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

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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.

**Key words:** Endocrine disruption, fish, freshwater, light pollution, thyroxine, triiodothyronine

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**Introduction**

Artificial light at night (ALAN) is an unprecedentedly increasing disturbance of natural nocturnal darkness (Gaston *et al.*, 2015, Hölker *et al.*, 2010). 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, Höcker 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., 2016) 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 et al., 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’-triiodo-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.

**Material and Methods**

**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).

**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).

**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.

(1) 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.

(2) Exposure to low intensities of ALAN (‘low ALAN experiment’)

*P. 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. Pre-acclimation 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 dusk 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).

**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 x 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.

**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 x 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 x g and 60 μL were transferred to an insert (250 μL) in an HPLC vial and stored at −20 °C until further use.

**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.

**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).

**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 homogeneously 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 only 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.

**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 1 (page 18). Full model specifications for each parameter as well as full results of the post-hoc tests are given in the supplementary material.

**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.

**Results**

**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 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 1).

In the low ALAN experiment, no ALAN effects were detected for T3 (LMM ALAN effects: LLR = 1.05, p = 0.79, Table 1, Figure 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).
Table 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.

| Experiment     | Parameter | Fixed variable | Fixed effects | Random effects | Goodness of fit |
|----------------|-----------|----------------|---------------|----------------|-----------------|
|                |           |                | LLR           | p-value        | LLR             | p-value        | $R^2_{\text{marginal}}$ | $R^2_{\text{conditional}}$ |
| High ALAN      | Log(T3)   | ALAN           | 11.94         | 0.008          | 2.08            | 0.35            | 0.2894           | 0.3548           |
|                | Sex       |                | 24.34         | <0.0001        | 12.47           | 0.0004          |                   |                 |
|                | Body mass |                | 1.96          | 0.58           | 2.83            | 0.24            | 0.0246           | 0.1648           |
|                | Log(T4)   | ALAN           | 6.58          | 0.09           | 2.16            | 0.34            | 0.0845           | 0.1957           |
|                | Log(T3/T4)| ALAN           | 1.05          | 0.79           | 15.52           | 0.0004          | 0.1546           | 0.4138           |
| Low ALAN       | Log(T3)   | ALAN           | 9.33          | 0.03           | 5.19            | 0.07            | 0.1306           | 0.3217           |
|                | Log(T4)   | ALAN + Body mass| 14.70        | 0.002          | 0.10            | 0.95            | 0.1633           | 0.1873           |
|                | Log(T3/T4)| ALAN + Body mass| 22.07        | <0.0001        | 0.09            | 0.67            | 0.1693           | 0.1873           |

$^1$ Log-likelihood ratio

Figure 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).

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 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).

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).

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.

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.

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 *tsb* 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., 2016), 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 char (*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.

**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 smolification, 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., 2018) 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).

**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., 2016, Zhao et al., 2016) or up to 1:20 (Arkoosh et al., 2017, Boujard and Leatherland, 1992, Chen et al., 2017) 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) and is also the most commonly reported T3/T4 ratio for teleost fish.

**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, Cyr and Eales, 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%).
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.

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Conflict of interest

None declared.

Supplementary material

Supplementary material is available at Conservation Physiology online.

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