community structure in the recipient ecosystem. *Front. Environ. Sci.* 5: 61. https://doi.org/10.3389/fenvs.2017.00061

Masuda T, Iigo M, Mizusawa K, Naruse M, Oishi T, Aida K, Tabata M (2003) Variations in plasma melatonin levels of the rainbow trout (*Oncorhynchus mykiss*) under various light and temperature conditions. *Zoolog. Sci.* 20: 1011-1016. https://doi.org/10.2108/zsj.20.1011

McCormick SD, Saunders RL (1990) Influence of ration level and salinity on circulating thyroid hormones in juvenile Atlantic salmon (*Salmo salar*). *Gen. Comp. Endocrinol.* 78: 224-230. https://doi.org/10.1016/0016-6480(90)90009-B

McCormick SD, Saunders RL, Henderson EB, Harmon PR (1987) Phototoperiod control of parr–smolt transformation in Atlantic salmon (*Salmo salar*): changes in salinity tolerance, gill Na^+, K^+-ATPase activity, and plasma thyroid hormones. *Can. J. Fish. Aquat. Sci.* 44: 1462-1468. https://doi.org/10.1139/f87-175

McElreath R (2020a) rethinking: Statistical Rethinking book package. R package version 2.01.

McElreath R (2020b) Statistical rethinking: A Bayesian course with examples in R and Stan. CRC press.

Melingen GO, Wergeland HI (2002) Physiological effects of an oil-adjuvanted vaccine on out-of-season Atlantic salmon (*Salmo salar* L.) smolt. *Aquaculture* 214: 397-409. https://doi.org/10.1016/S0044-8486(01)00867-5

Migaud H, Davie A, Taylor JF (2010) Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. *J. Fish Biol.* 76: 27-68. https://doi.org/10.1111/j.1095-8649.2009.02500.x

Migaud H, Fontaine P, Kestemont P, Wang N, Brun-Bellut J (2004) Influence of photoperiod on the onset of gonadogenesis in Eurasian perch *Perca fluviatilis*. *Aquaculture* 241: 561-574. https://doi.org/10.1016/j.aquaculture.2004.07.031

Migaud H, Wang N, Gardeur J-N, Fontaine P (2006) Influence of photoperiod on reproductive performances in Eurasian pech *Perca fluviatilis*. *Aquaculture* 252: 385-393. https://doi.org/10.1016/j.aquaculture.2005.07.029

Miwa S, Inui Y (1987) Effects of various doses of thyroxine and triiodothyronine on the metamorphosis of flounder (*Paralichthys olivaceus*). *Gen. Comp. Endocrinol.* 67: 356-363. https://doi.org/10.1016/0016-6480(87)90190-0

Moore MV, Pierce SM, Walsh HM, Kvalvik SK, Lim JD (2001) Urban light pollution alters the diel vertical migration of *Daphnia*. *Verh. Internat. Verein. Limnol.* 27: 1-4. https://doi.org/10.1080/03680770.1998.11901341

Morgan AL, Thompson KD, Auchinachie NA, Migaud H (2008) The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. *Fish Shellfish Immunol.* 25: 791-799. https://doi.org/10.1016/j.fsi.2008.05.011

Morin P-P, Hara TJ, Eales JG (1993) Thyroid hormone deiodination in brain, liver, gill, heart and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. I. Outer- and inner-ring thyroxine deiodination. *Gen. Comp. Endocrinol.* 90: 142-156. https://doi.org/10.1006/gcen.1993.1069

Mukherjee S, Maitra SK (2015) Gut melatonin in vertebrates: chronobiology and physiology. *Front. Endocrinol. (Lausanne)* 6: 112. https://doi.org/10.3389/fendo.2015.00112
Mukhi S, Patiño R (2007) Effects of prolonged exposure to perchlorate on thyroid and reproductive function in zebrafish. *Toxicol. Sci.* 96: 246-254. https://doi.org/10.1093/toxsci/kfm001
Muñoz-Perez JL, López-Patino MA, Álvarez-Otero R, Gesto M, Soengas JL, Míguez JM (2016) Characterization of melatonin synthesis in the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*): distribution, relation with serotonin, daily rhythms and photoperiod regulation. *J. Comp. Physiol. B* 186: 471-484. https://doi.org/10.1007/s00360-016-0966-4
Nakane Y, Ikegami K, Iigo M, Ono H, Takeda K, Takahashi D, Uesaka M, Kimijima M, Hashimoto R, Arai N, Suga T, Kosuge K, Abe T, Maeda R, Senga T, Amiya N, Azuma T, Amano M, Abe H, Yamamoto N, Yoshimura T (2013) The saccus vasculosus of fish is a sensor of seasonal changes in day length. *Nature Communications* 4: 2108. https://doi.org/10.1038/ncomms3108
Nakane Y, Yoshimura T (2014) Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Front. Neurosci.* 8: 115. https://doi.org/10.3389/fnins.2014.00115
Nakao N, Ono H, Yamamura T, Anraku T, Takagi T, Higashi K, Yasuo S, Katou Y, Kageyama S, Uno Y, Kasukawa T, Iigo M, Sharp PJ, Iwasawa A, Suzuki Y, Sugano S, Niimi T, Mizutani M, Namikawa T, Ebihara S, Ueda HR, Yoshimura T (2008) Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature* 452: 317-322. https://doi.org/10.1038/nature06738
Nakayama S, Doering-Arjes P, Linzmaier S, Briege J, Klefoth T, Pieterek T, Arlinghaus R (2018) Fine-scale movement ecology of a freshwater top predator, Eurasian perch (*Perca fluviatilis*), in response to the abiotic environment over the course of a year. *Ecol. Freshwat. Fish* 27: 798-812. https://doi.org/10.1111/eff.12393
Navara KJ, Nelson RJ (2007) The dark side of light at night: physiological, epidemiological, and ecological consequences. *J. Pineal Res.* 43: 215-224. https://doi.org/10.1111/j.1600-079X.2007.00473.x
Nayak PK, Singh TP (1987) Effect of melatonin and 5-methoxytryptamine on sex steroids and thyroid hormones during the prespawning phase of the annual reproductive cycle in the freshwater teleost, *Clarias batrachus*. *J. Pineal Res.* 4: 377-386. https://doi.org/10.1111/j.1600-079X.1987.tb00877.x
Nikaido Y, Ueda S, Takemura A (2009) Photic and circadian regulation of melatonin production in the Mozambique tilapia *Oreochromis mossambicus*. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 152: 77-82. https://doi.org/10.1016/j.cbpa.2008.09.001
Noyes PD, Lema SC, Roberts SC, Cooper EM, Stapleton HM (2014) Rapid method for the measurement of circulating thyroid hormones in low volumes of teleost fish plasma by LC-ESI/MS/MS. *Anal. Bioanal. Chem.* 406: 715-726. https://doi.org/10.1007/s00216-013-7528-3
O'Brien CS, Bourdo R, Bradshaw WE, Holzapfel CM, Cresko WA (2012) Conservation of the photoperiodic neuroendocrine axis among vertebrates: evidence from the teleost fish, *Gasterosteus aculeatus*. *Gen. Comp. Endocrinol.* 178: 19-27. https://doi.org/10.1016/j.ygcen.2012.03.010
O'Connor JJ, Fobert EK, Besson M, Jacob H, Lecchini D (2019) Live fast, die young: Behavioural and physiological impacts of light pollution on a marine fish during larval recruitment. *Mar. Pollut. Bull.* 146: 908-914. https://doi.org/10.1016/j.marpolbul.2019.05.038
OECD (2009) Test No. 231: Amphibian metamorphosis assay. *OECD Guidelines for the Testing of Chemicals, OECD Publishing, Paris* Section 2: https://doi.org/10.1787/9789264076242-en
OECD (2015) Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT). *OECD Guidelines for the Testing of Chemicals, OECD Publishing, Paris*: https://doi.org/10.1787/9789264242258-en
OECD (2019) Global biodiversity loss and the international context, Biodiversity: Finance and the Economic and Business Case for Action, Paris: OECD Publishing. https://doi.org/10.1787/a3147942-en
Ohlberger J, Mehner T, Staaks G, Hölker F (2008) Is ecological segregation in a pair of sympatric coregonines supported by divergent feeding efficiencies? *Can. J. Fish. Aquat. Sci.* 65: 2105-2113. https://doi.org/10.1139/f08-120
Okumoto N, Ikuta K, Aida K, Hanyu I, Hirano T (1989) Effects of photoperiod on smolting and hormonal secretion in masu salmon, *Oncorhynchus masou*. *Aquaculture* 82: 63-76. https://doi.org/10.1016/0044-8486(89)90396-7
Oliveira C, Duncan NJ, Pousão-Ferreira P, Mañanós E, Sánchez-Vázquez FJ (2010) Influence of the lunar cycle on plasma melatonin, vitellogenin and sex steroids rhythms in Senegal sole, *Solea senegalensis*. *Aquaculture* 306: 343-347. https://doi.org/10.1016/j.aquaculture.2010.05.003
Opitz R, Braunbeck T, Bogi C, Pickford DB, Nentwig G, Oehlmann J, Tooi O, Lutz I, Kloas W (2005) Description and initial evaluation of a *Xenopus* metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. *Environ. Toxicol. Chem.* 24: 653-664. https://doi.org/10.1897/04-214R.1
Oppedal F, Juell J-E, Taranger GL, Hansen T (2001) Artificial light and season affects vertical distribution and swimming behaviour of post-smolt Atlantic salmon in sea cages. *J. Fish Biol.* 58: 1570-1584. https://doi.org/10.1111/j.1095-8649.2001.tb02313.x

Orozco A, Valverde-R C (2005) Thyroid hormone deiodination in fish. *Thyroid* 15: 799-813. https://doi.org/10.1089/thy.2005.15.799
Osborn RH, Simpson TH (1978) Seasonal changes in thyroidal status in the plaice *Pleuronectes platessa* L. *J. Fish Biol.* 12: 519-526. https://doi.org/10.1111/j.1095-8649.1978.tb04197.x
Osborn RH, Simpson TH, Youngson AF (1978) Seasonal and diurnal rhythms of thyroidal status in the rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* 12: 531-540. https://doi.org/10.1111/j.1095-8649.1978.tb04199.x
Ouyang JQ, Davies S, Dominoni D (2018) Hormonally mediated effects of artificial light at night on behavior and fitness: linking endocrine mechanisms with function. *J. Exp. Biol.* 221: jeb156893. https://doi.org/10.1242/jeb.156893
Özeren SC, Kankılıç GB, Erkmen B, Polat H, Pehlivan E (2019) Effect of seasonal water temperature variation on the blood serums thyroid hormone levels of juvenile chub fishes (*Squalius cappadocicus*). *Biol. Rhythm Res.*: 1-6. https://doi.org/10.1080/09291016.2019.1566987
Park YJ, Park JG, Kim SJ, Lee YD, Saydur Rahman M, Takemura A (2006) Melatonin receptor of a reef fish with lunar-related rhythmicity: cloning and daily variations. *J. Pineal Res.* 41: 166-174. https://doi.org/10.1111/j.1600-079X.2006.00350.x
Park YJ, Park JG, Takeuchi Y, Hur SP, Lee YD, Kim SJ, Takemura A (2014) Influence of moonlight on mRNA expression patterns of melatonin receptor subtypes in the pineal organ of a tropical fish. *Mar. Genomics* 14: 67-70. https://doi.org/10.1016/j.margen.2013.10.006
Peirson SN, Halford S, Foster RG (2009) The evolution of irradiance detection: melanopsin and the non-visual opsins. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364: 2849-2865. https://doi.org/10.1098/rstb.2009.0050
Perkin EK, Hölker F, Tockner K (2014a) The effects of artificial lighting on adult aquatic and terrestrial insects. *Freshwat. Biol.* 59: 368-377. https://doi.org/10.1111/fwb.12270
Perkin EK, Hölker F, Tockner K, Richardson JS (2014b) Artificial light as a disturbance to light-naïve streams. *Freshwat. Biol.* 59: 2235-2244. https://doi.org/10.1111/fwb.12426
Persson L (1986) Effects of reduced interspecific competition on resource utilization in perch (*Perca fluviatilis*). *Ecology* 67: 355-364. https://doi.org/10.2307/1938578
Peter MCS (2011) The role of thyroid hormones in stress response of fish. *Gen. Comp. Endocrinol.* 172: 198-210. https://doi.org/10.1016/j.ygcen.2011.02.023
Peter VS, Joshua EK, Wendelaar Bonga SE, Peter MC (2007) Metabolic and thyroidal response in air-breathing perch (*Anabas testudineus*) to water-borne kerosene. *Gen. Comp. Endocrinol.* 152: 198-205. https://doi.org/10.1016/j.ygcen.2007.05.015
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: e45-e45. https://doi.org/10.1093/nar/29.9.e45
Philp AR, Garcia-Fernandez JM, Soni BG, Lucas RJ, Bellingham J, Foster RG (2000) Vertebrate ancient (VA) opsin and extraretinal photoreception in the Atlantic salmon (*Salmo salar*). *J. Exp. Biol.* 203: 1925-1936. https://doi.org/10.1242/jeb.203.12.1925
Pietsch C, Michel C, Kersten S, Valenta H, Dänicke S, Schulz C, Kloas W, Burkhardt-Holm P (2014) *In vivo* effects of deoxynivalenol (DON) on innate immune responses of carp (*Cyprinus carpio* L.). *Food Chem. Toxicol.* 68: 44-52. https://doi.org/10.1016/j.fct.2014.03.012
Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC (2018) nlme: Linear and nonlinear mixed effects models. R package version 3.1-137. https://CRAN.R-project.org/package=nlme.
Pohanka M, Pejchal J, Snopkova S, Havlickova K, Karasova JZ, Bostik P, Pikula J (2012) Ascorbic acid: an old player with a broad impact on body physiology including oxidative stress suppression and immunomodulation: a review. *Mini-Rev. Med. Chem.* 12: 35-43. https://doi.org/10.2174/138955712798868986
Porter MJR, Duncan NJ, Handeland SO, Stefansson SO, Bromage NR (2001) Temperature, light intensity and plasma melatonin levels in juvenile Atlantic salmon. *J. Fish Biol.* 58: 431-438. https://doi.org/10.1111/j.1095-8649.2001.tb02262.x
Poulin C, Bruyant F, Laprise M-H, Cockshutt AM, Marie-Rose Vandenhecke J, Huot Y (2014) The impact of light pollution on diel changes in the photophysiology of Microcystis aeruginosa. *J. Plankton Res.* 36: 286-291. https://doi.org/10.1093/plankt/fbt088
Power DM, Llewellyn L, Faustino M, Nowell MA, Björnsson BT, Einarsdottir IE, Canario AVM, Sweeney GE (2001) Thyroid hormones in growth and development of fish. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* 130: 447-459. https://doi.org/10.1016/S1532-0456(01)00271-X
Pun CSJ, So CW (2012) Night-sky brightness monitoring in Hong Kong: a city-wide light pollution assessment. *Environ. Monit. Assess.* 184: 2537-2557. https://doi.org/10.1007/s10661-011-2136-1
Puschnig J, Posch T, Uttenthaler S (2014a) Night sky photometry and spectroscopy performed at the Vienna University Observatory. *J. Quant. Spectros. Radiat. Transfer* 139: 64-75. https://doi.org/10.1016/j.jqsrt.2013.08.019
Puschnig J, Schwope A, Posch T, Schwarz R (2014b) The night sky brightness at Potsdam-Babelsberg including overcast and moonlit conditions. *J. Quant. Spectros. Radiat. Transfer* 139: 76-81. https://doi.org/10.1016/j.jqsrt.2013.12.011
R Core Team (2020) R: A language and environment for statistical computing. https://www.R-project.org/.

Raap T, Casasole G, Costantini D, AbdElgawad H, Asard H, Pinxten R, Eens M (2016) Artificial light at night affects body mass but not oxidative status in free-living nestling songbirds: an experimental study. *Sci. Rep.* 6: 35626. https://doi.org/10.1038/srep35626

Rahman MS, Kim BH, Takemura A, Park CB, Lee YD (2004) Effects of moonlight exposure on plasma melatonin rhythms in the seagrass rabbitfish, *Siganus canaliculatus*. *J. Biol. Rhythms* 19: 325-334. https://doi.org/10.1177/0748730404266712

Reading BJ, Sullivan CV, Schillling J (2017) The reproductive organs and processes: Vitellogenesis in fishes. In Reading BJ, Sullivan CV eds, Encyclopedia of Fish Physiology: Reference Module in Life Sciences, pp. 635-646. https://doi.org/10.1016/B978-0-12-809633-8.03076-4

Reddy PK, Leatherland JF (2003) Influences of photoperiod and alternate days of feeding on plasma growth hormone and thyroid hormone levels in juvenile rainbow trout. *J. Fish Biol.* 63: 197-212. https://doi.org/10.1046/j.1095-8649.2003.00144.x

Refinetti R, Lissen GC, Halberg F (2007) Procedures for numerical analysis of circadian rhythms. *Biol. Rhythm Res.* 38: 275-325. https://doi.org/10.1080/09291010600903692

Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PTJ, Kidd KA, MacCormack TJ, Olden JD, Ormerod SJ, Smol JP, Taylor WW, Tockner K, Vermaire JC, Dudgeon D, Cooke SJ (2018) Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biol. Rev. Camb. Philos. Soc.* 94: 849-873. https://doi.org/10.1111/brv.12480

Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PTJ, Kidd KA, MacCormack TJ, Olden JD, Ormerod SJ, Smol JP, Taylor WW, Tockner K, Vermaire JC, Dudgeon D, Cooke SJ (2019) Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biol. Rev. Camb. Philos. Soc.* 94: 849-873. https://doi.org/10.1111/brv.12480

Reiter RJ, Tan D-X, Korkmaz A, Erren TC, Piekarski C, Tamura H, Manchester LC (2007) Light at night, chronodisruption, melatonin suppression, and cancer risk: A review. *Critical ReviewsTM in Oncogenesis* 13: 303-328. https://doi.org/10.1615/CritRevOncog.v13.i4.30

Reiter RJ, Tan D-X, Osuna C, Gitto E (2000) Actions of melatonin in the reduction of oxidative stress - a review. *J. Biomed. Sci.* 7: 444-458. https://doi.org/10.1007/BF02253360

Renuka K, Joshi BN (2010) Melatonin-induced changes in ovarian function in the freshwater fish *Channa punctatus* (Bloch) held in long days and continuous light. *Gen. Comp. Endocrinol.* 165: 42-46. https://doi.org/10.1016/j.ygcen.2009.05.020

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. *Nature (London)* 418: 935-941. https://doi.org/10.1038/nature00965

Rich C, Longcore T (2006) Ecological consequences of artificial night lighting. Island Press, 1718 Connecticut Avenue, N.W., Suite 300, Washington, DC 20009, Washington, DC.

Riley WD, Bendall B, Ives MJ, Edmonds NJ, Maxwell DL (2012) Street lighting disrupts the diel migratory pattern of wild Atlantic salmon, *Salmo salar* L., smolts leaving their natal stream. *Aquaculture* 330-333: 74-81. https://doi.org/10.1016/j.aquaculture.2011.12.009

Riley WD, Davison PI, Maxwell DL, Bendall B (2013) Street lighting delays and disrupts the dispersal of Atlantic salmon (*Salmo salar*) fry. *Biol. Conserv.* 158: 140-146. https://doi.org/10.1016/j.biocon.2012.09.022

Rodríguez L, Begtashi I, Zanuy S, Carrillo M (2005) Long-term exposure to continuous light inhibits precocity in European male sea bass (*Dicentrarchus labrax*, L.): hormonal aspects. *Gen. Comp. Endocrinol.* 140: 116-125. https://doi.org/10.1016/j.ygcen.2004.10.011

Rodríguez R, Felip A, Cerqueira V, Hala E, Zanuy S, Carrillo M (2012) Identification of a photolabile period for reducing sexual maturation in juvenile male sea bass (*Dicentrarchus labrax*) by means of a continuous light regime. *Aquacult. Int.* 20: 1071-1083. https://doi.org/10.1007/s10499-012-9510-z

Rodríguez R, Felip A, Zanuy S, Carrillo M (2019) Advanced puberty triggered by bi-weekly changes in reproductive factors during the photolabile period in a male teleost fish, *Dicentrarchus labrax* L. *Gen. Comp. Endocrinol.* 275: 82-93. https://doi.org/10.1016/j.ygcen.2019.02.008

Rosenberg Y, Doniger T, Levy O (2019) Sustainability of coral reefs are affected by ecological light pollution in the Gulf of Aqaba/Eilat. *Commun Biol* 2: 289. https://doi.org/10.1038/s42003-019-0548-6

Saha S, Singh KM, Gupta BBP (2019) Melatonin synthesis and clock gene regulation in the pineal organ of teleost fish compared to mammals: Similarities and differences. *Gen. Comp. Endocrinol.* 279: 27-34. https://doi.org/10.1016/j.ygcen.2018.07.010

Saini C, Hutton P, Gao S, Simpson RK, Giraudeau M, Sepp T, Webb E, McGraw KJ (2019) Exposure to artificial light at night increases innate immune activity during development in a precocial bird. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 233: 84-88. http://doi.org/10.1016/j.cbpa.2019.04.002

Sakai K, Yamamoto Y, Ikeuchi T (2019) Vertebrates originally possess four functional subtypes of G protein-coupled melatonin receptor. *Sci. Rep.* 9: 9465. https://doi.org/10.1038/s41598-019-45925-2

Saligaut C, Linard B, Mañanos EL, Kah O, Breton B, Govoroun M (1998) Release of pituitary gonadotrophins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*): Modulation by estradiol and catecholamines. *Gen. Comp. Endocrinol.* 109: 302-309. https://doi.org/10.1006/gcen.1997.7033

Salmon M (2003) Artificial night lighting and sea turtles. *Biologist* 50: 163-168.
Salmon M, Witherington BE (1995) Artificial lighting and seafinding by Loggerhead hatchlings: Evidence for lunar modulation. *Copeia* 4: 931-938. https://doi.org/10.2307/1447042
Sanders D, Frago E, Kehoe R, Patterson C, Gaston KJ (2021) A meta-analysis of biological impacts of artificial light at night. *Nat Ecol Evol* 5: 74-81. https://doi.org/10.1038/s41559-020-01322-x
Sauzet S, Besseau L, Herrera-Pérez P, Covès D, Chatain B, Peyric E, Boeuf G, Muñoz-Cueto JA, Falcón J (2008) Cloning and retinal expression of melatonin receptors in the European sea bass, *Dicentrarchus labrax*. *Gen. Comp. Endocrinol.* 157: 186-195. https://doi.org/10.1016/j.ygcen.2008.04.008
Schligler J, Cortese D, Beldade R, Swearer SE, Mills SC (2021) Long-term exposure to artificial light at night in the wild decreases survival and growth of a coral reef fish. *Proc. Biol. Sci.* 288: 20210454. https://doi.org/10.1098/rspb.2021.0454
Schreiber AM (2006) Asymmetric craniofacial remodeling and lateralized behavior in larval flatfish. *J. Exp. Biol.* 209: 610-621. https://doi.org/10.1242/jeb.02056
Schroer S, Huggins BJ, Azam C, Hölker F (2020) Working with inadequate tools: legislative shortcomings in protection against ecological effects of artificial light at night. *Sustainability* 12: 2551. https://doi.org/10.3390/su12062551
Schulte-Römer N, Dannemann E, Meier J (2018). Light Pollution - A global discussion. In UFZ HCfERG ed, Leipzig, pp. 248
Schulz RW, de França LR, Lareyre JJ, Le Gac F, Chiarini-Garcia H, Nobrega RH, Miura T (2010) Spermatogenesis in fish. *Gen. Comp. Endocrinol.* 165: 390-411. https://doi.org/10.1016/j.ygcen.2009.02.013
Schulz RW, Miura T (2002) Spermatogenesis and its endocrine regulation. *Fish Physiol. Biochem.* 26: 43-56. https://doi.org/10.1023/A:1023303427191
Schwarzenberger A, Wacker A (2015) Melatonin synthesis follows a daily cycle in *Daphnia*. *J. Plankton Res.* 37: 636-644. https://doi.org/10.1093/plankt/fbv029
Sébert M-E, Legros C, Weltzien FA, Malpaux B, Chemineau P, Dufour S (2008) Melatonin activates brain dopaminergic systems in the eel with an inhibitory impact on reproductive function. *J. Neuroendocrinol.* 20: 917-929. https://doi.org/10.1111/j.1365-2826.2008.01744.x
Secombes CJ (1990) Isolation of salmonid macrophages and analysis of their killing activity. In Stolen JS, Fletcher TC, Anderson DP, Roberson BS eds, Techniques in fish immunology, Fair Haven, N.J., USA: SOS Publications, pp. 137-154,
Setiawan AN, Ozaki Y, Shoae A, Kazeto Y, Lokman PM (2012) Androgen-specific regulation of FSH signalling in the previtellogenic ovary and pituitary of the New Zealand shortfinned eel, *Anguilla australis*. *Gen. Comp. Endocrinol.* 176: 132-143. https://doi.org/10.1016/j.ygcen.2011.12.041
Shima JS, Osenberg CW, Noonburg EG, Alonzo SH, Swearer SE (2021) Lunar rhythms in growth of larval fish. *Proc. Biol. Sci.* 288: 20202609. https://doi.org/10.1098/rspb.2020.2609
Shin HS, Lee J, Choi CY (2011) Effects of LED light spectra on oxidative stress and the protective role of melatonin in relation to the daily rhythm of the yellowtail clownfish, *Amphiprion clarkii*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 160: 221-228. https://doi.org/10.1016/j.cbpa.2011.06.002
Spitschan M, Aguirre GK, Brainard DH, Sweeney AM (2016) Variation of outdoor illumination as a function of solar elevation and light pollution. *Sci. Rep.* 6: 26756. https://doi.org/10.1038/srep26756
Sreejith P, Beyo RS, Divya L, Vijayasree AS, Manju M, Oommen OV (2007) Triiodothyronine and melatonin influence antioxidant defense mechanism in a teleost *Anabas testudineus* (Bloch): *In vitro* study. *Indian J. Biochem. Biophys.* 44: 164-168. niscair.res.in
Stephan S (2021) Effects of artificial light at night, browning and eutrophication on freshwater phytoplankton. Dr. rer. nat., Technical University Berlin, Berlin
Stone T (2018) Re-envisioning the nocturnal sublime: On the ethics and aesthetics of nighttime lighting. *Topoi* 40: 481-491. https://doi.org/10.1007/s11245-018-9562-4
Sulistyo I, Fontaine P, Rinchard J, Gardeur J-N, Migaud H, Capdeville B, Kestemont P (2000) Reproductive cycle and plasma levels of steroids in male Eurasian perch *Perca fluviatilis*. *Aquat. Living Resour.* 13: 99-106. https://doi.org/10.1016/S0990-7440(00)00146-7
Sun J, Raap T, Pinxten R, Eens M (2017) Artificial light at night affects sleep behaviour differently in two closely related songbird species. *Environ. Pollut.* 231: 882-889. https://doi.org/10.1016/j.envpol.2017.08.098
Sun L, Zuo Z, Chen M, Chen Y, Wang C (2015) Reproductive and transgenerational toxicities of phenanthrene on female marine medaka (*Oryzias melastigma*). *Aquat. Toxicol.* 162: 109-116. https://doi.org/10.1016/j.aquatox.2015.03.013
Szczerbowski A, Kucharczyk D, Mamcarz A, Łuczýnski MJ, Targońska K, Kujawa R (2009) Artificial off-season spawning of Eurasian perch *Perca fluviatilis* L. *Arch. Pol. Fish./Arch. Ryb. Pol.* 17: 95-98.
Takemura A, Hiyakawa N, Niakido Y (2006) A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish, *Siganus guttatus*. *J. Pineal Res.* 40: 236-241. https://doi.org/10.1111/j.1600-079X.2005.00306.x

Takemura A, Rahman MS, Park YJ (2010) External and internal controls of lunar-related reproductive rhythms in fishes. *J. Fish Biol.* 76: 7-26. https://doi.org/10.1111/j.1095-8649.2009.02481.x
Tamir R, Lerner A, Haspel C, Dubinsky Z, Iluz D (2017) The spectral and spatial distribution of light pollution in the waters of the northern Gulf of Aqaba (Eilat). *Sci. Rep.* 7: 42329. https://doi.org/10.1038/srep42329
Tapia-Osorio A, Salgado-Delgado R, Angeles-Castellanos M, Escobar C (2013) Disruption of circadian rhythms due to chronic constant light leads to depressive and anxiety-like behaviors in the rat. *Behavioral Brain Research* 252: 1-9. https://doi.org/10.1016/j.bbr.2013.05.028
Thomas JR, James J, Newman RC, Riley WD, Griffiths SW, Cable J (2016) The impact of streetlights on an aquatic invasive species: Artificial light at night alters signal crayfish behaviour. *Appl. Anim. Behav. Sci.* 176: 143-149. https://doi.org/10.1016/j.applanim.2015.11.020
Thrush MA, Duncan NJ, Bromage NR (1994) The use of photoperiod in the production of out-of-season Atlantic salmon (*Salmo salar*) smolts. *Aquaculture* 121: 29-44. https://doi.org/10.1016/0044-8486(94)90005-1
Touzot M, Lefebure T, Lengagne T, Secondi J, Dumet A, Konecny-Dupre L, Veber P, Navratil V, Duchamp C, Mondy N (2021) Large-scale deregulation of gene expression by artificial light at night in tadpoles of common toads. *bioRxiv; non-peer-reviewed preprint*: https://doi.org/10.1101/2021.07.08.451570
Touzot M, Lengagne T, Secondi J, Desouhant E, Théry M, Dumet A, Duchamp C, Mondy N (2020) Artificial light at night alters the sexual behaviour and fertilisation success of the common toad. *Environ. Pollut.* 259: 113883. https://doi.org/10.1016/j.envpol.2019.113883
Touzot M, Teulier L, Lengagne T, Secondi J, Théry M, Libourel PA, Guillard L, Mondy N (2019) Artificial light at night disturbs the activity and energy allocation of the common toad during the breeding period. *Conserv. Physiol.* 7: coz002. https://doi.org/10.1093/conphys/coz002
Townsend CR, Risebow AJ (1982) The influence of light level on the functional response of a zooplanktonivorous fish. *Oecologia* 53: 293-295. https://doi.org/10.1007/BF00389002
Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test *Anal. Biochem.* 86: 271-278. https://doi.org/10.1016/0003-2697(78)90342-1
Underwood H (1989) The pineal and melatonin: Regulators of circadian function in lower vertebrates. *Experientia* 45: 914-922. https://doi.org/10.1007/BF01953048
Valenzuela AE, Silva VM, Klempau AE (2007) Some changes in the haematological parameters of rainbow trout (*Oncorhynchus mykiss*) exposed to three artificial photoperiod regimes. *Fish Physiol. Biochem.* 33: 35-48. https://doi.org/10.1007/s10695-006-9115-4
van der Heijden KB, Smits MG, van Someren EJ, Ridderinkhof KR, Gunning WB (2007) Effect of melatonin on sleep, behavior, and cognition in ADHD and chronic sleep-onset insomnia. *J. Am. Acad. Child Adolesc. Psychiatry* 46: 233-241. https://doi.org/10.1097/01.chi.0000246055.76167.0d
van der Kraak G (2009) The GnRH system and the neuroendocrine regulation of reproduction. In Bernier NJ, van der Kraak G, Farrell AP, Brauner CJ eds, Fish Neuroendocrinology Vol. 28, Amsterdam, The Naetherlands: Academic Press,
van der Kraak G, Donaldson EM, Chang JP (1986) Dopamine involvement in the regulation of gonadotropin secretion in coho salmon. *Can. J. Zool.* 64: 1245-1248. https://doi.org/10.1139/z86-185
van Doren BM, Willard DE, Hennen M, Horton KG, Stuber EF, Sheldon D, Sivakumar AH, Wang J, Farnsworth A, Winger BM (2021) Drivers of fatal bird collisions in an urban center. *PNAS* 118: https://doi.org/10.1073/pnas.2101666118
Vaughan MK, Hubbard GB, Champney TH, Vaughan GM, Little JC, Reiter RJ (1987) Splenic hypertrophy and extramedullary hematopoiesis induced in male Syrian hamsters by short photoperiod or melatonin injections and reversed by melatonin pellets or pinealectomy. *Am. J. Anat.* 179: 131-136. https://doi.org/10.1002/aja.1001790205
Vera LM, De Oliveira C, López-Olmeda JF, Ramos J, Mañanós E, Madrid JA, Sánchez-Vázquez FJ (2007) Seasonal and daily plasma melatonin rhythms and reproduction in Senegal sole kept under natural photoperiod and natural or controlled water temperature. *J. Pineal Res.* 43: 50-55. https://doi.org/10.1111/j.1600-079X.2007.00442.x
Vera LM, López-Olmeda JF, Bayarri MJ, Madrid JA, Sánchez-Vázquez FJ (2005) Influence of light intensity on plasma melatonin and locomotor activity rhythms in tench. *Chronobiol. Int.* 22: 67-78. https://doi.org/10.1081/cbi-200038157
Verma AK, Praksh S (2019) Impact of arsenic on haematology, condition factor, hepatosomatic and gastrosomatic index of a fresh water cat fish, *Mystus vittatus*. *International Journal on Biological Sciences* 10: 49-54. https://doi.org/10.13140/RG.2.2.31567.10409
Vivas Muñoz JC, Bierbach D, Knopf K (2019) Eye fluke (*Tylodelphys clavata*) infection impairs visual ability and hampers foraging success in European perch. *Parasitol. Res.* 118: 2531-2541. https://doi.org/10.1007/s00436-019-06389-5
Vowles AS, Kemp PS (2021) Artificial light at night (ALAN) affects the downstream movement behaviour of the critically endangered European eel, *Anguilla anguilla*. *Environ. Pollut.* 274: 116585. https://doi.org/10.1016/j.envpol.2021.116585

Vriend J, Reiter RJ, Anderson GR (1979) Effects of the pineal and melatonin on thyroid activity of male golden hamsters. *Gen. Comp. Endocrinol.* 38: 189-195. https://doi.org/10.1016/0016-6480(79)90206-5
Walker AM, Godard MJ, Davison P (2014) The home range and behaviour of yellow-stage European eel *Anguilla anguilla* in an estuarine environment. *Aquat. Conserv.: Mar. Freshwat. Ecosyst.* 24: 155-165. https://doi.org/10.1002/aqc.2380
Walker WH, 2nd, Bumgarner JR, Walton JC, Liu JA, Melendez-Fernandez OH, Nelson RJ, DeVries AC (2020) Light pollution and cancer. *Int. J. Mol. Sci.* 21: 9360. https://doi.org/10.3390/ijms21249360
Walles TJW (2020) Mesozooplankton studies through automation and machine learning. Dr. rer. nat., Technical University Berlin, Berlin
Walpita CN, Crawford AD, Janssens ED, Van der Geyten S, Darras VM (2009) Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. *Endocrinology* 150: 530-539. https://doi.org/10.1210/en.2008-0457
Walpita CN, Van der Geyten S, Rurangwa E, Darras VM (2007) The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (*Danio rerio*) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. *Gen. Comp. Endocrinol.* 152: 206-214. https://doi.org/10.1016/j.ygcen.2007.02.020
Wang D, Stapleton HM (2010) Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* 397: 1831-1839. https://doi.org/10.1007/s00216-010-3705-9
Wang J, Shi G, Yao J, Sheng N, Cui R, Su Z, Guo Y, Dai J (2020) Perfluoropolyether carboxylic acids (novel alternatives to PFOA) impair zebrafish posterior swim bladder development via thyroid hormone disruption. *Environ. Int.* 134: 105317. https://doi.org/10.1016/j.envint.2019.105317
Wang N, Eckmann R (1994) Distribution of perch (*Perca fluviatilis* L.) during their first year of life in Lake Constance. *Hydrobiologia* 277: 135-143. https://doi.org/10.1007/BF00007295
Weber GM, Okimoto DK, Richman NHr, Grau EG (1992) Patterns of thyroxine and triiodothyronine in serum and follicle-bound oocytes of the tilapia, *Oreochromis mossambicus*, during oogenesis. *Gen. Comp. Endocrinol.* 85: 392-404. https://doi.org/10.1016/0016-6480(92)90084-w
Weltzien F-A, Andersson E, Andersen Ø, Shalchian-Tabrizi K, Norberga B (2004) The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 137: 447-477. https://doi.org/10.1016/j.cbpb.2003.11.007
Whitmore D, Foulkes NS, Sassone-Corsi P (2000) Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* 404: 87-91. https://doi.org/10.1038/35003589
Wickham H (2016) ggplot2: Elegant graphics for data analysis
Witherington BE (1992) Behavioral responses of nesting sea turtles to artificial lighting. *Herpetologica* 48: 31-39. https://www.jstor.org/stable/3892916
Witherington BE (1997) The problem of photopollution for sea turtles and other nocturnal animals. In Clemmons JR, Buchholz R eds, Behavioral approaches to conservation in the wild, Cambridge, U.K.: Cambridge University Press, pp. 303-328,
Wittkowski W, Bergmann M, Hoffmann K, Pera F (1988) Photoperiod-dependent changes in TSH-like immunoreactivity of cells in the hypophysial pars tuberalis of the Djungarian hamster, *Phodopus sungorus*. *Cell Tissue Res.* 251: 183-187. https://doi.org/10.1007/BF00215463
Wood S, Loudon A (2014) Clocks for all seasons: unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. *J. Endocrinol.* 222: R39-R59. https://doi.org/10.1530/JOE-14-0141
Wright ML (2002) Melatonin, diel rhythms, and metamorphosis in anuran amphibians. *Gen. Comp. Endocrinol.* 126: 251-254. https://doi.org/10.1016/S0016-6480(02)00012-6
Wright ML, Bruni NK (2004) Influence of the photocycle and thermocycle on rhythms of plasma thyroxine and plasma and ocular melatonin in late metamorphic stages of the bullfrog tadpole, *Rana catesbeiana*. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* 139: 33-40. https://doi.org/10.1016/j.cbpb.2004.06.012
Wuertz S, Nitsche A, Jastroch M, Gessner J, Klingenspor M, Kirschbaum F, Kloas W (2007) The role of the IGF-I system for vitellogenesis in maturing female sterlet, *Acipenser ruthenus* Linnaeus, 1758. *Gen. Comp. Endocrinol.* 150: 140-150. https://doi.org/10.1016/j.ygcen.2006.07.005
Yam KM, Yoshiura Y, Kobayashi M, Ge W (1999) Recombinant goldfish activin B stimulates gonadotropin-Iβ but Inhibits gonadotropin-IIβ expression in the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* 116: 81-89. https://doi.org/10.1006/gcen.1999.7339
Yoshimura T (2010) Neuroendocrine mechanism of seasonal reproduction in birds and mammals. *Animal Science Journal* 81: 403-410. https://doi.org/10.1111/j.1740-0929.2010.00777.x
Yuen CW, Ge W (2004) Follistatin suppresses FSHβ but increases LHβ expression in the goldfish – evidence for an activin-mediated autocrine/paracrine system in fish pituitary. *Gen. Comp. Endocrinol.* 35: 108-115. https://doi.org/10.1016/j.ygcen.2003.08.012

Yumnamcha T, Khan ZA, Rajiv C, Devi SD, Mondal G, Sanjita Devi H, Bharali R, Chattoraj A (2017) Interaction of melatonin and gonadotropin-inhibitory hormone on the zebrafish brain-pituitary-reproductive axis. *Mol. Reprod. Dev.* 84: 389-400. https://doi.org/10.1002/mrd.22795

Zachmann A, Falcón J, Knijff SCM, Bolliet V, Ali MA (1992) Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the white sucker (*Catostomus commersoni*) *in vitro*. *Gen. Comp. Endocrinol.* 86: 26-33. https://doi.org/10.1016/0016-6480(92)90122-Z

Zhao HJ, Xu JK, Yan ZH, Ren HQ, Zhang Y (2020) Microplastics enhance the developmental toxicity of synthetic phenolic antioxidants by disturbing the thyroid function and metabolism in developing zebrafish. *Environ. Int.* 140: 105750. https://doi.org/10.1016/j.envint.2020.105750

Zhao X, Ren X, Ren B, Luo Z, Zhu R (2016) Life-cycle exposure to BDE-47 results in thyroid endocrine disruption to adults and offsprings of zebrafish (*Danio rerio*). *Environ. Toxicol. Pharmacol.* 48: 157-167. https://doi.org/10.1016/j.etap.2016.10.004

Zhou T, John-Alder HB, Weis JS, Weis P (2000) Endocrine disruption: thyroid dysfunction in mummichogs (*Fundulus heteroclitus*) from a polluted habitat. *Mar. Environ. Res.* 50: 393-397. https://doi.org/10.1016/S0141-1136(00)00042-8

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O (2010) Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.* 165: 438-455. https://doi.org/10.1016/j.ygcen.2009.04.017

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in ecology with R. Springer Science+Business Media, LLC, New York, USA.

Appendices

Appendix A – Abbreviations and Glossary

11 KT	11-keto-testosterone; main sexual steroid in male fish
AANAT	Arylalkyl -N- aminotransferase; key enzyme in the production of melatonin
Adverse effect	*"Change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences"* (IPCS, 2004)
ALAN	Artificial light at night
BMAL, *bmal*	Brain and Muscle ARNT-Like (PROTEIN, *gene*)
Climate chamber	A temperature-controlled room for experimental setups
CLOCK, *clock*	Circadian Locomotor Output Cycles Kaput (PROTEIN, *gene*)
CRY, *cry*	Cryptochrome (PROTEIN, *gene*)
DIO, *dio*	Deiodinase
DVM	Diel vertical migration; a behavioral pattern of aquatic animals with distinct hours of the day with upward and downward movement
E2	17-β-estradiol; main sexual steroid in female fish
Ecophysiology	Physiological (e.g., molecular, cellular, metabolic) processes in an ecological context
Environmental stressor	Anthropogenically introduced factors which alter natural ecosystems *"beyond its natural limits of variation"* (Carrier-Belleau *et al.*, 2021)
FSH, *fshβ*	Follicle-stimulating hormone (PROTEIN, *gene*); gonadotropin
GnIH	Gonadotropin-inhibitory hormone
GnRH	Gonadotropin-releasing hormone
HPG axis	Hypothalamic-pituitary-gonadal axis
HPT axis	Hypothalamic-pituitary-thyroid axis
I_G	Gonadosomatic index (gonad mass relative to body mass)
I_H	Hepatosomatic index (liver mass relative to body mass)
I_S	Splenosomatic index (spleen mass relative to body mass)

IDA	International Dark Sky Association
IEA	International Energy Agency
IUCN	International Union for Conservation of Nature
K	Condition index (standard length relative to body mass)
LakeLab	Large lake enclosure research facility by the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB)
LH, *lhβ*	Luteinizing hormone (PROTEIN, *gene*); gonadotropin
LL	Experimental setting with continuous illumination
LOEL	Lowest-observed-effect level
Molecular clock	Oscillator of the circadian system; involves oscillating expression of a variety of genes (mainly CLOCK, BMAL, PER, and CRY)
NOEL	No-observed-effect level
PER, *per*	Period (PROTEIN, *gene*)
Photoperiod (*x* L:*y* D)	length of daytime (*x* L indicates hours of daytime or bright light in artificial conditions, *y* D refers to the hours of darkness)
rT3	3,3',5'-reverse triiodothyronine;
T2	3,3'- or 3,5-diiodothyronine; metabolically less active thyroid hormone with two bound iodine (see TH)
T3	3,3',5-triiodothyronine; thyroid hormone with three bound iodine (see TH)
T4	Thyroxine; thyroid hormone with four bound iodine
TBARS	Thiobarbituric acid reactive substances; a physiological indicator for lipid peroxidation
TH	Thyroid hormones; vital hormones produced in thyroid follicles and fundamental for normal development and metabolism
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
Skyglow	A form of light pollution where light is scattered in the air by particles that has rather low light intensities, but illuminates large areas (km to hundreds of km) due to scattering of light in the atmosphere away from the original light source
Smoltification	A transition process of juvenile anadromous salmonids in which physiological adjustments from life in freshwater to life in salt water occur
WHO	World Health Organization

Appendix B – Supplementary material to Chapter 1

Table S1.1
Model specifications of the linear mixed models for absolute (A) and relative (B) melatonin concentrations with treatment and time as fixed factors in an interaction term and tanks nested in run as a random factor. A weight term was added for treatment to account for different variances among treatments. A cosine function with a period of 21 h as a covariate of time was included to linearize the relationship (costime). Post-hoc tests compared every treatment to every other treatment with Tukey's adjustment.

A) Melatonin concentration in tank water

R^2_m = 0.6772 and R^2_c = 0.7139

Fixed effects	**Estimate ± SE**	**DF**	**T-value**	**LLR**	***p*-value**
Intercept (0 lx at 11 am)	16.40 ± 0.83	188	19.71		
treat 0.01 lx	–7.59 ± 0.63	19	–12.05		
treat 0.1 lx	–9.68 ± 0.61	19	–15.84		
treat 1 lx	–12.13 ± 0.62	19	–19.71		
costime	4.85 ± 0.66	188	7.31		
treat 0.01 lx : costime	–3.00 ± 0.73	188	–4.12	37.58	<0.0001
treat 0.1 lx : costime	–3.49 ± 0.69	188	–5.06		
treat 1 lx : costime	–4.30 ± 0.70	188	–6.15		
Random effect	**Estimate**			**LLR**	***p*-value**
Run	$\sigma^2 = 0.91^2$			47.43	<0.0001
tank in run	$\sigma^2 = 0.70^2$				
residual	$\sigma^2 = 3.22^2$				

Post-hoc testing of melatonin concentrations in tank water

Contrast	**Estimate ± SE**	**df**	**T-value**	***p*-value**
0 lx – 0.01 lx	3.00 ± 0.73	188	4.12	0.0003
0 lx – 0.1 lx	3.49 ± 0.69	188	5.06	<0.0001
0 lx – 1 lx	4.30 ± 0.70	188	6.16	<0.0001
0.01 lx – 0.1 lx	0.49 ± 0.35	188	1.40	0.5034
0.01 lx – 1 lx	1.30 ± 0.37	188	3.52	0.0030
0.1 lx – 1 lx	0.81 ± 0.29	188	2.83	0.0265

continued **Table S.1.1**

B) Melatonin concentration relative to baseline					
R^2_m = 0.4430 and R^2_c = 0.5578					
Fixed effects	**Estimate ± SE**	**DF**	**T-value**	**LLR**	***p*-value**
Intercept (0 lx at 11 am)	163.12 ± 7.80	188	20.92		
treat 0.01 lx	–14.65 ± 10.72	19	–1.37		
treat 0.1 lx	–39.01 ± 10.42	19	–3.74		
treat 1 lx	–58.06 ± 10.82	19	–5.37		
costime	48.62 ± 6.46	188	7.52		
treat 0.01 lx : costime	–16.25 ± 8.27	188	–1.96	17.83	0.0005
treat 0.1 lx : costime	–23.74 ± 7.33	188	–3.24		
treat 1 lx : costime	–35.67 ± 8.55	188	–4.17		
Random effect	**Estimate**			**LLR**	***p*-value**
Run	σ^2 = 0.0049²			28.35	<0.0001
tank in run	σ^2 = 15.97²				
residual	σ^2 = 31.34²				
Post-hoc testing of melatonin concentrations relative to baseline					
Contrast	**Estimate ± SE**	**df**	**T-value**		***p*-value**
0 lx – 0.01 lx	16.2 ± 8.27	188	1.96		0.2055
0 lx – 0.1 lx	23.7 ± 7.33	188	3.24		0.0077
0 lx – 1 lx	35.7 ± 8.55	188	4.17		0.0003
0.01 lx – 0.1 lx	7.5 ± 6.22	188	1.21		0.6243
0.01 lx – 1 lx	19.4 ± 7.61	188	2.55		0.0555
0.1 lx – 1 lx	11.9 ± 6.58	188	1.81		0.2703

Table S1.2
Relative MESOR and acrophase of daily melatonin rhythms in the tank water of Eurasian perch under control conditions with dark nights (0 lx) and three different nocturnal light intensities. The best-fit values (range of 95% confidence intervals) of the non-linear regression using the single-component cosine analysis (Equation 1.1) are graphically displayed in Figure 1.2. The base for the acrophase values is 8 am prior to the sampling period.

	MESOR (%)	**Acrophase (h)**
Initial values	200	20
0 lx	164.0 (155.1 to 172.8)	21.30 (20.26 to 22.35)
0.01 lx	149.2 (140.2 to 158.1)	21.60 (19.90 to 23.31)
0.1 lx	124.9 (120.1 to 129.7)	21.65 (20.43 to 22.88)
1 lx	106.2 (96.91 to 115.6)	19.81 (16.70 to 22.92)

Table S1.3
Model specifications of the linear mixed model analysis on the sums of daily and nocturnal melatonin concentrations in the tank water (Figure 1.3) with treatment and "time of day" (tod) as fixed factors in an interaction term and tanks nested in runs as a random factor. A weight term was added for treatment to account for different variances among treatments. Post-hoc tests compared every treatment to every other treatment with Tukey's adjustment.

Sums of daily and nocturnal melatonin concentrations in the tank water					
R^2_m = 0.8671 and R^2_c = 0.9222					
Fixed effects	**Estimate ± SE**	**DF**	**t-value**	**LLR**	***p*-value**
Intercept (0 lx at 11 am)	55.06 ± 3.84	20	–14.34		
treat 0.01 lx	–24.46 ± 3.13	19	–7.81		
treat 0.1 lx	–31.41 ± 3.06	19	–10.26		
treat 1 lx	–39.29 ± 3.09	19	–12.72		
tod night	22.62 ± 3.40	20	6.64		
treat 0.01 lx : tod night	–12.99 ± 3.59	20	–3.61	27.02	<0.0001
treat 0.1 lx : tod night	–15.72 ± 3.47	20	–4.53		
treat 1 lx : tod night	–19.66 ± 3.52	20	–5.59		
Random effect	**Estimate**			**LLR**	***p*-value**
Run	σ^2 = 3.81²			24.05	<0.0001
tank in run	σ^2 = 3.17²				
residual	σ^2 = 5.90²				
Post-hoc testing (Tukey adjustment) of daily and nocturnal sums					
Contrast (treatments)	**Estimate ± SE**	**df**	**t-value**		***p*-value**
0 lx – 0.01 lx (day)	24.46 ± 3.13	19	7.81		<0.0001
0 lx – 0.1 lx (day)	31.41 ± 3.06	19	10.26		<0.0001
0 lx – 1 lx (day)	39.29 ± 3.09	19	12.72		<0.0001
0.01 lx – 0.1 lx (day)	6.95 ± 2.06	19	3.38		0.0154
0.01 lx – 1 lx (day)	14.83 ± 2.10	19	7.07		<0.0001
0.1 lx – 1 lx (day)	7.88 ± 1.99	19	3.96		0.0043
0 lx – 0.01 lx (night)	37.45 ± 3.13	19	11.96		<0.0001
0 lx – 0.1 lx (night)	47.13 ± 3.06	19	15.40		<0.0001
0 lx – 1 lx (night)	58.95 ± 3.09	19	19.09		<0.0001
0.01 lx – 0.1 lx (night)	9.68 ± 2.06	19	4.70		0.0008
0.01 lx – 1 lx (night)	21.50 ± 2.10	19	10.25		<0.0001
0.1 lx – 1 lx (night)	11.82 ± 1.99	19	5.94		0.0001
day – night (0 lx)	–22.62 ± 3.41	20	–6.64		<0.0001
day – night (0.01 lx)	–9.63 ± 1.15	20	–8.35		<0.0001
day – night (0.1 lx)	–6.91 ± 0.66	20	–10.42		<0.0001
day – night (1 lx)	–2.96 ± 0.88	20	–3.38		0.0030

Appendix C – Supplementary material to Chapter 2

Table S2.1
Kruskal-Wallis and Mann-Whitney U-test analyses of *rpL8* dependencies on Treatment for each sex in the two different experiments. For the climate chamber experiment, Ct values were corrected by a calibrator sample which was measured on each plate. For the field experiment, Ct values were taken directly as all samples were measured on the same plate. For more information see Material and methods section.

Kruskal-Wallis test	**Explanatory variable**	**Sex**	**Chi²**	**df**	***p*-value**
climate chamber experiment	ALAN	male	4.63	3	0.20
	ALAN	female	5.43	3	0.14
Mann-Whitney *U*-tests	**Explanatory variable**	**Sex**	***U***		***p*-value**
climate chamber experiment	Sex	males vs. females	395		0.08
field experiment	Sex	males vs. females	25		0.95
	ALAN	males	NA†		NA†
	ALAN	female	27.5		0.30

† not enough data points for meaningful statistics (only n = 2 individuals for each treatment)

Table S2.2
Full linear model (LM) specifications for female Eurasian perch *Perca fluviatilis* in the climate chamber experiment. Uncertainties of estimates are expressed as standard errors (SE) as well as 95% confidence intervals (CI). LMs for each dependent variable (relative pituitary expressions of *fshβ*, *lhβ*, and the ratio of their change relative to control, as well as plasma 11-keto-testosterone) included ALAN and body mass as fixed factors. For more information see Material and methods section.

***fshβ* climate chamber experiment ($R^2_{multiple}$ = 0.6508, $R^2_{adjusted}$ = 0.5344)**					
Fixed factors	**Estimate ± SE**	**95% CI**	**t-value**	**F-statistic**	***p*-value**
Intercept †	–5.89 ± 1.48	–9.12 – –2.66	–3.98		
ALAN 0.01 lx	0.05 ± 0.83	–1.75 – 1.85	0.06	0.10	0.96
ALAN 0.1 lx	–0.09 ± 0.82	–1.87 – 1.70	–0.10		
ALAN 1 lx	0.23 ± 0.76	–1.93 – 1.36	–0.38		
Body mass	0.23 ± 0.05	0.11 – 0.35	4.33	18.75	0.001
***lhβ* climate chamber experiment ($R^2_{multiple}$ = 0.7682, $R^2_{adjusted}$ = 0.691)**					
Fixed factors	**Estimate ± SE**	**95% CI**	**t-value**	**F-statistic**	***p*-value**
Intercept †	–3.00 ± 0.59	–4.29 – –1.72	–5.08		
ALAN 0.01 lx	0.31 ± 0.33	–0.40 – 1.03	0.95	3.21	0.06
ALAN 0.1 lx	0.12 ± 0.33	–0.60 – 0.83	0.35		
ALAN 1 lx	0.77 ± 0.30	0.11 – 1.42	2.55		
Body mass	0.12 ± 0.02	0.07 – 0.16	5.54	30.66	0.0001
***fshβ*-change/*lhβ*-change climate chamber experiment ($R^2_{multiple}$ = 0.4133, $R^2_{adjusted}$ = 0.2178)**					
Fixed factors	**Estimate ± SE**	**95% CI**	**t-value**	**F-statistic**	***p*-value**
Intercept †	–2.89 ± 1.55	–6.28 – 0.49	–1.86		
ALAN 0.01 lx	–0.26 ± 0.87	–2.15 – 1.63	–0.31	0.91	0.47
ALAN 0.1 lx	–0.20 ± 0.86	–2.07 – 1.67	–0.23		
ALAN 1 lx	–1.05 ± 0.79	–2.78 – 0.67	–1.33		
Body mass	0.11 ± 0.06	–0.01 – 0.23	2.03	4.11	0.07
11-keto-testosterone climate chamber experiment ($R^2_{multiple}$ = 0.24, $R^2_{adjusted}$ = –0.06)					
Fixed factors	**Estimate ± SE**	**95% CI**	**t-value**	**F-statistic**	***p*-value**
Intercept †	–32 ± 100	–254 – 190	–032		
ALAN 0.01 lx	7 ± 43	–89 – 103	0.17	0.54	0.67
ALAN 0.1 lx	–6 ± 46	–108 – 94	–0.12		
ALAN 1 lx	37 ± 39	–49 – 123	0.95		
Body mass	5 ± 4	–3 – 13	1.39	1.92	0.19

† Intercept taken at Controls (“0 lx”)

Table S2.3
Full linear mixed model (LMM) specifications for male Eurasian perch *Perca fluviatilis* in the climate chamber experiment. Uncertainties of estimates are expressed as standard errors (SE) as well as 95% confidence intervals (CI). Log-likelihood ratios (LLRs) and *p*-values refer to overall effects from model comparisons. LMMs for each dependent variable (relative pituitary expressions of *fshβ*, *lhβ*, and the ratio of their change relative to control, as well as plasma 11-keto-testosterone) included ALAN and body mass as fixed factors and runs and aquaria as random terms including a weight term for variation across aquaria. For more information see Material and methods section.

***fshβ* climate chamber experiment ($R^2_{marginal}$ = 0.1512, $R^2_{conditional}$ = 0.1512)**

Fixed effects	Estimate ± SE	95% CI	df	t-value	LLR	*p*-value
Intercept †	–1.98 ± 0.42	–2.83 – 1.13	37	–4.73		
ALAN 0.01 lx	–0.30 ± 0.25	–0.82 – 0.21	19	–1.24	6.08	0.11
ALAN 0.1 lx	–0.53 ± 0.26	–1.07 – –0.02	19	–2.01		
ALAN 1 lx	–0.01 ± 0.19	–0.40 – 0.38	19	–0.05		
Body mass	0.11 ± 0.02	0.07 – 0.15	37	5.57	22.33	<0.0001
Random effect	**Estimate**				**LLR**	***p*-value**
Runs	σ^2 = 2.2e05²					
Aquaria in runs	σ^2 = 1.0e-05²				1.4e-08	1
Residual	σ^2 = 1.15²					

***lhβ* climate chamber experiment ($R^2_{marginal}$ = 0.1045, $R^2_{conditional}$ = 0.2208)**

Fixed effects	Estimate ± SE	95% CI	df	t-value	LLR	*p*-value
Intercept †	–1.22 ± 0.34	–1.90 – 0.53	37	–3.58		
ALAN 0.01 lx	0.32 ± 0.20	–0.10 – 0.74	19	1.58	4.12	0.25
ALAN 0.1 lx	–0.09 ± 0.24	–0.56– 0.09	19	–0.39		
ALAN 1 lx	0.01 ± 0.24	–0.50 – 0.52	19	0.03		
Body mass	0.05 ± 0.01	0.05 – 0.08	37	3.73	7.07	0.008
Random effect	**Estimate**				**LLR**	***p*-value**
Runs	σ^2 = 0.08²					
Aquaria in runs	σ^2 = 0.29²				0.88	0.26
Residual	σ^2 = 0.78²					

continued **Table S2.3**

***fshβ*-change/*lhβ*-change climate chamber experiment ($R^2_{marginal}$ = 0.0491, $R^2_{conditional}$ = 0.1119)**						
Fixed effects	**Estimate ± SE**	**95% CI**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	–1.81 ± 0.38	–2.59 – 1.04	37	–4.73		
ALAN 0.01 lx	–0.39 ± 0.21	–0.83 – 0.05	19	–1.85	9.09	0.03
ALAN 0.1 lx	–0.23 ± 0.27	–0.80 – 0.33	19	–0.87		
ALAN 1 lx	0.15 ± 0.17	–0.21 – 0.51	19	0.86		
Body mass	0.09 ± 0.02	0.06 – 0.13	37	5.24	17.70	<0.0001
Random effect	**Estimate**				**LLR**	***p*-value**
Runs	σ^2 = 1.4e-05²					
Aquaria in runs	σ^2 = 1.0e-08²				8.0e-09	1
Residual	σ^2 = 0.58²					
11-keto-testosterone climate chamber experiment ($R^2_{marginal}$ = 0.5020, $R^2_{conditional}$ = 0.5020)						
Fixed effects	**Estimate ± SE**	**95% CI**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	–2184 ± 490	–3173 – –1195	43	–4.45		
ALAN 0.01 lx	–374 ± 241	–878 – 129	19	–1.56	7.93	0.048
ALAN 0.1 lx	193 ± 265	–362 – 749	19	0.73		
ALAN 1 lx	377 ± 308	–267 – 1021	19	1.22		
Body mass	182 ± 21	129 – 225	43	8.52	29.81	<0.0001
Random effect	**Estimate**				**LLR**	***p*-value**
Runs	σ^2 = 0.03²					
Aquaria in runs	σ^2 = 0.03²				8.3e-08	1
Residual	σ^2 = 682²					

† Intercept taken at Controls (“0 lx”)

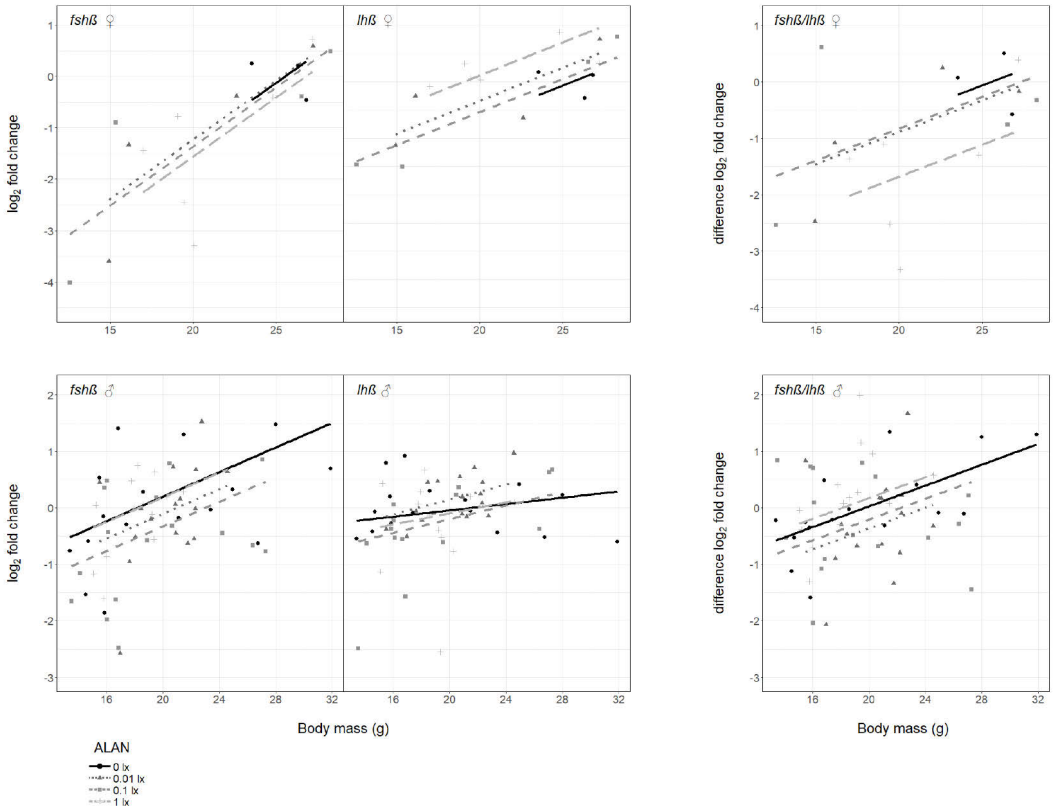

Figure S2.1 Correlations of the expression of *fshβ* and *lhβ*, and the ratio of their change relative to control with body mass of female and male Eurasian perch *Perca fluviatilis* after two weeks of exposure to artificial light at night (ALAN) at different illuminances (0.01 lx, 0.1 lx, 1 lx) or controls without nocturnal illumination (< 0.00167 lx, "0 lx"). Body mass significantly explained variance in the data for all dependent variables ($p < 0.05$ in LMs for females, LMMs for males), except for the ratio of females (upper right panel).

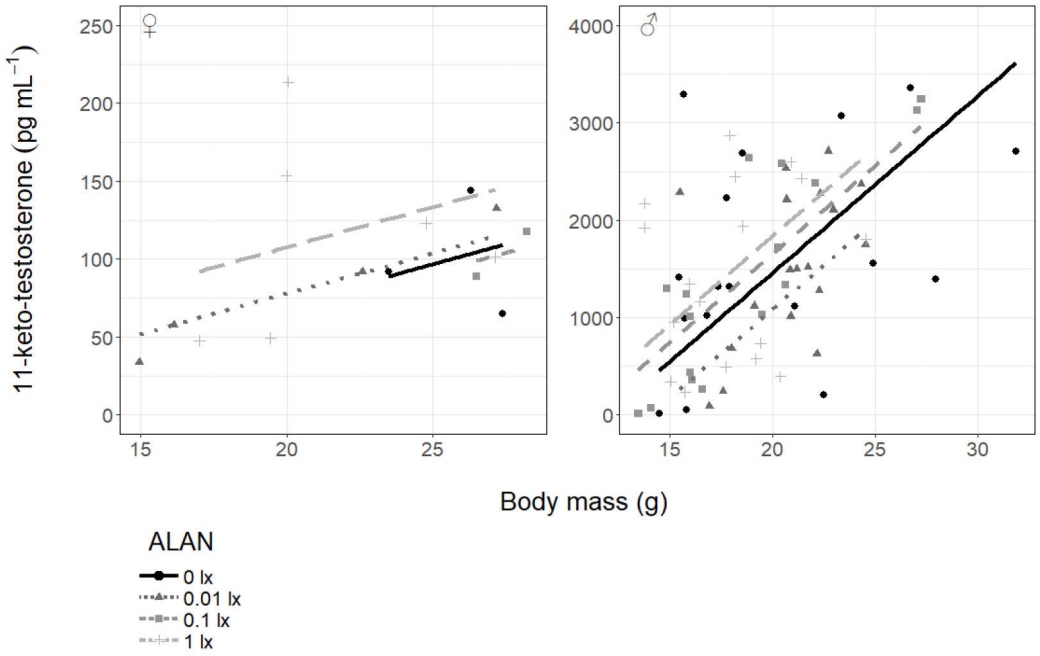

Figure S2.2 Correlations of the expression of 11-keto-testosterone (11 KT) with body mass of female and male Eurasian perch *Perca fluviatilis* after two weeks of exposure to artificial light at night (ALAN) at different illuminances (0.01 lx, 0.1 lx, 1 lx) or controls without nocturnal illumination (< 0.00167 lx, "0 lx") in the climate chamber experiment. Body mass significantly explained variance in the data for males (LMM, Body mass-factor: $p < 0.0001$), but not for females (LMs, Body mass-factor: $p = 0.19$).

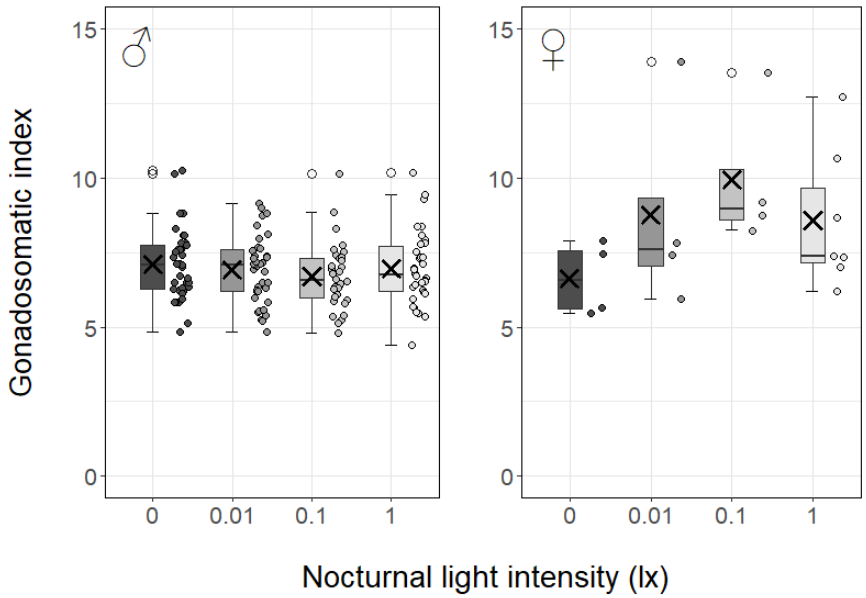

Figure S2.3 Gonadosomatic index (gonad mass relative to body mass, %) for maturing male and female Eurasian perch *Perca fluviatilis* after two weeks of exposure to artificial light at night (ALAN) at different illuminances (0.01 lx, 0.1 lx, 1 lx) or controls without nocturnal illumination (< 0.00167 lx, "0 lx") in the climate chamber experiment.

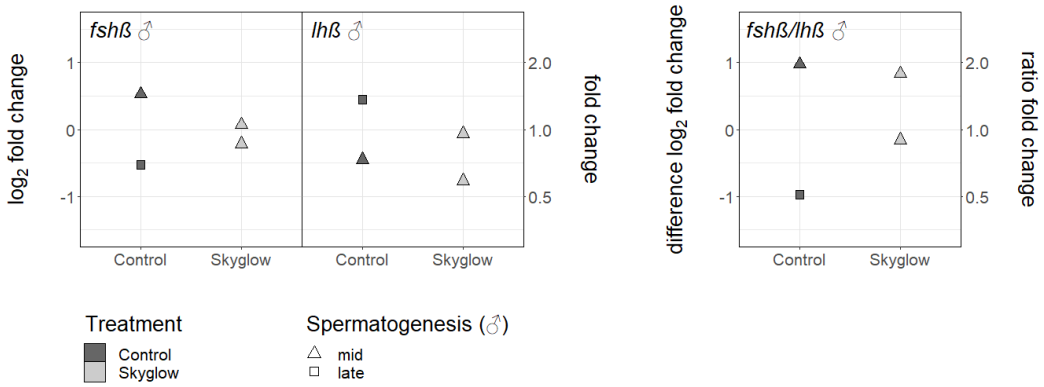

Figure S2.4 Relative mRNA expression of the *β*-subunits of the gonadotropins follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*) of male Eurasian perch *Perca fluviatilis* exposed to natural conditions (Control, dark gray) or artificial illumination (Skyglow; light gray) for 1 – 2 months in the field experiment (outdoor enclosures). Samples of the pituitary were taken throughout the day. Individual points represent the values of each individual (n = 2 for Control and Skyglow) with triangles or squares indicating mid- or late spermatogenesis, respectively. On the left, both y-axes refer to both genes. The right graph shows the ratio of *fshβ*-change/*lhβ*-change. No statistical analysis was performed due to low sample size.

Table S2.4 Full data for the field experiment. Run – Temporal replication (Oct – October, Nov – November), Date – Sampling date, Std length – standard length (cm), Body mass (g), Gonad – mass of gonads (g), GSI – gonadosomatic index (gonad mass relative to body mass, %), 11KT – 11-keto-testosterone (pg mL^{-1}), rpL8 – ribosomal protein L8, FSH – follicle-stimulating hormone, LH – luteinizing hormone, Ct – Cycle threshold, Ctcorr – Ct-value corrected for efficiencies of primers, dCt/ddCt/2ddCt – delta Ct-values/delta delta Ct-values/2^{ddCt}-values (see "Relative mRNA quantification by RT-qPCR").

Treatment	Aquarium	Run	Date	Std length	Body mass	Sex	Gonad	GSI	11KT	rpL8_CalcorrCt	LH_dCt	LH_ddCt	LH_2ddCt	FSH_dCt	FSH_ddCt	FSH_2ddCt
Control ("0 lx")	A3	Oct	19.10.2017	11.8	23	m	1.72	7.36	3067	19.74	2.81	-0.44	0.74	-1.52	-0.03	0.98
Control ("0 lx")	A3	Oct	19.10.2017	12.2	27	f	1.55	5.65	65							
Control ("0 lx")	A1	Oct	19.10.2017	12.7	32	m	2.08	6.51	2710	19.64	2.65	-0.60	0.66	-0.79	0.70	1.62
Control ("0 lx")	A1	Oct	19.10.2017	12.4	27	f	2.00	7.46		20.57	3.51	0.12	1.08	0.02	-0.46	0.73
Control ("0 lx")	A1	Oct	19.10.2017	12.0	27	m	2.36	8.84	3361	20.09	2.73	-0.52	0.70	-2.11	-0.63	0.65
Control ("0 lx")	A1	Oct	19.10.2017	11.9	26	f	2.08	7.89	144	20.16	3.10	-0.29	0.82	0.70	0.21	1.16
Control ("0 lx")	A1	Oct	19.10.2017	10.3	15	m	1.26	8.28								
Control ("0 lx")	A1	Oct	19.10.2017	10.2	14	m	0.97	7.01								
Control ("0 lx")	A1	Oct	19.10.2017	10.4	17	m	1.68	10.15								
Control ("0 lx")	A1	Oct	19.10.2017	11.5	21	m	2.17	10.27	1119	20.00	3.39	0.14	1.10	-1.66	-0.18	0.88
Control ("0 lx")	A2	Oct	20.10.2017	10.4	18	m	1.35	7.60	2223							
Control ("0 lx")	A2	Oct	20.10.2017	11.0	22	m	1.69	7.83								
Control ("0 lx")	A2	Oct	20.10.2017	10.7	18	m	1.37	7.58								
Control ("0 lx")	A2	Oct	20.10.2017	12.0	25	m	1.67	6.70	1558	18.60	3.66	0.41	1.33	-1.16	0.33	1.26
Control ("0 lx")	A2	Oct	20.10.2017	12.0	28	m	2.47	8.81	1396	18.73	3.48	0.23	1.17	-0.01	1.48	2.79
Control ("0 lx")	A2	Oct	20.10.2017	10.4	17	m	1.37	8.09								
Control ("0 lx")	A2	Oct	20.10.2017	11.7	24	f	1.29	5.46	92	19.59	3.57	0.17	1.13	0.73	0.25	1.19
Control ("0 lx")	A3	Nov	29.11.2017	10.2	16	m	1.28	8.09								
Control ("0 lx")	A3	Nov	29.11.2017	11.4	16	m	0.76	4.82	51	19.01	2.97	-0.27	0.83	-3.35	-1.86	0.28
Control ("0 lx")	A3	Nov	29.11.2017	11.5	23	m	1.43	6.35	210							
Control ("0 lx")	A3	Nov	29.11.2017	11.2	17	m	1.13	6.50	1314	20.28	3.16	-0.09	0.94	-1.78	-0.29	0.82
Control ("0 lx")	A3	Nov	29.11.2017	10.9	16	m	0.99	6.25								
Control ("0 lx")	A3	Nov	29.11.2017	11.9	22	m	1.38	6.21								
Control ("0 lx")	A3	Nov	29.11.2017	11.1	17	m	1.15	6.63								
Control ("0 lx")	A3	Nov	29.11.2017	11.7	19	m	1.48	7.81								
Control ("0 lx")	A3	Nov	29.11.2017	10.9	18	m			1320							
Control ("0 lx")	A2	Nov	30.11.2017	11.2	19	m	1.17	6.32	2684	18.57	3.55	0.30	1.23	-1.21	0.28	1.21
Control ("0 lx")	A2	Nov	30.11.2017	10.8	16	m	1.20	7.43								
Control ("0 lx")	A2	Nov	30.11.2017	10.7	16	m	1.12	7.13	988	18.90	3.45	0.20	1.15	-1.63	-0.15	0.90
Control ("0 lx")	A2	Nov	30.11.2017	10.5	16	m	1.11	7.07	3295							
Control ("0 lx")	A2	Nov	30.11.2017	10.3	13	m	0.84	6.26		18.93	2.70	-0.54	0.69	-2.25	-0.77	0.59
Control ("0 lx")	A2	Nov	30.11.2017	10.6	15	m	0.75	5.12								
Control ("0 lx")	A2	Nov	30.11.2017	10.0	15	m	1.05	7.13		18.93	3.18	-0.06	0.96	-2.08	-0.59	0.66
Control ("0 lx")	A2	Nov	30.11.2017	11.3	21	m	1.63	7.59		18.76	3.20	-0.05	0.96	-0.19	1.30	2.46
Control ("0 lx")	A2	Nov	30.11.2017	10.2	13	m	0.86	6.49								

Treatment	Aquarium	Run	Date	Std length	Body mass	Sex	Gonad	GSI	11KT	rpL8_CalcorrCt	LH_dCt	LH_ddCt	LH_2ddCt	FSH_dCt	FSH_ddCt	FSH_2ddCt
Control (“0 lx“)	A1	Nov	30.11.2017	11.3	20	m	1.53	7.53								
Control (“0 lx“)	A1	Nov	01.12.2017	10.2	15	m	0.90	5.84	1410	19.00	4.04	0.80	1.74	-0.95	0.54	1.45
Control (“0 lx“)	A1	Nov	01.12.2017	10.7	15	m	0.85	5.84	10	18.19	2.83	-0.42	0.75	-3.03	-1.54	0.34
Control (“0 lx“)	A1	Nov	01.12.2017	10.5	17	m	0.98	5.83	1020	18.36	4.17	0.92	1.89	-0.08	1.41	2.65
Control (“0 lx“)	A1	Nov	01.12.2017	11.1	18	m	1.12	6.13								
Control (“0 lx“)	A1	Nov	01.12.2017	11.0	17	m	1.30	7.75								
Control (“0 lx“)	A1	Nov	01.12.2017	10.0	15	m	0.92	5.94								
0.01 lx	B1	Oct	19.10.2017	11.1	21	m	1.29	6.21								
0.01 lx	B1	Oct	19.10.2017	11.2	23	f	1.77	7.81	92	19.51	2.76	-0.63	0.64	0.10	-0.39	0.77
0.01 lx	B1	Oct	19.10.2017	11.4	22	m	1.99	9.14	1517	21.26	3.96	0.71	1.64	-2.11	-0.63	0.65
0.01 lx	B1	Oct	19.10.2017	10.9	21	m	1.81	8.74	2532							
0.01 lx	B1	Oct	19.10.2017	12.2	28	m	2.08	7.56								
0.01 lx	B1	Oct	19.10.2017	11.3	24	m	1.69	6.95	2371							
0.01 lx	B1	Oct	19.10.2017	10.2	17	m	1.24	7.17								
0.01 lx	B3	Oct	19.10.2017	11.5	21	m	1.33	6.34	1014	18.37	3.45	0.20	1.15	-1.93	-0.45	0.73
0.01 lx	B3	Oct	19.10.2017	11.4	17	m	0.93	5.48	93	18.68	2.74	-0.50	0.70	-4.06	-2.58	0.17
0.01 lx	B3	Oct	19.10.2017	12.0	27	f	1.61	5.94	133	20.13	4.15	0.76	1.69	1.07	0.59	1.51
0.01 lx	B3	Oct	19.10.2017	10.8	19	m	1.35	7.03	1119	18.88	3.72	0.47	1.39	-1.69	-0.20	0.87
0.01 lx	B3	Oct	19.10.2017	9.9	15	m	1.07	7.36								
0.01 lx	B2	Oct	20.10.2017	11.3	21	m	1.66	7.95	1489	18.90	3.14	-0.10	0.93	-1.42	0.07	1.05
0.01 lx	B2	Oct	20.10.2017	11.2	22	m	2.01	9.01	2276	18.42	3.70	0.45	1.37	-0.81	0.68	1.60
0.01 lx	B2	Oct	20.10.2017	10.8	18	m	1.02	5.81	244	18.89	3.19	-0.06	0.96	-2.44	-0.95	0.52
0.01 lx	B2	Oct	20.10.2017	10.8	18	m	1.63	8.83		19.13	3.70	0.45	1.37	-1.50	-0.01	0.99
0.01 lx	B2	Oct	20.10.2017	11.6	24	m	1.54	6.50								
0.01 lx	B2	Oct	20.10.2017	10.6	16	f	1.20	7.41	58	19.45	3.14	-0.25	0.84	-0.84	-1.33	0.40
0.01 lx	B2	Oct	20.10.2017	11.0	20	m	1.66	8.40								
0.01 lx	B1	Nov	29.11.2017	11.8	22	m	1.65	7.38	1277	18.80	3.33	0.08	1.06	-1.50	-0.02	0.99
0.01 lx	B1	Nov	29.11.2017	12.0	23	m	1.10	4.85	2711	19.12	3.11	-0.14	0.91	0.04	1.53	2.89
0.01 lx	B1	Nov	29.11.2017	10.9	16	m	0.84	5.41	2282	20.33	2.87	-0.38	0.77	-1.03	0.45	1.37
0.01 lx	B1	Nov	29.11.2017	10.7	17	m	1.20	7.11								
0.01 lx	B1	Nov	29.11.2017	10.9	16	m	0.93	5.98								
0.01 lx	B1	Nov	30.11.2017	10.7	15	m	0.80	5.23								
0.01 lx	B1	Nov	30.11.2017	10.8	17	m	1.22	7.10								
0.01 lx	B1	Nov	30.11.2017	10.2	15	m	0.91	6.25								
0.01 lx	B3	Nov	30.11.2017	12.2	21	m	1.51	7.13	1501	18.44	3.09	-0.16	0.90	-1.33	0.16	1.12
0.01 lx	B3	Nov	30.11.2017	10.9	18	m	1.46	8.12	687	18.08	3.02	-0.22	0.86	-2.00	-0.52	0.70
0.01 lx	B3	Nov	30.11.2017	10.5	16	m	1.26	7.69								
0.01 lx	B3	Nov	30.11.2017	12.1	25	m	1.55	6.31	1749	17.83	4.22	0.97	1.96	-0.84	0.65	1.57
0.01 lx	B3	Nov	30.11.2017	10.8	18	m	1.00	5.55								
0.01 lx	B3	Nov	30.11.2017	11.8	21	m	1.18	5.56								
0.01 lx	B3	Nov	30.11.2017	10.7	18	m	1.38	7.64								
0.01 lx	B3	Nov	30.11.2017	10.2	15	m	1.23	7.97								
0.01 lx	B2	Nov	01.12.2017	11.5	21	m	1.49	7.20	2211	18.60	3.80	0.55	1.47	-0.76	0.73	1.65
0.01 lx	B2	Nov	01.12.2017	11.8	23	m	1.19	5.19	2109	19.32						

Treatment	Aquarium	Run	Date	Std length	Body mass	Sex	Gonad	GSI	11KT	rpL8_CalcorrCt	LH_dCt	LH_ddCt	LH_2ddCt	FSH_dCt	FSH_ddCt	FSH_2ddCt
0.01 lx	B2	Nov	01.12.2017	10.8	16	m	1.05	6.49								
0.01 lx	B2	Nov	01.12.2017	11.8	22	m	1.38	6.21	626	18.80	3.50	0.25	1.19	-2.03	-0.55	0.69
0.01 lx	B2	Nov	01.12.2017	12.0	20	m	1.49	7.33								
0.01 lx	B2	Nov	01.12.2017	11.2	16	m	1.07	6.88								
0.01 lx	B2	Nov	01.12.2017	11.1	17	m	1.22	7.31								
0.01 lx	B2	Nov	01.12.2017	10.7	15	f	2.08	13.91	34	18.18	2.27	-1.12	0.46	-3.11	-3.59	0.08
0.1 lx	G3	Oct	18.10.2017	11.9	26	m	1.98	7.51		19.20	2.87	-0.37	0.77	-2.15	-0.66	0.63
0.1 lx	G3	Oct	18.10.2017	12.3	27	m	2.77	10.16	3246	20.10	3.92	0.68	1.60	-2.26	-0.77	0.58
0.1 lx	G3	Oct	18.10.2017	10.9	19	m	1.40	7.43	2643	19.73	3.16	-0.09	0.94	-2.06	-0.57	0.67
0.1 lx	G3	Oct	18.10.2017	11.5	21	m	1.49	6.93								
0.1 lx	G3	Oct	18.10.2017	11.1	20	m	1.42	7.00	1720							
0.1 lx	G2	Oct	19.10.2017	12.2	27	f	2.32	8.74	89	20.37	3.75	0.36	1.28	0.09	-0.40	0.76
0.1 lx	G2	Oct	19.10.2017	12.3	28	f	2.33	8.25	118	20.16	4.19	0.80	1.74	0.97	0.48	1.40
0.1 lx	G2	Oct	19.10.2017	10.3	16	m	1.17	7.30	1014	20.34	3.02	-0.22	0.86	-1.00	0.48	1.40
0.1 lx	G2	Oct	19.10.2017	10.2	16	m	1.12	7.04	1244	19.88	2.87	-0.38	0.77	-1.14	0.35	1.28
0.1 lx	G1	Oct	20.10.2017	11.7	22	m	1.95	8.84	2385							
0.1 lx	G1	Oct	20.10.2017	12.2	27	m	2.24	8.29	3127	18.87	3.88	0.63	1.55	-0.63	0.86	1.81
0.1 lx	G1	Oct	20.10.2017	11.4	20	m	1.43	7.35	1026	19.19	2.64	-0.61	0.66	-1.30	0.19	1.14
0.1 lx	G1	Oct	20.10.2017	11.9	24	m	1.60	6.60		18.11	3.33	0.09	1.06	-1.93	-0.45	0.73
0.1 lx	G1	Oct	20.10.2017	11.5	23	m	1.75	7.60								
0.1 lx	G1	Oct	20.10.2017	11.2	20	m	1.57	7.74								
0.1 lx	G1	Nov	30.11.2017	11.8	21	m	1.32	6.37	1337	18.23	3.61	0.36	1.29	-1.81	-0.32	0.80
0.1 lx	G1	Nov	30.11.2017	11.6	17	m	1.09	6.35								
0.1 lx	G1	Nov	30.11.2017	11.5	20	m	1.32	6.46	2586	20.28	3.48	0.23	1.17	-0.70	0.78	1.72
0.1 lx	G1	Nov	30.11.2017	11.5	23	m	1.34	5.89								
0.1 lx	G1	Nov	30.11.2017	11.2	21	m	1.42	6.85								
0.1 lx	G1	Nov	30.11.2017	10.9	14	m	0.68	4.81	69	19.03	2.62	-0.63	0.65	-2.65	-1.16	0.45
0.1 lx	G1	Nov	30.11.2017	10.4	14	m	0.88	6.07								
0.1 lx	G1	Nov	30.11.2017	10.2	15	m	0.97	6.52								
0.1 lx	G1	Nov	30.11.2017	10.4	15	f	1.41	9.20		21.89	1.88	-1.51	0.35	-0.41	-0.90	0.54
0.1 lx	G2	Nov	30.11.2017	10.9	14	m	0.79	5.88	11	19.41	0.76	-2.49	0.18	-3.14	-1.65	0.32
0.1 lx	G2	Nov	30.11.2017	11.7	17	m	1.05	6.31	259	20.90	2.69	-0.55	0.68	-3.12	-1.63	0.32
0.1 lx	G2	Nov	30.11.2017	10.6	15	m			1300							
0.1 lx	G2	Nov	30.11.2017	9.4	13	f	1.71	13.54		18.65	1.92	-1.47	0.36	-3.53	-4.01	0.06
0.1 lx	G2	Nov	30.11.2017	10.1	14	m										
0.1 lx	G3	Nov	01.12.2017	10.7	16	m	1.17	7.25	356	19.04	2.72	-0.53	0.69	-1.92	-0.43	0.74
0.1 lx	G3	Nov	01.12.2017	11.1	17	m	0.88	5.25		18.86	1.68	-1.57	0.34	-3.97	-2.48	0.18
0.1 lx	G3	Nov	01.12.2017	10.7	16	m	0.93	5.80	431	20.12	3.30	0.06	1.04	-3.47	-1.98	0.25
0.1 lx	G3	Nov	01.12.2017	10.8	14	m	0.77	5.39								
0.1 lx	G3	Nov	01.12.2017	11.1	20	m	1.19	6.02								
0.1 lx	G3	Nov	01.12.2017	10.8	16	m	0.87	5.37								
0.1 lx	G3	Nov	01.12.2017	11.3	17	m	1.06	6.26								
0.1 lx	G3	Nov	01.12.2017	11.7	18	m	1.19	6.80								
0.1 lx	G3	Nov	01.12.2017	11.1	19	m	0.96	5.12								

Treatment	Aquarium	Run	Date	Std length	Body mass	Sex	Gonad	GSI	11KT	rpL8_CalcorrCt	LH_dCt	LH_ddCt	LH_2ddCt	FSH_dCt	FSH_ddCt	FSH_2ddCt
1 lx	R1	Oct	18.10.2017	11.5	20	m	1.88	9.30		20.48	2.48	-0.77	0.59	-1.29	0.19	1.14
1 lx	R1	Oct	18.10.2017	10.8	16	m	0.93	5.63	1167							
1 lx	R1	Oct	18.10.2017	10.1	14	m	0.93	6.62								
1 lx	R1	Oct	18.10.2017	10.5	15	m	0.96	6.36	343	19.92	2.12	-1.13	0.46	-2.65	-1.17	0.45
1 lx	R1	Oct	18.10.2017	9.7	14	m	1.10	7.95	1924							
1 lx	R3	Oct	19.10.2017	11.5	19	m	1.36	6.98	735	19.08	2.73	-0.52	0.70	-0.85	0.64	1.55
1 lx	R3	Oct	19.10.2017	11.1	20	f	1.73	8.67	154							
1 lx	R3	Oct	19.10.2017	10.9	19	m	1.32	6.80		19.24	0.70	-2.55	0.17	-2.04	-0.56	0.68
1 lx	R3	Oct	19.10.2017	10.8	19	f	1.40	7.34		18.46	3.72	0.33	1.26	-0.29	-0.78	0.58
1 lx	R2	Oct	19.10.2017	12.2	27	f	2.01	7.40	102	18.62	3.72	0.33	1.26	1.21	0.72	1.65
1 lx	R2	Oct	19.10.2017	11.6	25	m	1.80	7.32	1805	18.84	3.35	0.10	1.07	-0.80	0.68	1.61
1 lx	R2	Oct	19.10.2017	10.6	18	m	1.32	7.36	2873	18.23	3.53	0.29	1.22	-1.50	-0.01	0.99
1 lx	R2	Oct	19.10.2017	10.8	19	m	1.56	8.37	1938	18.96	3.13	-0.12	0.92	-1.42	0.06	1.05
1 lx	R2	Oct	19.10.2017	11.7	25	f	1.54	6.20	123	20.00	4.28	0.89	1.85	0.07	-0.41	0.75
1 lx	R1	Nov	29.11.2017	10.6	18	m	1.19	6.55	2449	18.48	3.92	0.67	1.59	-0.74	0.75	1.68
1 lx	R1	Nov	29.11.2017	10.7	17	f	1.19	7.00	48	19.15	3.31	-0.08	0.95	-0.96	-1.44	0.37
1 lx	R1	Nov	29.11.2017	11.2	20	m	1.55	7.81								
1 lx	R1	Nov	29.11.2017	10.8	16	m	1.01	6.25								
1 lx	R1	Nov	29.11.2017	10.8	18	m	1.23	6.95	494	19.23	3.32	0.07	1.05	-1.01	0.48	1.39
1 lx	R1	Nov	29.11.2017	10.8	16	m	0.86	5.45	235	19.23	2.95	-0.30	0.81	-3.09	-1.60	0.33
1 lx	R1	Nov	29.11.2017	11.0	18	m	1.11	6.16								
1 lx	R1	Nov	29.11.2017	9.2	13	m	0.77	5.94								
1 lx	R3	Nov	30.11.2017	11.3	21	m	1.79	8.36	2431	18.79	3.45	0.20	1.15	-1.20	0.29	1.22
1 lx	R3	Nov	30.11.2017	11.1	16	m	1.07	6.71	1345	18.89	3.10	-0.15	0.90	-2.34	-0.86	0.55
1 lx	R3	Nov	30.11.2017	11.3	21	m	1.57	7.52	2597							
1 lx	R3	Nov	30.11.2017	11.2	15	m	0.64	4.40								
1 lx	R3	Nov	30.11.2017	10.3	16	m	1.60	10.19								
1 lx	R3	Nov	30.11.2017	11.3	19	m	1.34	7.12								
1 lx	R3	Nov	30.11.2017	11.0	17	m	1.56	9.45								
1 lx	R3	Nov	30.11.2017	10.5	14	m	0.93	6.54								
1 lx	R3	Nov	30.11.2017	11.5	21	m	1.29	6.14								
1 lx	R3	Nov	30.11.2017	11.8	19	m	1.04	5.35								
1 lx	R3	Nov	30.11.2017	10.1	13	m	0.82	6.43								
1 lx	R2	Nov	01.12.2017	11.6	19	f	2.08	10.68	50	17.95	3.46	0.06	1.05	-1.97	-2.46	0.18
1 lx	R2	Nov	01.12.2017	12.0	20	m	1.59	7.79	395	20.81						
1 lx	R2	Nov	01.12.2017	11.8	19	m	1.41	7.34	577	18.59	2.86	-0.39	0.76	-1.60	-0.11	0.92
1 lx	R2	Nov	01.12.2017	10.8	15	m	0.86	5.66	952	18.50	3.68	0.43	1.35	-1.44	0.05	1.03
1 lx	R2	Nov	01.12.2017	11.2	20	f	2.55	12.73	214	20.84	3.43	0.04	1.03	-2.80	-3.29	0.10
1 lx	R2	Nov	01.12.2017	10.2	14	m	0.76	5.50								
1 lx	R2	Nov	01.12.2017	10.9	16	m	1.28	8.07								
1 lx	R2	Nov	01.12.2017	11.2	18	m	1.17	6.49								
1 lx	R2	Nov	01.12.2017	10.3	14	m			2174							

Table S2.5 Full data for the field experiment. Treat – treatment (Con – Control, Sky – Skyglow), Encl – Enclosure, Hist – histological status of vitellogenesis/spermatogenesis, Release – Release date, Re-catch – date of re-catchment, start – body mass before release (g), End – body mass at the day of re-catchment (g), Std length – standard length (cm), Gonad – mass of gonads (g), GSI – gonadosomatic index (gonad mass relative to body mass, %), rpL8 – ribosomal protein L8, FSH – follicle-stimulating hormone, LH – luteinizing hormone, Ct – Cycle threshold, Ctcorr – Ct-value corrected for efficiencies of primers, dCt/ddCt/2ddCt – delta Ct-values/delta delta Ct-values/2^{ddCt}-values (see "Relative mRNA quantification by RT-qPCR")

Treat	Encl	Sex	Hist	Tag	Release	Re-catch	Start bm	End bm	Std length	Gonad	GSI	rpL8 Ct	rpL8 Ctcorr	FSH Ct	FSH Ctcorr	FSH dCt	FSH ddCt	FSH 2ddCt	LH Ct	LH Ctcorr	LH dCt	LH ddCt	LH 2ddCt
Con	E3	f	early	DST	2018-0-07	2018-10-04	146	145	19.9	1.84	1.61	19.80	18.82	17.18	16.09	2.73	-0.61	0.66	15.58	14.67	4.15	-0.19	0.88
Con	E5	f	mid	DST	2018-0-17	2018-10-05	175	170	22.1	4.65	2.74	18.12	17.23	15.27	14.30	2.93	-0.41	0.75	13.85	13.04	4.19	-0.15	0.90
Con	E9	f	mid	DST	2018-0-07	2018-10-04	193	190	22.5	6.59	3.47	17.94	17.06	14.27	13.37	3.69	0.35	1.28	13.73	12.93	4.13	-0.21	0.87
Con	E9	f	pre	DST	2018-0-17	2018-10-04	122	115	19.0	0.72	0.63												
Con	E3	f	early	PIT	2018-0-08	2018-10-04	43	46	14.0	1.55	3.37	19.01	18.07	15.68	14.68	3.39	0.05	1.04	14.31	13.47	4.60	0.26	1.20
Con	E5	f	mid	PIT	2018-0-08	2018-10-05	55	58	15.2	1.62	2.78	20.96	19.93	17.06	15.98	3.95	0.61	1.53	16.26	15.31	4.62	0.28	1.21
Con	E15	f	pre	PIT	2018-0-08	2018-10-05	83	64	17.5	0.33	0.51												
Con	E15	f	pre	PIT	2018-08-08	2018-10-05	61	56	15.9	0.52	0.93												
Con	E5	m	late	DST	2018-09-07	2018-10-04	155	111	19.3	8.04	7.26	18.68	17.76	16.38	15.34	2.42	-0.53	0.69	14.04	13.22	4.53	0.45	1.37
Con	E11	m	mid	DST	2018-09-07	2018-10-05	227	222	23.5	13.99	6.31	17.65	16.78	14.21	13.30	3.47	0.53	1.44	13.96	13.14	3.63	-0.45	0.73
Sky	E10	f	early	DST	2018-09-07	2018-10-05	181	167	21.8	6.95	4.15	17.90	17.02	13.81	12.93	4.09	0.75	1.68	14.18	13.36	3.66	-0.68	0.63
Sky	E4	f	mid	DST	2018-09-07	2018-10-04	136	134	19.5	3.03	2.26	18.82	17.89	15.55	14.56	3.33	-0.01	0.99	14.89	14.02	3.87	-0.47	0.72
Sky	E4	f	mid	DST	2018-09-07	2018-10-04	192	183	22.0	4.14	2.26	19.00	18.07	14.97	14.02	4.05	0.71	1.63	15.14	14.26	3.81	-0.53	0.69
Sky	E10	f	mid	DST	2018-08-17	2018-10-05	230	210	24.4	4.48	2.13	17.95	17.06	15.28	14.31	2.75	-0.59	0.67	14.71	13.85	3.21	-1.13	0.46
Sky	E13	f	mid	DST	2018-08-17	2018-10-05	98	95	18.6	1.84	1.95	20.09	19.10	16.19	15.16	3.94	0.60	1.51	15.70	14.78	4.32	-0.02	0.99
Sky	E19	f	mid	DST	2018-09-07	2018-10-05	222	220	23.4	9.75	4.43	17.94	17.06	13.96	13.07	3.98	0.64	1.56	13.81	13.00	4.06	-0.28	0.82
Sky	E4	f	pre	DST	2018-08-17	2018-10-04	159	147	20.6	0.72	0.49												
Sky	E10	f	pre	DST	2018-09-07	2018-10-05	105	101	18.1	0.44	0.44												
Sky	E19	f	pre	DST	2018-08-17	2018-10-05	141	122	20.3	0.61	0.50												
Sky	E4	f	mid	PIT	2018-08-08	2018-10-04	49	61	14.8	1.84	3.00												
Sky	E13	f	mid	PIT	2018-08-08	2018-10-05	66	59	15.4	0.92	1.55	18.06	17.17	15.11	14.15	3.02	-0.32	0.80	13.71	12.91	4.25	-0.08	0.94
Sky	E18	f	mid	PIT	2018-08-08	2018-10-05	71	75	16.6	2.95	3.92	18.70	17.78	14.51	13.59	4.20	0.86	1.81	14.82	13.96	3.82	-0.51	0.70
Sky	E10	f	pre	PIT	2018-08-08	2018-10-05	55	56	15.5	0.28	0.50												
Sky	E10	f	pre	PIT	2018-08-08	2018-10-05	62	62	16.7	0.37	0.59												
Sky	E13	f	pre	PIT	2018-08-08	2018-10-05	59	50	15.4	0.23	0.46												
Sky	E19	f	pre	PIT	2018-08-08	2018-10-05	65	56	15.9	0.26	0.47												
Sky	E19	f	pre	PIT	2018-08-08	2018-10-05	76	69	16.8	0.20	0.29												
Sky	E13	m	mid	DST	2018-09-07	2018-10-05	120	119	19.2	7.22	6.09	19.92	18.94	17.01	15.93	3.01	0.07	1.05	16.59	15.63	3.32	-0.77	0.59
Sky	E18	m	mid	PIT	2018-08-08	2018-10-05	29	37	12.6	3.81	10.35	19.49	18.53	16.88	15.80	2.73	-0.22	0.86	15.41	14.51	4.02	-0.06	0.96

Appendix D – Supplementary material to Chapter 3

Abbreviations for tables S3.1 to S3.3

ALAN – Artificial light at night

LMM – Linear mixed model

R^2_m – marginal R^2

R^2_c – conditional R^2

SE – standard error

df – degrees of freedom

LLR – log-likelihood ratio

f – female

m – male

nd – not differentiated (premature fish)

Table S3.1
LMM specifications and post-hoc results (Tukey's correction) for significant fixed effects in plasma triiodothyronine (T3) of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

log(T3) high ALAN experiment (R^2_m = 0.2894, R^2_c = 0.3548)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept ‡	1.231 ± 0.171	83	7.18		
ALAN 1 lx	–0.097 ± 0.085	15	–1.14	11.94	0.0076
ALAN 10 lx	0.075 ± 0.086	15	–0.87		
ALAN 100 lx	–0.309 ± 0.085	15	–3.63		
Sex m	0.377 ± 0.079	83	4.74	24.34	<0.0001
Sex nd	0.062 ± 0.074	83	0.83		
Body mass	0.007 ± 0.002	83	3.65	12.47	0.0004
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.08^2$				
Aquaria nested in runs	$\sigma^2 = 0.05^2$			2.08	0.3536
Residual	$\sigma^2 = 0.29^2$				
Post-hoc treatment effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
0 lx – 1 lx	0.097 ± 0.086	15	1.14		0.6722
0 lx – 10 lx	0.075 ± 0.086	15	0.87		0.8202
0 lx – 100 lx	0.309 ± 0.085	15	3.63		0.0118
1 lx – 10 lx	–0.022 ± 0.085	15	–0.26		0.9934
1 lx – 100 lx	0.212 ± 0.085	15	2.50		0.0999
10 lx – 100 lx	0.234 ± 0.085	15	2.74		0.0652
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–0.377 ± 0.080	83	–4.74		<0.0001
f – nd	–0.062 ± 0.074	83	0.84		0.6825
m – nd	0.315 ± 0.073	83	4.33		0.0001
log(T3) low ALAN experiment (R^2_m = 0.1546, R^2_c = 0.4138)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	–0.608 ± 0.260	115	–2.34		
ALAN 0.01 lx	–0.025 ± 0.083	19	–0.30	1.05	0.7886
ALAN 0.1 lx	–0.020 ± 0.082	19	–0.24		
ALAN 1 lx	–0.080 ± 0.083	19	–0.96		
Body mass	0.044 ± 0.089	115	4.76	22.07	<0.0001
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.23^2$				
Aquaria nested in runs	$\sigma^2 = 9.03e\text{-}06^2$			15.52	0.0004
Residual	$\sigma^2 = 0.34^2$				

‡ Intercept taken at 0 lx for females
† Intercept taken at 0 lx

Table S3.2
LMM specifications and post-hoc results (Tukey's correction) for significant fixed effects for plasma thyroxine (T4) of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

log(T4) high ALAN experiment (R^2_m = 0.0246, R^2_c = 0.1648)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	0.962 ± 0.108	84	8.93		
ALAN 1 lx	0.063 ± 0.152	15	0.41	1.96	0.5809
ALAN 10 lx	0.156 ± 0.152	15	1.03		
ALAN 100 lx	0.164 ± 0.152	15	1.08		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 2.4e-05^2				
Aquaria nested in runs	σ^2 = 0.16^2			2.83	0.2429
Residual	σ^2 = 0.40^2				
log(T4) low ALAN experiment (R^2_m = 0.1633, R^2_c = 0.1873)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	1.388 ± 0.471	73	2.95		
ALAN 0.01 lx	–2.411 ± 0.804	19	–3.00		
ALAN 0.1 lx	–1.986 ± 0.743	19	–2.67		
ALAN 1 lx	–0.041 ± 0.782	19	–0.05		
Body mass	–0.044 ± 0.022	73	–2.00		
ALAN 0.01 lx : Body mass	0.117 ± 0.039	73	2.97	14.70	0.0021
ALAN 0.1 lx : Body mass	0.101 ± 0.036	73	2.80		
ALAN 1 lx : Body mass	–0.008 ± 0.038	73	–0.22		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 1.64e-05^2				
Aquaria nested in runs	σ^2 = 0.08^2			0.10	0.9498
Residual	σ^2 = 0.49^2				
Post-hoc treatment effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
0 lx – 0.01 lx	–0.117 ± 0.039	73	–2.97		0.0203
0 lx – 0.1 lx	–0.101 ± 0.036	73	–2.80		0.0326
0 lx – 1 lx	0.008 ± 0.038	73	0.22		0.9960
0.01 lx – 0.1 lx	0.016 ± 0.043	73	0.36		0.9835
0.01 lx – 1 lx	0.125 ± 0.045	73	2.81		0.0315
0.1 lx – 1 lx	0.110 ± 0.042	73	2.62		0.0513

† Intercept taken at 0 lx

Table S3.3
LMM specifications and post-hoc results (Tukey's correction) for significant fixed effects for the ratio of triiodothyronine (T3) and thyroxine (T4) in the plasma of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

log(T3/T4) high ALAN experiment (R^2_m = 0.0845, R^2_c = 0.1957)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	0.884 ± 0.141	84	6.26		
ALAN 1 lx	–0.124 ± 0.200	15	–0.62	6.58	0.0867
ALAN 10 lx	–0.254 ± 0.200	15	–1.28		
ALAN 100 lx	–0.478 ± 0.200	15	–2.39		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 1.82e-05^2				
Aquaria nested in runs	σ^2 = 0.20^2			2.16	0.3393
Residual	σ^2 = 0.55^2				
log(T3/T4) low ALAN experiment (R^2_m = 0.1306, R^2_c = 0.3217)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept †	–1.348 ± 0.612	73	–2.20		
ALAN 0.01 lx	1.815 ± 0.924	19	1.96		
ALAN 0.1 lx	0.955 ± 0.855	19	1.12		
ALAN 1 lx	–0.978 ± 0.898	19	–1.09		
Body mass	0.061 ± 0.027	73	2.26		
ALAN 0.01 lx : Body mass	–0.090 ± 0.045	73	–2.00	9.33	0.0252
ALAN 0.1 lx : Body mass	–0.053 ± 0.042	73	–1.27		
ALAN 1 lx : Body mass	0.051 ± 0.043	73	1.18		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 0.28^2				
Aquaria nested in runs	σ^2 = 0.10^2			5.19	0.0747
Residual	σ^2 = 0.56^2				
Post-hoc treatment effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
0 lx – 0.01 lx	0.091 ± 0.045	73	2.00		0.1968
0 lx – 0.1 lx	0.053 ± 0.042	73	1.27		0.5867
0 lx – 1 lx	–0.051 ± 0.043	73	–1.18		0.6423
0.01 lx – 0.1 lx	–0.038 ± 0.050	73	–0.76		0.8741
0.01 lx – 1 lx	–0.142 ± 0.051	73	–2.76		0.0359
0.1 lx – 1 lx	–0.104 ± 0.048	73	–2.16		0.1449

† Intercept taken at 0 lx

Table S3.4
Sample numbers per ALAN treatment, per sex, and per hormone (T3 or T4) of analyzed plasma samples of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments. Numbers in brackets are samples that were extracted and measured but with values below the limit of quantification.

high ALAN experiment

	0 lx		**1 lx**		**10 lx**		**100 lx**		**all ALAN treatments**	
	T3	T4	T3	T4	T3	T4	T3	T4	**T3**	**T4**
females	9	9	8	8	8	8	9	9	**34**	**34**
males	11	11	10	10	8	8	11	10 (+1)	**40**	**39 (+1)**
not differentiated	6	6	9	8 (+1)	10	10	7	7	**32**	**31 (+1)**
all sexes	**26**	**26**	**27**	**26 (+1)**	**26**	**26**	**27**	**26 (+1)**	**106**	**104 (+2)**

low ALAN experiment

	0 lx		**0.01 lx**		**0.1 lx**		**1 lx**		**all ALAN treatments**	
	T3	T4	T3	T4	T3	T4	T3	T4	**T3**	**T4**
females	3	2 (+1)	1	0 (+1)	1	0 (+1)	3	3	**8**	**5**
males	17	12 (+5)	19 (+1)	16 (+4)	15	13 (+2)	15	11 (+4)	**66 (+1)**	**52 (+15)**
not differentiated	13 (+1)	11 (+3)	15	9 (+6)	20	15 (+5)	15	9 (+6)	**63 (+1)**	**44 (+20)**
not available	1	1	0	0	0	0	2	0 (+2)	**3**	**1 (+2)**
all sexes	**34 (+1)**	**26 (+9)**	**35 (+1)**	**25 (+11)**	**36**	**28 (+8)**	**35**	**23 (+12)**	**140 (+2)**	**102 (+38)**

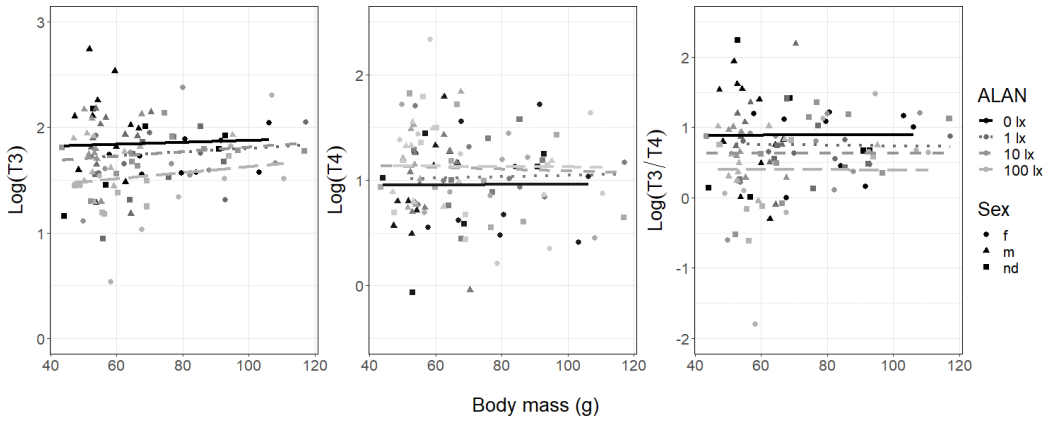

Figure S3.1 Logarithmized T3 (ng mL^{-1}), T4 (ng mL^{-1}), or ratio of T3/T4 against body mass (g) of *Perca fluviatilis* exposed to different nocturnal light intensities (different shades of grey) for different sexes (shapes) ("high ALAN experiment"). Lines depict the predictions of LMM analyses for each ALAN treatment (see tables S3.1-S3.3 above). Artificial light at night (ALAN) and sex, as well as body mass significantly explained variance only of log(T3) (ALAN effects: LLR = 11.94, $p = 0.008$; Sex effect: LLR = 24.34, $p < 0.0001$; Body mass effect: LLR = 12.47, $p = 0.0004$). ALAN did not significantly explain variance of log(T4) or log(T3/T4) (log(T4): LLR = 1.96, $p = 0.58$; log(T3/T4): LLR = 6.38, $p = 0.09$).

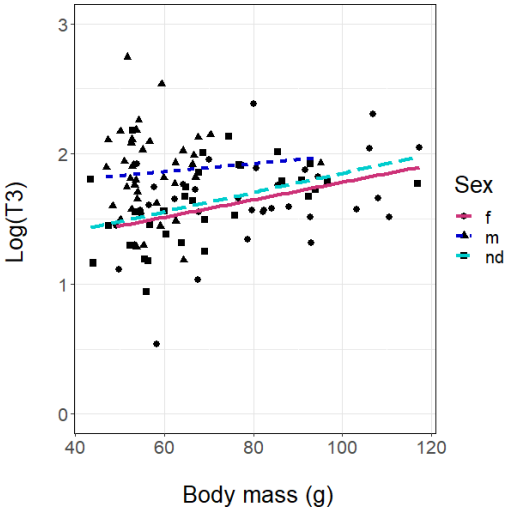

Figure S3.2 Logarithmized T3 (ng mL^{-1}) against body mass (g) of *Perca fluviatilis* exposed to different nocturnal light intensities for different sexes (shapes) ("high ALAN experiment"). Lines depict the predictions of LMM analyses for females (f; solid), males (m; dotted) and not differentiated (nd; dashed). Sex significantly explained T3 variance (fixed effect of sex: LLR = 24.34, $p < 0.0001$).

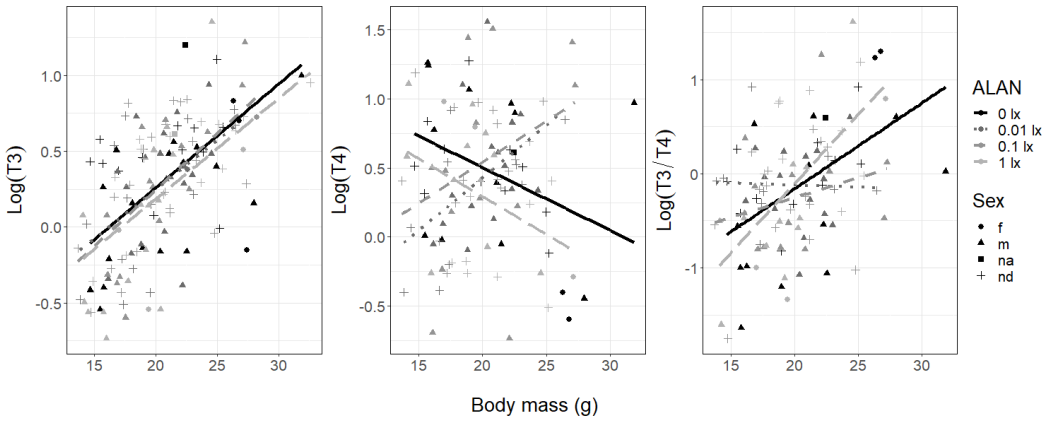

Figure S3.3 Logarithmized T3 (ng mL^{-1}), T4 (ng mL^{-1}), or ratio of T3/T4 against body mass (g) of *Perca fluviatilis* exposed to different nocturnal light intensities (different shades of grey) for different sexes (shapes) ("low ALAN experiment"). Lines depict the predictions of LMM analyses for each ALAN treatment (see tables S3.1-S3.3 above). Artificial light at night (ALAN) did not significantly explain variance of log(T3) (ALAN effect: LLR = 1.05, $p = 0.79$). Body mass significantly explained variance of log(T3) (Body mass effect: LLR = 22.07, $p < 0.0001$). An interaction of ALAN and body mass significantly explained variance of log(T4) or log(T3/T4) (log(T4): LLR = 14.70, $p = 0.002$; log(T3/T4): LLR = 9.33, $p = 0.03$).

Appendix E – Supplementary material to Chapter 4

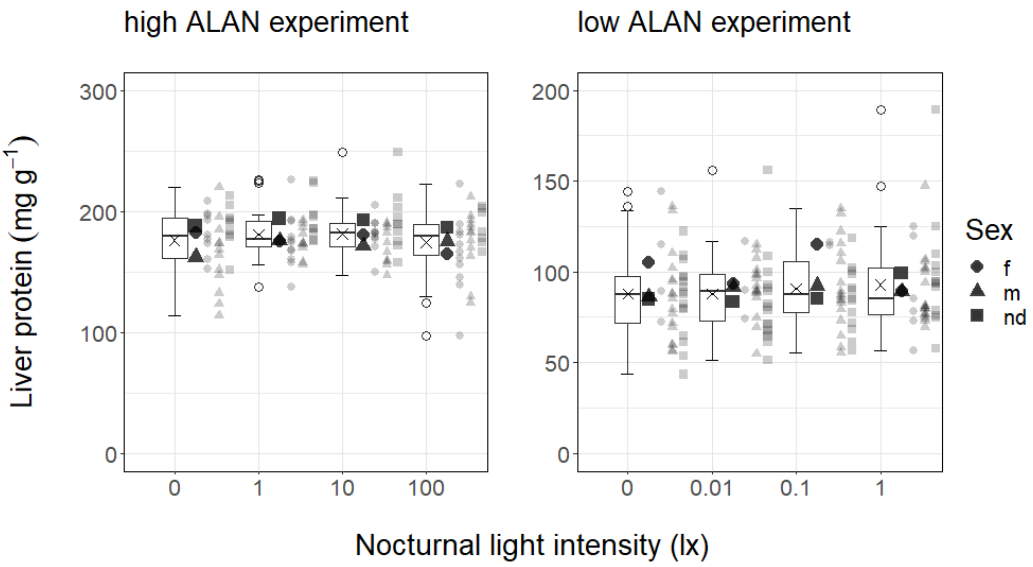

Figure S4.1 Protein content in liver extracts in *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for two weeks. Protein content is expressed per g liver mass. Boxplots display data for each treatment and "X"s inside the boxes indicate the mean (high ALAN experiment: n = 30; low ALAN experiment: n = 37 for 0 lx and 1 lx, n = 35 for 0.01 lx, n = 33 for 0.1 lx). Different shapes of big points indicate the mean of each sex (m – males, f – females, nd – not differentiated (premature fish)), whereas small points represent individuals. There were no significant differences across treatments in either experiment (Linear mixed models, ALAN effect in high ALAN experiment: log-likelihood ratio (LLR) = 1.61, p = 0.66; ALAN effect in low ALAN experiment: LLR = 1.21, p = 0.75).

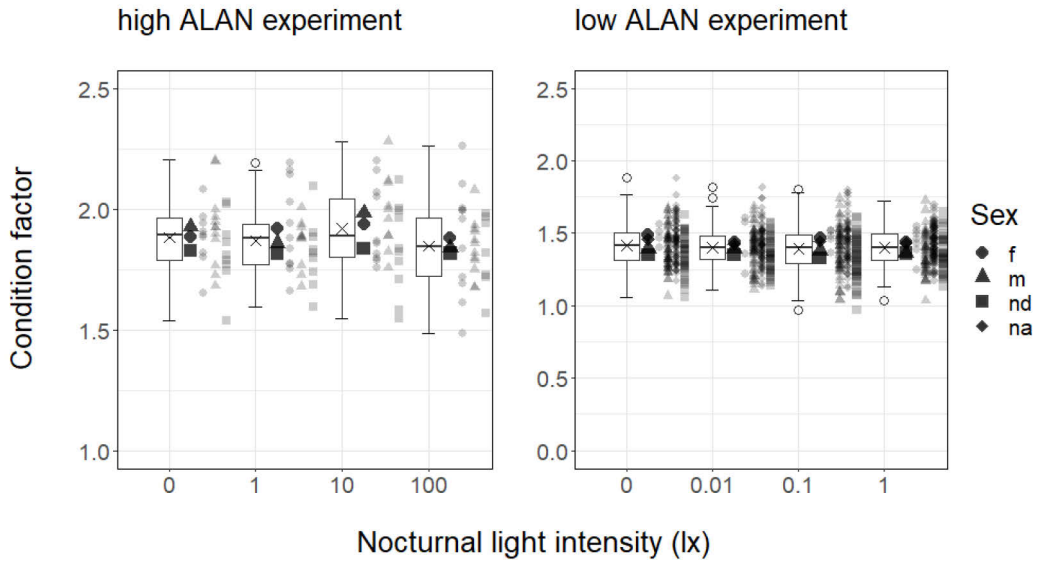

Figure S4.2 Condition factor (*K*) of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for two weeks in two different experiments. Boxplots display data for each treatment and ”X”s inside the boxes indicate the mean (high ALAN experiment: n = 30; low ALAN experiment: n = 180). Different shapes of big points indicate the mean of each sex (m – males, f – females, nd – not differentiated (premature fish), na – not available (sex not determined)), whereas small points represent individuals. There were no significant differences across treatments in either experiment (Linear mixed models, ALAN effect in high ALAN experiment: log-likelihood ratio (LLR) = 3.64, p = 0.30; ALAN effect in low ALAN experiment: LLR = 1.40, p = 0.71).

Abbreviations for tables S4.1 to S4.9

LMM – Linear mixed model

R^2_m – marginal R^2

R^2_c – conditional R^2

SE – standard error

df – degrees of freedom

LLR – log-likelihood ratio

Treat – treatment

f – female

m – male

nd – not differentiated (premature fish)

na – not available (sex not determined)

“in” (random effects, e.g., “Individuals in aquaria in runs”) – “nested in” (e.g., “Individuals nested in aquaria nested in runs”)

PMA – phorbol 12-myristate 13-acetate

no stim – cell cultures without PMA stimulation

PMA stim – cell cultures with PMA stimulation

Table S4.1
LMM specifications for the respiratory burst activity (RBA) of head kidney leucocyte cultures of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

RBA high ALAN experiment (R^2_m = 0.9955, R^2_c = 0.9988)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f/no stim)	0.09 ± 0.005	837	19.46		
Treat 1 lx	0.0005 ± 0.004	15	0.12	2.05	0.5613
Treat 10 lx	0.0005 ± 0.004	15	0.12		
Treat 100 lx	–0.004 ± 0.004	15	–1.01		
PMA stim	0.402 ± 0.008	837	50.28	874.42	<0.0001
Sex male	–0.002 ± 0.002	98	–1.08	5.96	0.0509
Sex nd	0.003 ± 0.002	98	1.32		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.0046^2$				
Aquaria in runs	$\sigma^2 = 0.0047^2$			400.03	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 0.0095^2$				
Residual	$\sigma^2 = 0.0069^2$				

Table S4.2
LMM specifications and post-hoc results (Bonferroni correction) for lysozyme activity in the plasma of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

Lysozyme high ALAN experiment (R^2_m = 0.0684, R^2_c = 0.9511)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	257.16 ± 18.75	235	13.71		
Treat 1 lx	11.57 ± 21.85	15	0.53	3.07	0.3809
Treat 10 lx	–3.90 ± 21.83	15	–0.18		
Treat 100 lx	28.53 ± 21.98	15	1.30		
Sex m	30.37 ± 16.38	96	1.85	6.15	0.0462
Sex nd	41.66 ± 17.32	96	2.41		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 0.0073²				
Aquaria in runs	σ^2 = 16.50²			621.02	<0.0001
Individuals in aquaria in runs	σ^2 = 72.91²				
Residual	σ^2 = 17.60²				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–30.4 ± 16.4	96	–1.854		0.2004
f – nd	–41.7 ± 17.3	96	–2.406		0.0542
m – nd	–11.3 ± 16.9	96	–0.667		1.0000

Lysozyme low ALAN experiment (R^2_m = 0.0308, R^2_c = 0.9290)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/na)	219.82 ± 24.57	213	8.95		
Treat 0.01 lx	12.03 ± 13.69	19	0.88	1.66	0.6448
Treat 0.1 lx	7.41 ± 13.15	19	0.56		
Treat 1 lx	–2.48 ± 13.51	19	–0.18		
Sex f	–51.50 ± 21.35	193	–2.41		
Sex m	–12.79 ± 15.19	193	–0.84	6.44	0.0919
Sex nd	–19.52 ± 15.04	193	–1.30		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 26.33 ²				
Aquaria in runs	σ^2 = 11.59²			275.87	<0.0001
Individuals in aquaria in runs	σ^2 = 58.00²				
Residual	σ^2 = 18.21²				

Table S4.3
LMM specifications and post-hoc results (Bonferroni correction) for thiobarbituric acid reactive substances (TBARS) in liver homogenates of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

TBARS high ALAN experiment (R^2_m = 0.4172, R^2_c = 0.9990)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	95.25 ± 11.60	240	8.21		
Treat 1 lx	17.34 ± 13.50	15	1.28	7.61	0.0549
Treat 10 lx	–0.62 ± 13.49	15	–0.05		
Treat 100 lx	30.79 ± 13.50	15	2.28		
Sex m	79.87 ± 9.49	98	8.42	59.77	<0.0001
Sex nd	16.10 ± 10.05	98	1.60		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 4.27^2$				
Aquaria in runs	$\sigma^2 = 12.12^2$			1237.5	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 42.94^2$				
Residual	$\sigma^2 = 1.88^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–79.9 ± 9.5	98	–8.417		<0.0001
f – nd	–16.1 ± 10.1	98	–1.602		0.3372
m – nd	–63.8 ± 10.0	98	6.411		<0.0001

TBARS low ALAN experiment (R^2_m = 0.1710, R^2_c = 0.9914)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	183.07 ± 54.38	285	3.37		
Treat 0.01 lx	–15.77 ± 51.49	19	–0.31	0.38	0.9435
Treat 0.1 lx	1.58 ± 51.89	19	0.03		
Treat 1 lx	13.88 ± 51.35	19	0.27		
Sex m	235.69 ± 44.04	117	5.35	29.42	<0.0001
Sex nd	143.85 ± 44.44	117	3.24		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 21.62^2$				
Aquaria in runs	$\sigma^2 = 66.04^2$			1003.6	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 145.96^2$				
Residual	$\sigma^2 = 16.52^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–235.7 ± 44.0	117	–5.351		<0.0001
f – nd	–143.9 ± 44.4	117	–3.237		0.0047
m – nd	91.8 ± 27.1	117	3.388		0.0029

Table S4.4
LMM specifications and post-hoc results (Bonferroni correction) for protein content in liver homogenates of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

Liver protein high ALAN experiment (R^2_m = 0.1149, R^2_c = 0.9355)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	172.86 ± 6.56	120	26.33		
Treat 1 lx	4.36 ± 6.38	15	0.68	1.61	0.6567
Treat 10 lx	5.58 ± 6.39	15	0.87		
Treat 100 lx	–0.54 ± 6.40	15	–0.08		
Sex m	–2.67 ± 4.74	98	–0.56	14.22	0.0008
Sex nd	15.45 ± 5.03	98	3.07		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 5.29^2$				
Aquaria in runs	$\sigma^2 = 4.92^2$			217.71	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 21.15^2$				
Residual	$\sigma^2 = 6.27^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	2.67 ± 4.74	98	0.562		0.8405
f – nd	–15.45 ± 5.03	98	–3.073		0.0082
m – nd	–18.12 ± 4.96	98	–3.650		0.0013
Liver protein low ALAN experiment (R^2_m = 0.0120, R^2_c = 0.9923)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	88.18 ± 13.73	142	6.42		
Treat 0.01 lx	–0.34 ± 5.43	19	–0.06	1.21	0.7506
Treat 0.1 lx	1.85 ± 5.50	19	0.34		
Treat 1 lx	4.64 ± 5.39	19	0.86		
Sex m	1.95 ± 5.56	116	0.35	1.49	0.4752
Sex nd	–2.15 ± 5.59	116	–0.39		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 17.42^2$				
Aquaria in runs	$\sigma^2 = 5.56^2$			432.32	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 18.34^2$				
Residual	$\sigma^2 = 2.30^2$				

Table S4.5
LMM specifications and post-hoc results (Bonferroni correction) for superoxide dismutase (SOD) activity in liver extracts of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

SOD high ALAN experiment (R^2_m = 0.0584, R^2_c = 0.9798)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	8.44 ± 0.99	120	8.50		
Treat 1 lx	–0.60 ± 0.99	15	–0.61	1.61	0.6254
Treat 10 lx	–0.85 ± 0.99	15	0.87		
Treat 100 lx	0.21 ± 0.99	15	–0.08		
Sex m	1.01 ± 0.47	98	2.13	5.27	0.0717
Sex nd	0.21 ± 0.50	98	0.42		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.91^2$				
Aquaria in runs	$\sigma^2 = 1.30^2$			138.38	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 1.99^2$				
Residual	$\sigma^2 = 0.38^2$				
SOD low ALAN experiment (R^2_m = 0.0788, R^2_c = 0.9017)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	22.36 ± 2.86	143	7.82		
Treat 0.01 lx	0.15 ± 1.99	19	0.07	1.68	0.6422
Treat 0.1 lx	2.07 ± 2.03	19	1.02		
Treat 1 lx	–0.11 ± 2.04	19	–0.05		
Sex m	2.41 ± 1.94	117	1.25	9.68	0.0079
Sex nd	5.16 ± 1.97	117	2.62		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 2.56^2$				
Aquaria in runs	$\sigma^2 = 2.34^2$			108.01	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 5.33^2$				
Residual	$\sigma^2 = 2.20^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–2.41 ± 1.94	117	–1.246		0.6455
f – nd	–5.16 ± 1.97	117	–2.617		0.0301
m – nd	–2.75 ± 1.12	117	–2.453		0.0469

Table S4.6
LMM specifications and post-hoc results (Bonferroni correction) for catalase (CAT) activity in liver extracts of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

CAT high ALAN experiment (R^2_m = 0.1071, R^2_c = 0.9308)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	37.82 ± 9.00	221	4.20		
Treat 1 lx	8.77 ± 12.11	15	0.72	1.73	0.6299
Treat 10 lx	10.67 ± 12.07	15	0.88		
Treat 100 lx	–0.89 ± 12.06	15	–0.07		
Sex m	19.38 ± 4.96	97	3.91	15.06	0.0005
Sex nd	6.69 ± 5.05	97	1.32		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.14^2$				
Aquaria in runs	$\sigma^2 = 16.96^2$			260.88	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 20.17^2$				
Residual	$\sigma^2 = 7.64^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	–19.38 ± 4.96	97	–3.906		0.0005
f – nd	–6.69 ± 5.05	97	–1.323		0.5663
m – nd	12.69 ± 5.15	97	2.462		0.0469

CAT low ALAN experiment (R^2_m = 0.0067, R^2_c = 0.7895)

Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	107.01 ± 22.29	217	4.80		
Treat 0.01 lx	1.83 ± 19.52	17	0.09	0.55	0.9087
Treat 0.1 lx	–9.49 ± 20.53	17	–0.46		
Treat 1 lx	–4.99 ± 19.34	17	–0.26		
Sex m	10.48 ± 16.83	94	0.62	0.61	0.7362
Sex nd	5.16 ± 16.92	94	0.31		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 15.04^2$				
Aquaria in runs	$\sigma^2 = 21.16^2$			304.48	<0.0001
Individuals in aquaria in runs	$\sigma^2 = 53.53^2$				
Residual	$\sigma^2 = 30.85^2$				

Table S4.7
LMM specifications and post-hoc results (Bonferroni correction) for the condition factor (*K*) of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

***K* high ALAN experiment (R^2_m = 0.0929, R^2_c = 0.1142)**					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	1.91 ± 0.035	98	54.82		
Treat 1 lx	–0.016 ± 0.040	15	–0.40	3.64	0.3034
Treat 10 lx	0.041 ± 0.043	15	0.95		
Treat 100 lx	–0.039 ± 0.043	15	–0.92		
Sex m	–0.0032 ± 0.034	98	–0.095	6.01	0.0495
Sex nd	–0.081 ± 0.036	98	–2.25		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 2.41e-06²				
Aquaria in runs	σ^2 = 0.021²			0.05	0.9737
Residual	σ^2 = 0.14²				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	0.003 ± 0.034	98	0.095		1.0000
f – nd	0.081 ± 0.036	98	2.255		0.0792
m – nd	0.078 ± 0.035	98	2.191		0.0924
***K* low ALAN experiment (R^2_m = 0.0366, R^2_c = 0.4522)**					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	1.43 ± 0.071	693	20.12		
Treat 0.01 lx	–0.015 ± 0.017	19	–0.86	1.40	0.7057
Treat 0.1 lx	–0.017 ± 0.018	19	–0.96		
Treat 1 lx	–0.016 ± 0.017	19	–0.92		
Sex f	0.0073 ± 0.026	693	0.28	45.05	<0.0001
Sex m	–0.012 ± 0.010	693	–1.17		
Sex nd	–0.075 ± 0.011	693	–6.56		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 0.099²				
Aquaria in runs	σ^2 = 0.022²			209.44	<0.0001
Residual	σ^2 = 0.12²				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
na – f	–0.007 ± 0.026	693	–0.280		1.0000
na – m	0.012 ± 0.010	693	1.169		1.0000
na – nd	0.075 ± 0.011	693	6.559		<0.0001
f – m	0.020 ± 0.027	693	0.726		1.0000
f – nd	0.082 ± 0.027	693	3.023		0.0156
m – nd	0.063 ± 0.012	693	5.115		<0.0001

Table S4.8
LMM specifications and post-hoc results (Bonferroni correction) for the splenosomatic index (I_S) of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

I_S high ALAN experiment (R^2_m = 0.0409, R^2_c = 0.0751)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	0.106 ± 0.008	98	13.23		
Treat 1 lx	–0.012 ± 0.007	15	–1.68	3.21	0.3608
Treat 10 lx	–0.006 ± 0.009	15	–0.67		
Treat 100 lx	–0.005 ± 0.008	15	–0.62		
Sex m	–0.013 ± 0.006	98	–2.16	11.39	0.0034
Sex nd	0.004 ± 0.007	98	0.54		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.0045^2$				
Aquaria in runs	$\sigma^2 = 0.0059^2$			1.62	0.4449
Residual	$\sigma^2 = 0.38^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	0.013 ± 0.006	98	2.161		0.0994
f – nd	–0.004 ± 0.007	98	–0.537		1.0000
m – nd	–0.016 ± 0.005	98	–3.052		0.0088
I_S low ALAN experiment (R^2_m = 0.1384, R^2_c = 0.2815)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	–1.97 ± 0.089	231	–22.06		
Treat 0.01 lx	–0.030 ± 0.085	19	–0.35	2.36	0.5008
Treat 0.1 lx	0.093 ± 0.083	19	1.12		
Treat 1 lx	–0.004 ± 0.081	19	–0.055		
Sex m	–0.067 ± 0.077	231	–0.87	19.89	<0.0001
Sex nd	0.13 ± 0.078	231	1.71		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	$\sigma^2 = 0.00062^2$				
Aquaria in runs	$\sigma^2 = 0.11^2$			6.32	0.0425
Residual	$\sigma^2 = 0.25^2$				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	0.067 ± 0.077	231	0.866		1.0000
f – nd	–0.133 ± 0.078	231	–1.709		0.2665
m – nd	–0.199 ± 0.042	231	–4.770		<0.0001

Table S4.9
LMM specifications and post-hoc results (Bonferroni correction) for the hepatosomatic index (I_H) of *Perca fluviatilis* exposed to different nocturnal light intensities in two different experiments.

I_H high ALAN experiment (R^2_m = 0.1943, R^2_c = 0.3762)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	1.95 ± 0.12	98	16.12		
Treat 1 lx	–0.14 ± 0.094	15	–1.50	8.93	0.0303
Treat 10 lx	–0.13 ± 0.094	15	–1.34		
Treat 100 lx	–0.29 ± 0.094	15	–3.08		
Sex m	–0.31 ± 0.065	98	–4.72	20.49	<0.0001
Sex nd	–0.20 ± 0.069	98	–2.96		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 0.13²				
Aquaria in runs	σ^2 = 0.087²			9.61	0.0082
Residual	σ^2 = 0.29²				
Post-hoc treatment effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
0 lx – 1 lx	0.1403 ± 0.0936	15	1.499		0.9272
0 lx – 10 lx	0.1257 ± 0.0936	15	1.343		1.0000
0 lx – 100 lx	0.2884 ± 0.0936	15	3.081		0.0457
1 lx – 10 lx	–0.0147 ± 0.0935	15	–0.157		1.0000
1 lx – 100 lx	0.1481 ± 0.0936	15	1.582		0.8075
10 lx – 100 lx	0.1628 ± 0.0937	15	1.737		0.6173
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	0.306 ± 0.065	98	4.721		<0.0001
f – nd	–0.203 ± 0.069	98	2.955		0.0117
m – nd	–0.103 ± 0.068	98	–1.521		0.3944
I_H low ALAN experiment (R^2_m = 0.1422, R^2_c = 0.1422)					
Fixed effects	**Estimate ± SE**	**df**	**t-value**	**LLR**	***p*-value**
Intercept (at 0 lx/f)	1.00 ± 0.04	76	22.82		
Treat 0.01 lx	–0.04 ± 0.04	19	–0.99	2.25	0.5222
Treat 0.1 lx	–0.04 ± 0.03	19	–1.25		
Treat 1 lx	–0.01 ± 0.04	19	–0.30		
Sex m	–0.22 ± 0.04	76	–5.50	25.92	<0.0001
Sex nd	–0.19 ± 0.04	76	–4.71		
Random effect	**Estimate**			**LLR**	***p*-value**
Runs	σ^2 = 1.47e-06²				
Aquaria in runs	σ^2 = 2.72e-07²			6.1e-09	1
Residual	σ^2 = 0.16²				
Post-hoc sex effects	**Estimate ± SE**	**df**	**t-ratio**		***p*-value**
f – m	0.220 ± 0.040	76	5.505		<0.0001
f – nd	0.186 ± 0.040	76	4.713		<0.0001
m – nd	–0.033 ± 0.024	76	–1.387		0.5086

Table S4.10
Mann-Whitney-U-test for parameters of the innate immune system, proxies for oxidative stress and body indices to test for differences between *Perca fluviatilis* with or without parasitic infection by *Triaenophorus nodulosus* in the liver in the low ALAN experiment.

Parameter	Mean ± SD (no parasites)	Mean ± SD (parasites)	U	*p*-value
Lysozyme	208.16 ± 56.81	204.55 ± 66.88	3134.5	0.5118
TBARS	331.50 ± 132.51	377.23 ± 206.01	2375	0.4685
Liver protein	90.28 ± 22.65	89.19 ± 23.11	2665	0.5569
Superoxide dismutase	26.51 ± 7.16	25.38 ± 6.48	2706	0.4504
Catalase	112.33 ± 63.39	114.31 ± 65.61	1655	0.8035
Condition factor	1.39 ± 0.13	1.35 ± 0.14	8678.5	0.0561
Splenosomatic index	0.16 ± 0.06	0.15 ± 0.07	8830.5	0.0151

Table S4.11
R packages (in bold) with references used for statistical analysis and visualization of data.

R package	Used for
Anguie, B. (2017). **gridExtra**: Miscellaneous functions for "Grid" graphics. R package version 2.3. URL: https://CRAN.R-project.org/package=gridExtra	data visualization
Barton, K. (2020). **MuMIn**: Multi-model inference. R package version 1.43.17. URL: https://CRAN.R-project.org/package=MuMIn	calculations of marginal and conditional R^2 values of the LMMs
Lenth, R. (2020). **emmeans**: Estimated marginal means, aka Least-squares means. R package version 1.4.7. URL: https://CRAN.R-project.org/package=emmeans	post-hoc testing
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team (2018). **nlme**: Linear and nonlinear mixed effects models. R package version 3.1-148. URL: https://CRAN.R-project.org/package=nlme.	linear mixed effect modelling
Wickham, H. (2016). **ggplot2**: Elegant graphics for data analysis. Springer-Verlag New York.	data visualization

Statement of academic integrity

I hereby declare that the submitted thesis “Effects of skyglow on the physiology of the Eurasian perch *Perca fluviatilis*” 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 contribution 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, …….....

Franziska Kupprat