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Studying effects of light pollution and aquacultural light regimes using the teleost model medaka (*Oryzias latipes*)

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Biology

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by

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Acknowledgements

Abstract

Light is the main cue in synchronizing daily circadian rhythm in most animals, in addition to synchronizing the perception of the year for some species. Due to the steady increase of light pollution around the world, and especially near water bodies, this study aims to investigate the effects of different light regimes. Those regimes include light pollution and the continuous light regime used in aquaculture, mainly to increase growth and delay sexual maturation, is also studied.

Firstly, light pollution levels were measured in Oslo's main river, and the harbor area. I found that urban light pollution is severe in some places and can reach relatively deep as I detected it at 5 meters depth in the fjord. These results suggest that aquatic animals may be exposed to light pollution in the Oslo area and thus the effects should be studied.

I then investigated in a laboratory setup the effect of light pollution on the teleost model Japanese medaka (*Oryzias latipes*). In addition to the continuous light regime was also studied. I found several effects of the altered light regimes. The reproductive cycle was found to be desynchronized for both light regimes. The fish's behavior was also found to be altered. Additionally, fish development was found to be affected with promoted growth and altered brain and heart morphology. The artificial light regimes were also found to affect neurochemistry and the gene expression of one pituitary gene. A clear noradrenergic response was found, with the control fish having higher noradrenergic activity. Fish exposed to light pollution had a higher serotonergic brain activity. The pituitary gonadotropin *lhb* was found to be decreased in the continuous light regime.

All together these results demonstrate that light pollution and continuous light clearly affect the fish, having welfare implications for aquaculture production, and suggesting that light pollution indeed affects wild fish.

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1. Introduction

1.1   Role of Light for Biological Rhythms

In living organisms, light is considered the main environmental cue which is used to synchronize biological rhythms with daytime or season. In teleost fish for instance, other cues also can be used to perceive time, such as the cycle of temperature, food availability, water composition, social cues, rainfall, tides and lunar cycles (Falcón & Zohar, 2018). Together, they all work as "time givers" for predicting upcoming fitness-deciding events. However, the 24-hour light/dark photoperiod on earth is often considered the main cue in synchronizing the biological rhythms, as it is the most predictable and stable cue through the day and night, and even year.

The phenomenon of keeping track of the day, using daily timed events, is called circadian rhythms. The circadian rhythms work endogenously to optimize the timing for individuals, in relation to ecosystem interactions. Physiological and behavioral actions are guided by the diurnal variation in light, as a cue to control and uphold a circadian rhythm. The light regime is therefore very important for many species. Indeed, certain fish behaviors are dependent on specific light intensities and duration to active or maintain, such as foraging, schooling and migration (Nightingale et al., 2006). Salmonids are well-known for migrating at night from their spawning sites, and into the ocean. Mature salmon also migrate back at night, into their home river to avoid predation (Nightingale et al., 2006).

The circadian rhythm can be altered by disrupting the natural 24 light/dark photoperiod (Vitaterna et al., 2001). Therefore, the stable rhythmicity of light/dark (L/D) cycle is therefore essential for most animals, including teleost fish. Predator-prey interactions have adapted to the duration of light and could be skewed in favor of either predator or prey, if the time of light in the day is extended (Vitaterna et al., 2001). Salmonids, for example, can be exposed to higher levels of predation if exposed to light during the dark phase, thus minimizing their migratory success (Nightingale et al., 2006). An extended photoperiod is also found to delay spawning and increase somatic growth in the teleost gilthead seabream (*Sparus aurata*) (Kissil et al., 2001). Therefore, altering the environmental light cue can mess with the ecological, but also the physiological adaptations for fish.

1.2   Light pollution at night

Artificial Light is used to extend the duration of the day, during the dark phase, for safety measures, advertising, and art, among others. This artificial light use unfortunately produce what is commonly called light pollution at night. Light pollution is steadily increasing. The effects are vast, as light pollution from cities can be detected more than 150 kilometers from a city (Duriscoe et al., 2007). That means light pollution from a big city can be seen all the way to the otherwise untouched areas.

Near cities, the effects can have dramatic consequences. Several animals have a natural interest in pursuing light. With the addition of artificial lights, it can result in so-called "fatal attraction". During migration, insects use the moon to navigate, but they are also attracted to other light sources which can mimic the moon, leading them astray. Unfortunately, one third of insects that become attracted to these artificial light sources do not survive until dawn, either by starvation or predation (Owens et al., 2020). A lot of insects also crash into cars, because they are pursuing the headlights, with death as a result (Frank et al., 2006). This mechanism is also found in birds, as short-tailed shearwater (*Ardenna tenuirostris*) fledglings have a higher chance on finding themselves grounded on the road when the traffic lights are turned on (Rodríguez et al., 2014). Therefore, it is important to properly identify the effects of light pollution, as there is still a knowledge gap regarding the overview of ecological consequences tied with artificial light at night (ALAN) (Grubisic et al., 2019; Longcore & Rich, 2004).

Putting efforts into studying light pollution can have huge rewards, since the detrimental effects can be reduced by turning off or changing the light bulb or angle. While short-tailed shearwaters was found to have their fatal attraction reduced by using high pressure sodium lights (Rodríguez et al., 2017), this varies between animal groups and species within. There is therefore a need to study several types of artificial light, to see which solution could reduce the effects of light pollution.

Although an upwards trend in research on light pollution is present, the range of studied effects and animal groups are limited. It has been clearly stated that there is a need for increased study of the effects of ALAN on underwater life (Longcore & Rich, 2004). Humanity tends to settle close to water, as it has always been an essential part of human life. For example, 50 % of the human population live within 3 kilometers of inland water (Kummu et al., 2011), resulting in human light sources, in close proximity to freshwater. The coastline is found to be highly affected by artificial light at night (ALAN), as 22.2 % of the coastal regions are exposed to it

(Davies et al., 2014). The cities are the most important source of light pollution, and urban waters are therefore exposed to the highest levels of light pollution. Light is also affecting aquatic wildlife directly, by cargo ships, oil platform lighting and the constant light used at aquaculture sea cages (Longcore & Rich, 2004).

1.3   Measurements of light pollution at night

On land light pollution has been measured satisfactorily, with the use of satellites. Satellites have also been used to study marine light pollution. Freshwater light pollution has also been studied, by using aerial recording (Kuechly et al., 2012). However, for satellite/aerial measurements, bridges obstruct the detection of light pollution, as light often is being used under bridges, straight into the water. This is, among other things, done to avoid reflection back to the sky, as a measure to minimize sky glow and astronomical light pollution. Sky glow is the increased effect light pollution can have, as it reflects to the skies, which deflect the light back down to the land and nearby waters. The sky glow effect on freshwater systems is impossible to detect since clouds will obstruct the detection. When looking into the effects of light pollution, on urban and suburban rivers, the measurements should be done at multiple points or preferably in a transect (Jechow & Hölker, 2019).

Measuring light pollution underwater can be done with marine light sensors in the form of a PAR-sensor (Photosynthetically Active Radiation). Using a PAR-sensor enables progress in underwater light pollution research, as it can measure light at low levels and offers high radiometric sensitivity and dynamic range. The downsides are that using a PAR-sensor usually provides minimal spectral selection and does not provide sufficient spectral resolution to discriminate ALAN from natural ambient light signals (Tidau et al., 2021).

1.4   Aquatic life in the Oslo fjord and nearby rivers

Marine life in the Oslo fjord has been struggling, after increased fishing and eutrophication, due to nutrient supply from agriculture. In addition, the introduction of bottom trawling one hundred years ago, for shrimp fishing, has had detrimental effects on the ecosystem and marine life. This is due to the ruination of the sea floor, as it takes away the variation in sea floor habitat. It is also problematic due to by-catch, as the trawling does not select for one species, but can take out vulnerable species from the ecosystem. Even if Atlantic cod (*Gadus morhua*), on a national level, is listed as least concern (LC), the local stock of Coastal cod is struggling. There is a

negative fish stock trend in inner Oslo fjord, with the resulting ban of recreational cod fishing in inner Oslo fjord, since 2019 (Moland et al., 2021). The anadromous salmonids Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*) use the lower parts in Akerselva, Oslo's main river, as spawning site. Atlantic salmon went from being categorized as least concern to near threatened (NT) in 2021, as the population has decreased (Hesthagen et al., 2021). Therefore, there is a critical need for protecting coastal cod and Atlantic Salmon. It is thus necessary to measure the light pollution levels they are exposed to and to look at the effects light pollution has on these populations. Because of the steady increase in light pollution this issue should be addressed quickly.

1.5   The aquaculture use of continuous light

Norway is the world's largest producer of farmed Atlantic salmon and the second largest nation of exporting aquatic products (Food and Agriculture Organization of the United Nations, 2022). As a measure to increase growth and postponing maturation, salmonids are normally exposed to continuous light in aquaculture (Berg et al., 1992; Hansen et al., 1992). For example largemouth bass (*Micropterus salmoides)* had greater growth and food intake in continuous light, than a 12 hour light and 12 hour dark regime (Petit et al., 2003).

A good control of the fish production, with growth and meat quality optimized, and with lower mortality ratio, results in greater profit. As discussed, this is among other factors done by tweaking the light regime, as it is a cheap way of controlling the production of fish. As the market has high standards for meat quality, early sexual maturation is a concern, as the meat quality often can be reduced. Additionally, continuous light is used to activate the parr-smolt transformation (Björnsson et al., 2000) and manipulate the spawning time for the broodfish (Taranger et al., 1998).

The use of artificial photoperiods comes across as an environmentally friendly and practical tool, in the control of sexual maturation and fish development. Although this may be true and beneficial to the industry, fish welfare should also be considered. Fish welfare should be measured by biological and not economic factors (Volpato et al., 2007). Even if artificial light regimes work well to ensure adequate food quality and high yields, but the welfare implications must be examined.

Since fish are extremely diverse as a group, not all fish benefit growth-wise from continuous light. For example, bottom-dwelling or nocturnal fish prefer lower levels of light (Lee et al.,

2017; Luchiari et al., 2006). Since these have adapted to low light levels, elevated levels of light might be harmful to the eye, and thus the fish. Consequently, controlling the fish production and growth with artificial photoperiods should be designed around the environmental conditions of the fish's natural life.

The literature on the isolated effects of light regimes in fish is narrowed to growth and feed conversion, and welfare studies of chickens raised for food, broiler chickens (*Gallus gallus domesticus*), will be compared. Broilers are raised under continuous light since this allows them to grow faster, with a better conversion rate from feeding to growth. Still, development of a circadian rhythm is known to be important for welfare of domestic animals (Bessei, 2006). This has not been possible when the broilers were exposed to near continuous light L23:D1 (Rutten et al., 2002).

The metabolic disease ascites is found to affect broiler chickens and the industry due to prominent levels of mortality and reduced weight. Ascites is a disease that can both develop and cause heart failure. It can also lead to infections and kidney failure (Julian, 1993). Compared to near continuous light L23:D1, having intermittent periods of darkness during the natural night, was found to reduce ascites-induced mortality (Buys et al., 1998; Hassanzadeh et al., 2000).

Interestingly, heart failure is also a big problem in the aquaculture of salmonids, and can be explained by intensive growth of fish, in combination with other factors (Poppe et al., 2003). Abnormal cardiac morphology can be applied to look at development of heart sickness and has been found less prevalent in slow growing fish than in intensively raised fish. Intensive growth of fish includes selective breeding programs and artificial environmental conditions. Selective breeding programs are targeting increased growth and survival, which are both economic factors. Artificial environmental conditions include the management of constant light regimes with higher temperatures and little to no time with darkness. On the other side slow growing fish experience natural yearly fluctuating light regimes and temperatures. Isolated, the most decisive factor is not known and possible factors should be investigated separately (Brijs et al., 2020).

Regarding welfare, fish are the only vertebrate production animal in Norway that is allowed raised with continuous light. The law states that aquaculture fish have the right to be treated well, including proper welfare. It does not, however, regulate the use of light regimes (Akvakulturdriftsforskriften, 2008). Superior animals have better welfare regulations, such as

the previously discussed poultry. The law for superior animals is stricter with the use of light regimes. For instance, broiler chickens, have a statutory right to a daily light regime with at least 6 hours of darkness per day (Forskrift om hold av høns og kalkun, 2001).

1.6   Teleost light perception

Fish receive light primarily through photoreceptors in the retina of the eye and the pineal gland, with likelihood of also existing elsewhere in the brain (Falcón et al., 2007). Furthermore, studies on zebrafish (*Danio rerio*) suggest that every cell in their tissues possess circadian clocks which respond to light (Steindal & Whitmore, 2019). Studies on zebrafish cannot be generalized as a consequence of the diversity of teleost, but for example medaka (*Oryzias latipes*) have been found to inhabit circadian clock genes in the eyes, brain, fins and heart (Cuesta et al., 2014). As the tissues tested all showed a rhythmicity in gene expression timed to the light regime, it is highly likely that other tissues also inhabit the rhythmic clock genes.

In response to environmental changes in the amount and photoperiodic duration of light, the retina and pineal gland in fish signal through two distinct types of mechanisms. Chemo-electric information from the retina and pineal gland is transmitted to the brain via the retinohypothalamic and pineal tracts. The information given provides the brain with day length and subtle variations in ambient illumination. Hormonal signaling serves as the other signaling mechanism, with the pineal gland producing melatonin. The melatonin production, which is released into the bloodstream and cerebrospinal fluid, is regulated by the photoperiod, and melatonin levels in the bloodstream provide the central nervous system with information about the time of day and season. In the retina, melatonin acts as a factor that affects the cells in the retina itself. In the pineal gland melatonin acts on specific targets, through melatonin receptors (Falcón et al., 2007).

Interestingly, melatonin receptors have been found in many brain areas, including the preoptic area (POA), which is known to influence pituitary function. The pituitary gland is a key regulator of many physiological processes, which includes growth, metabolism, and reproduction. Melatonin can modulate the production of hormones in the pituitary gland, by binding to specific receptors, thereby influencing the release of hormones into the bloodstream (Falcón et al., 2007). The pituitary regulation of hormonal genes can therefore be interesting to study on the teleost response to different light regimes.

To conclude, the pineal gland plays a central role in regulating the response to environmental changes. By signaling, both neurally and hormonally, the pineal gland provides the brain with important information on the time of day, season, and levels of ambient light.

1.7 The Japanese medaka as a model fish?

The Japanese medaka (*Oryzias latipes*) is a small freshwater fish (3 - 4 cm) with short generation time as they mature sexually after two to three months. The species is robust with high tolerance to changes in temperature and salinity. It also has high resistance towards common fish diseases (Wittbrodt et al., 2002). For these reasons, medaka is an emerging model species that is applied to study physiology, behavior, and development. It is also well suited in a laboratory setup due to its high tolerance to inbreeding, which is a recurring issue in fish facilities (Kirchmaier et al., 2015).

I want to use medaka as a model for the local fish species Atlantic salmon (*S. salar*) and coastal cod (*Gadus morhua*), as they are likely affected by the light pollution from the cities. Zebrafish is a more established model species, as is medaka. In comparison to zebrafish (*Danio* rerio) medaka shares a closer evolutionary ancestor in to both Atlantic salmon (*S. salar*) and cod (*G. morhua*) (Davidson, 2013). With its low cost, in comparison to physically larger species like most salmonids, it is possible to dive quite deep into research questions, even with limited funding. Since teleost fish are highly diverse, our findings should be confirmed with another model. A laboratory setup is good to identify biological effects but should be replicated in the wild. However, using a small model fish, in a controlled setup, is excellent to target the research and make new discoveries.

1.8 Aim

This study's aim is to explore the effects of light pollution and the use of continuous light in aquaculture. To achieve this aim, we first went into the field to measure light pollution underwater, to identify relevant light pollution levels.

Next, medaka was raised in a laboratory setup, using the light pollution conditions observed in the field and continuous light to mimic aquaculture conditions, to study the effects of the light regimes on fish biology. I looked at the behavior of the fish and morphology. Following up, anxiety was assessed using the established Open Field Test, while brains and hearts were morphologically studied.

The laboratory methods HPLC and qPCR will be applied to support the potential behavioral and morphological findings. Brain chemistry, for the different light treatments, will be researched with HPLC, while qPCR on pituitary genes regulating hormones will be used to look at basal development.

This study is meant as a pilot to uncover new effects of light pollution and the use of continuous light. If the findings are alarming, the studies should be replicated in wild fish and aquaculture species.

Hypotheses:

1. Light pollution in Oslo is highly present.
2. Light pollution desynchronizes spawning.
3. Type of light regimes affect fish behavior, development and/or morphology.
4. Light regimes affect brain chemistry and/or gene regulation of hormones.

2.   Methods

In my master thesis I investigated how the fish respond to the different light regimes. Three groups of our model fish medaka were raised in the laboratory: Control, ALAN, and Continuous Light. They were raised to study various behaviors, fish morphology and gene expression of pituitary hormones and neurochemistry studying monoamines, to investigate the effects of light pollution and continuous light on the teleost medaka.

2.1   Light measuring in the field

As a contribution in the mapping of light pollution penetrating urban waters, and additionally implementing biologically relevant light pollution levels into this laboratory study, the first part of this project consisted of on-site light pollution measurements. These measurements were taken during the period from January to March 2022 in a non-systematic transect alongside Oslo harbor and up the capital's main river; "Akerselva". These areas were chosen with the goal of representing different levels of light pollution, based on location and directionality of light.
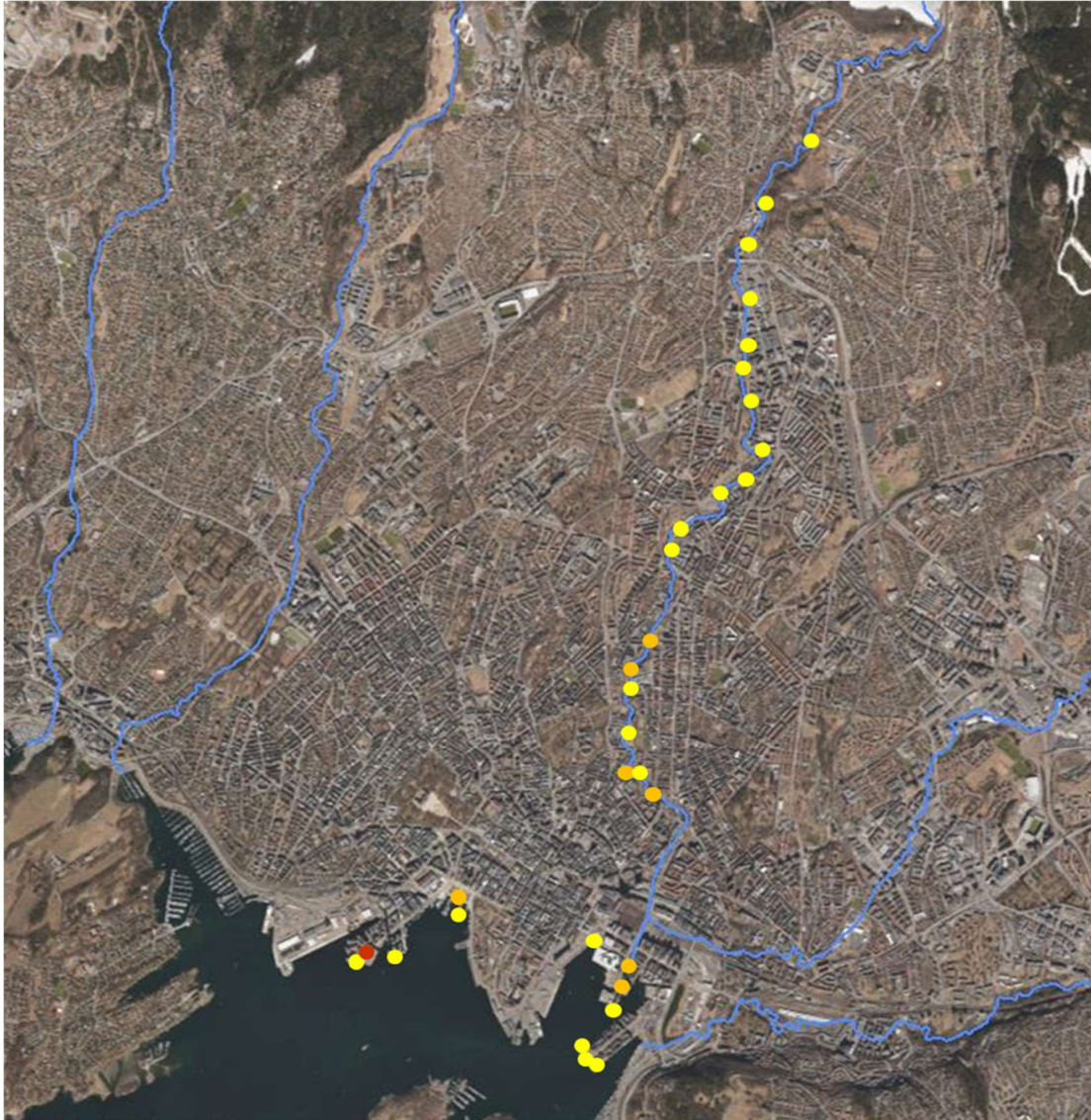
Figure 1. Map of Oslo (NVE, 2023) showing the sampling sites in Akerselva and Oslo harbor. At some points several samples were taken closely due to variation, which is shown by the color codes. The color codes are yellow for one sample, orange for two samples, and red for three samples.

The reason for us using this specific PAR-sensor (SQ-640-SS, Apogee Instruments, USA) was that it can detect a wider spectral range of light, than both a standard PAR-sensor and lux-meter. It was additionally sensitive to low levels of light and waterproof. The PAR-sensor was connected to a Bluetooth data logger (AT-100 microCache, Apogee Instruments, USA) to quantify light levels over time, and we specifically measured light at each point for at least 3 minutes to calculate the mean. The PAR-sensor was attached to a platform facing upwards to the surface of the water, in which the sensor measured lights penetrating downwards, to provide standardized measurements. The different depths were measured using different lengths of

thread connected to cabin hooks, that was lastly hooked onto a pyramidic structure with the sensor attached. The depths measured in the main river were 25 and 50 cm. In Oslo harbor one meter was also measured. Additionally, spots at five meters depth were measured where possible.

Figure 2. On the left the PAR-sensor (Apogee Instruments, USA) is shown, which was used to quantify underwater light. On the right the constructed float with the submersed PAR-sensor connected to the float with carabin hooks and thread is shown.

2.2   Laboratory experiments

Experimental setup

We used medaka (*Oryzias latipes)* from the HdrR strain as the model teleost for this study in the established model fish facility at the School of Veterinary Science, NMBU. They were raised in a re-circulating water system at 28 degrees Celsius, with the water holding a pH of 7.6, same as for the parallel study (Closs et al., 2023). Once the larvae had hatched, they were raised in three different cabinets with differing light regimes. Within the strain, I used two different populations, one wild type (WT) and one double transgenic population tg(lhb:hrGfpII/fshb:DsRed2) (DTG). The reason was that we wanted to count gonadotropin cells with fluorescent microscope, but due to time limitation this was not done.

1. The control group fish were raised in a regime of 14 hours light and 10 hours darkness as recommended by the book "Medaka: biology, management, and experimental protocols" (Kinoshita et al., 2009). The onset of light was at 8 am and lights were turned off at 22 pm.

2. Like our control group, the artificial light at night (ALAN) group was exposed to 14:10 LD in addition to ALAN (0.223 μmol m^{-2} s^{-1}, ~14 lux) to mimic light pollution in urban waters. Even though the ALAN we implement is a high value, the underwater urban light pollution was equal at some points.

3. The third group of fish is exposed to continuous light to mimic the common light condition protocol used in aquaculture. The use of continuous light can be seen as an extreme ALAN condition, which makes it relevant to analyze the three groups together.

The cabinet light values were measured with both a digital lux-meter (TES-1337, TES Electrical Electronic Corp), in addition to the PAR-sensor (SQ-640-SS, Apogee Instruments, USA) that was used in the urban waters. When measuring the tanks (Table 1), the light values were measured within the tanks, towards the light, at several places. When measuring with the PAR-sensor, the tank had water, to mimic the light levels the fish were exposed to. The lux-meter was, however, not waterproof, and the values were measured in the tanks without water. The actual lux values the fish were exposed to were therefore most likely lower. These values are good for this pilot to show the possible effects of light pollution. In addition to the nighttime light, the ALAN group followed the same daylight regime times as the control group.

Daytime light values were high, as is recommended when raising medaka (Hamilton et al., 1969). Three months old medaka, was raised without tank lids, due to several ongoing

experiments, and therefore a lack of lids. After four months, lids were put on to avoid fish jumping out of the tank, and thereby reducing the lights. For the last group of fish, that was only used to look at reproduction, lids were on from the start of their life. Because of this, light values with and without lids are presented.

The light intensity of the replicated nighttime light pollution for ALAN went from being 14.25 lux (0.223 μmol m^{-2} s^{-1}) before 4 months, and 7.75 lux (0.126 μmol m^{-2} s^{-1}) after, which is only 55 % of the light pollution exposed to earlier.

Table 1. Presenting the cabinet values as mean ± SEM.

Light Regime	With/without lid	Daytime lux values	Daytime PAR-values (μmol m^{-2} s^{-1})	Nighttime lux values	Nighttime PAR values (μmol m^{-2} s^{-1})
Control	Without	1431.88 ± 149.01	23.84 ± 2.65	0	0
Control	With	845.63 ± 100.98	13.88 ± 1.61	0	0
ALAN	Without	1431.88 ± 149.01	23.84 ± 2.65	14.25 ± 1.11	0.223 ± 0.03
ALAN	With	845.63 ± 100.98	13.88 ± 1.61	7.75 ± 1.03	0.126 ± 0.02
Continuous Light	Without	1431.88 ± 149.01	23.84 ± 2.65	1431.88 ± 149.01	23.84 ± 2.65
Continuous Light	With	845.63 ± 100.98	13.88 ± 1.61	845.63 ± 100.98	13.88 ± 1.61

After approximately three weeks the number of fish per tank was reduced to 12.

2.3 Female reproduction analysis

In laboratory conditions, medaka are spawning once a day (Hamilton et al., 1969) within an hour after the onset of light (Kinoshita et al., 2009). Therefore, we investigated whether the different light regimes influence the spawning synchronization by tracking the timing of the spawning, together with the quantity and quality of female gametes by counting eggs and larval survival.

Timing of spawning

Egg laying trends were analyzed by counting spawning females at the timepoints; 7:30 am (30 min before light onset), 11:30 am and 3:30 pm. After counting the females, in a measure to avoid multiple counts of egg-laying time points from the same fish, the eggs were removed from the abdomen.

Females' gametes quantity and quality

Eggs were collected, counted, and put for each spawning females separately in a 6-well dish. The dishes were filled with egg-water containing a low concentration of trypan blue dye, to identify and take out dead eggs, thus preventing the potential spread of disease (Tilton et al., 2005). Egg-water was changed every third day. After 10 days the successfully hatched larvae were counted.

2.4   Behavioral analysis

Behavioral analysis were done to assess the effects of the three different light regimes on stress as behavior can be a good indicator of stress in fish (Braithwaite & Ebbesson, 2014). For the dive and open field test a recording system was set up, and the software Ethovision XT 13 (Noldus, Wageningen, The Netherlands) was used to track the fish and quantify the behavior with a camera (acA1300-60gm Basler) from either the top (Open Field Test) or in front (Dive Test) of the tanks. For the behavioral recordings, a UV background panel was used to increase the contrast between the fish and the background, thus making it easier for the software to track the fish. The automated tracking software could measure several parameters for one test.

Dive Test

As we believed fish were using the water column differently from the light columns, a dive test (Fig. 3.) was set up to quantify the possible behavior seen. Due to the tank being transparent, the tracking software sometimes followed the mirror image of the fish. To solve this problem, the tanks were stuffed with fish safe plastic pillows on the sides as seen in figure 3. In addition to the time spent in the three water column zones (top, middle, bottom), the distance swum was also quantified. 4 fish were released into the experimental tank and recorded for 10 minutes simultaneously.

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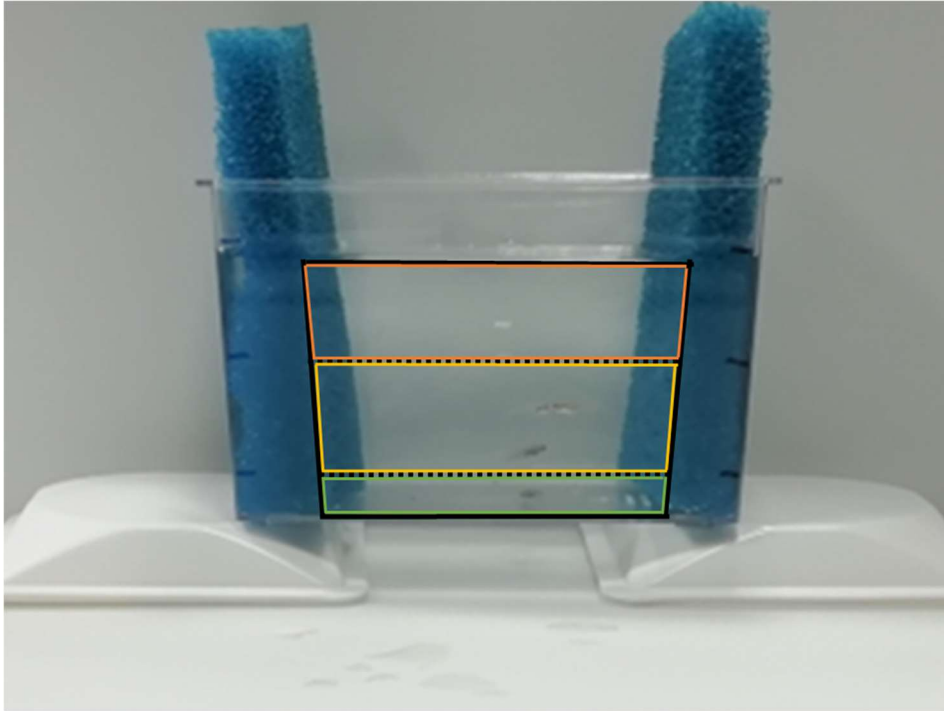
Figure 3. Showing the dive test with the three different water column zones. The zones bottom (green), middle (yellow) and top (orange) are outlined. The bottom area was 2 cm, middle area 6 cm, and the top area 9.5 cm high. The average width of the tank field where the fish could swim freely was 12 cm.

Open Field Test

Further assessing the explorative behavior and possible anxiety, we used the standardized Open Field Test. One by one, the fish were released into the open field to explore the novel tank for 5 minutes. A 30*30 cm square box was used and filled with 4 liters of water. The open field test assesses how a fish reacts to being alone in a relatively small, but open water space, and this is here measured by time spent in the center area, distance moved, and time spent stationary (defined as time spent moving less than 1.75 cm per second).
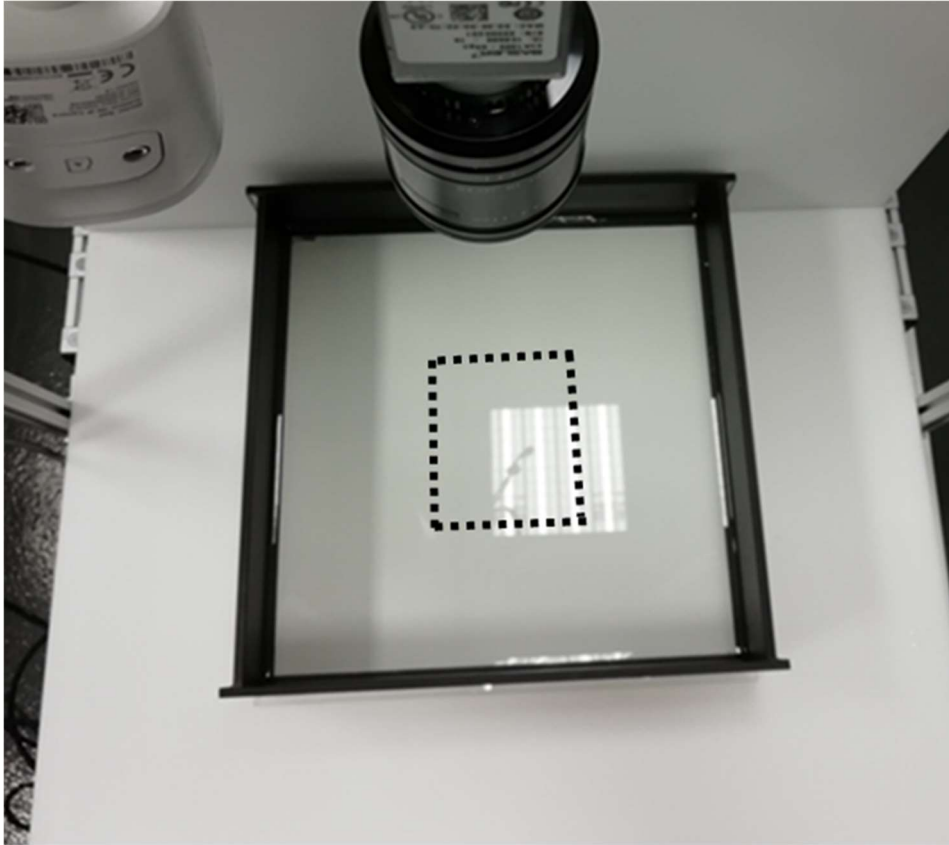
Figure 4. The open field test is shown with the zones inside and outside separated by the line, and the camera filmed from the top of the tank.

2.5 Sampling and morphometric measurements

Fish euthanasia

As blood can crystallize when frozen, we used the more common Tricaine methane sulfonate (MS-222) for euthanasia when needing to sample blood. To prepare a lethal dose of MS-222 one tube of 0,6% stock solution was diluted with 10 mL aquarium water. The fish were then euthanized by being put in a beaker with the prepared 0,06% MS-222 for around 30 seconds. Fish were then dept in ice water for hardening the tissues before sampling.

External fish body measurements

Following euthanasia, the body length and weight of the fish were measured. Fish development can be studied by using growth parameters. Fulton's condition factor was later calculated based on the length and weight. The formula used was $K=100*W/L^3$, W being weight in mg and L being length in mm.

Blood sampling

Quickly after euthanasia, a glass needle connected to an aspirator tube was used to collect blood from the caudal vein, a method developed by our lab (Royan et al., 2020). Needles were prepared by inserting a 90 mm long glass capillary into a needle puller machine which stretched the glass capillary following heating until it broke, providing two needles. The tip of each needle was then broken using rough dissecting forceps to make an opening before insertion into the aspirator tube. The needle was coated with sodium heparin by suction and blowing, to prevent coagulation of blood.

After all preparation, the fish was gently dried with a paper towel and put under the dissection microscope. The scales covering the caudal peduncle vein were scraped off before needle insertion into the caudal vein. The mouthpiece of the aspirator tube was then suctioned until at least 1 µL of blood had collected in the needle. The needle was then removed, and its content was collected into a tube by centrifugation for one second, before storage at -80 degrees Celsius until use.

Brain sampling and morphology

The teleost brain controls behavior and cognitive functions, and brain morphometry was therefore studied. Brains from double transgenic fish was collected in toto under a dissection microscope with a technique previously described (Ager-Wick et al., 2018) and quickly fixed in 4% PFA (PBS-T). Following two washes with PBS, pictures of the brain in the dorsal, lateral, and ventral views were taken using a stereomicroscope (Nikon, SMZ25). Calibration of metric values was done by using pictures of 0.01 mm scale in the different magnifications used.
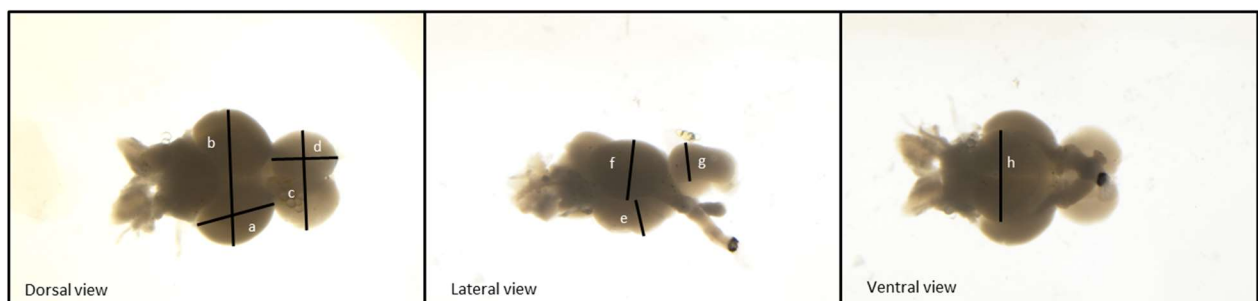
Figure 5. Measurements taken of the brains sampled.

Brains from WT fish were also sampled the same way, but brain parts were split and stored in sodium acetate buffer for later use in HPLC. The brains from the WT population were sampled the same way, only for them the brain parts were split and stored in sodium acetate buffer for the later use in quantifying the monoaminergic neurochemistry.

Heart sampling and morphology

The heart pumps venous blood collected from all fish organs, to again be oxygenized by the gills in the branchial apparatus (Yamauchi, 1980). Heart function therefore has a high impact on blood flow and general fish performance (Sanchez-Quintana et al., 1995), and good heart morphology has additionally been found to be positively correlated with cardiac function (Sande & Poppe, 1995).

Hearts were dissected using fine forceps under a dissection microscope. Carefully, the pericardiac sac was opened, before the heart was exposed. The sinus venosus was first cut, to safely detach the heart. Secondly, a point as high as possible from the bulbus arteriosus and towards the ventral aorta, were cut while lifting the heart carefully out.

Each individual heart was rinsed in phosphate buffered saline (PBS) for approximately 30 seconds, to get the heart clean of blood. Thereafter, each heart was washed 20 mmol/L Potassium Chloride (KCl), mixed with PBS for approximately 30 seconds. The excess KCL stopped the heart from contracting and that way locking it in diastole (Icardo et al., 2005). Lastly, they were stored in Paraformaldehyde (PFA) 4% at 4 degrees Celsius until analysis.

Before analysis, I washed the hearts twice with PBS to safely work with them, as PFA is hazardous. Agarose gel was made and poured onto a petri dish, functioning as a translucent heart holder. The hearts were placed in the petri dish together with PBS and moved with the help of forceps into the two standardized projection angles lateral left and ventrodorsal (Frisk et al., 2020) used for studying the cardiac morphology. Pictures were taken by a stereomicroscope (Nikon, SMZ25), and were saved as in the file format TIFF (Tagged Image File Format).

The saved TIFF-images were analyzed in the ImageJ 1.53k software (National Institutes of Health, Maryland, U.S.A.). To measure the alignment of bulbus arteriosus (α) on the lateral left projection, a straight line was drawn between the middle of the base until the apex. A second

line was drawn through the bulbus arteriosus to the longitudinal axis. The alignment angle (α) was then measured in the analysis software using the angle tool (Figure 6).

The ventricular width and length were measured from the ventrodorsal projection to calculate the width:length ratio. The height/width ratio was measured by drawing alongside base. The ventricular width was measured by going parallel down the ventricle until the widest area was detected. The ventricular height was then measured 90 degrees to the width line from middle of apex to the start-point of bulbus arteriosus (Figure 6).
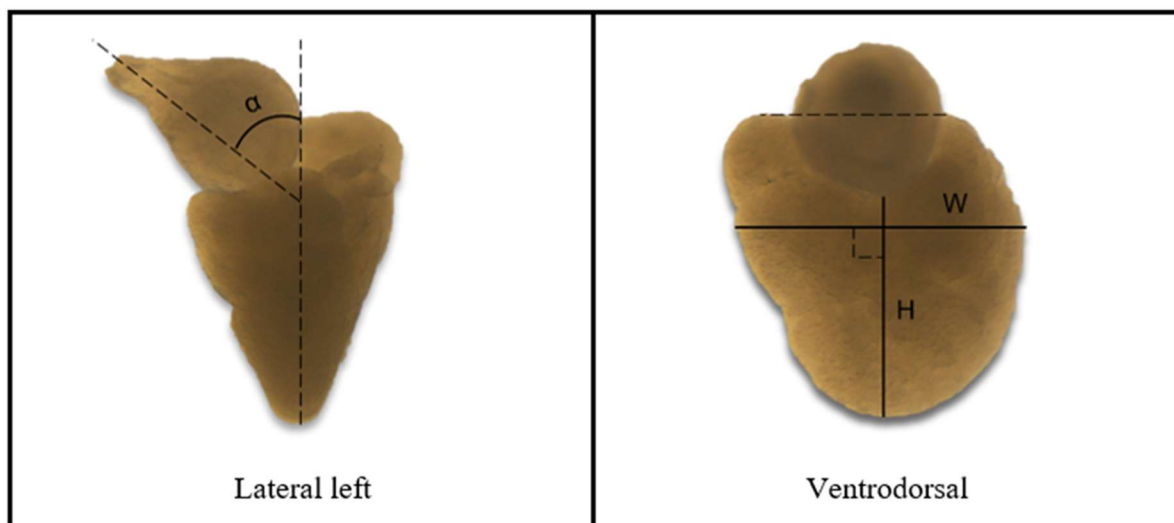
Figure 6. Medaka hearts in the projection lateral left and ventrodorsal. Here the lateral left angle is done according to a study on Atlantic salmon and rainbow trout (Poppe et al., 2003) and the ventrodorsal ventricular height:width ratio according to a study on Atlantic salmon (Frisk et al., 2020).

Sampling of gonads and analysis of GSI

The gonads were dissected and used for sex determination. The gonads were also weighed to assess the gonadosomatic index (GSI) (gonad weight/body weight x 100), to look at the light regimes possible effect on gonadal growth.

Sampling of brain and pituitary

For HPLC and possibly later gene expression, brains were dissected using sharp forceps under a microscope and split into five parts: Telencephalon, Hypothalamus, Optic tectum, Brain stem and Cerebellum. These parts were put into RNase free 1,5 mL tubes filled with 100 μL sodium acetate buffer including Internal Standard. The sodium acetate buffer contains 3 grams sodium acetate, 4,3 mL 100% glacial acetic acid and 16 sodium hydroxide pellets in 1000 mL Milli-Q

water. To serve as an internal standard, 10 mL of 94.2 ng/ml^{-1} 3,4-dihydroxybenzyl amine hydrobromide (DHBA) was added. Lastly the pH was adjusted to 5 by titration with concentrated phosphoric acid (H$_3$PO$_4$). The tubes were weighed before and after tissue sampling.

Pituitaries were taken from the ventral point of the inferior lobe (Ager-Wick et al., 2018) and then collected in 2 mL tubes containing 300 μL TRIzol^{TM} reagent (Invitrogen) and 5 - 6 zirconium oxide beads (Bertin Technologies; diameter 1.4 mm) before being rapidly frozen on dry ice and stored at –80 degrees Celsius until use.

2.6 Hormone quantification

To supply another perspective on the behavioral study, possible hormonal and neural effects of the light treatments are studied. For this part, only the wild type fish were used. Neural activity was quantified by studying the monoaminergic activity in the brain. Hormones are studied by looking at genes in the pituitary which regulate hormone production.

2.7 High-performance liquid chromatography (HPLC)

HPLC was done according to (Vindas, Johansen, et al., 2014) and (Höglund et al., 2023). The HPLC machine is used to study the monoaminergic activity in the brain, by looking at the ratios between the metabolite and its parent monoamine ratio. The parent monoamines serotonin (5-HT), dopamine (DA), and their metabolites 5-hydroxy indole acetic acid (5-HIAA) and dihydroxyphenylacetic acid (DOPAC) were analyzed. In addition, Norepinephrine (NE) was analyzed, but not its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG). To save time MHPG was not analyzed. Additionally, I decided not to run the cerebellum samples, as it is a area of the brain with low monoaminergic activity, and rarely analyzed. Not analyzing MHPG and cerebellum was a choice done to save time, as the HPLC-machine is quite time-consuming and were in high demand by researchers.

Homogenizing brain parts in Sodium acetate buffer

Preparing the samples for HPLC-analysis the brain parts was homogenized, by the use of a Q55/CL-188 Sonicator (model CL-188, QSonica Sonicators, Connecticut Newtown, USA). After the sample had thawed, the samples were one by one sonicated at the amplitude of 15 in

3 second bouts, and intermittently put on regular ice. This was repeated 1-3 times for each sample, until the solution was visibly homogenous. The ice was used to avoid degrading of the monoamines, as they are heat sensitive and thus degrade at RT. After sonicating they were put back on dry ice. To avoid hearing damage ear protection was used when sonicating the samples.

At the end of homogenizing the batch of samples, they were run in the centrifuge for 15 minutes at 4 degrees Celsius and 20 000 G (VWR® Mega Star 600R, VWR International BVBA, Leuven Belgium). After centrifuging, the liquid was transferred to a new Eppendorf tube and put in -80 degrees Celsius. The old Eppendorf tube with the pellet was also kept at -80 degrees Celsius, for possible RNA extraction.

Monoaminergic neurochemistry (HPLC)

The mobile phase that the machine was run with consisted of 86.27 mM sodium dihydrogen phosphate (NaH_2PO_4), 0.81 mM sodium octyl sulfate ($C_8H_{17}NaO_4S$) and 3.7 µL ethylenediaminetetraacetic acid (EDTA) in deionized water (18.2 MW resistance) holding 7% acetonitrile brought to a pH of 3.1 with phosphoric acid. This machine consisted of a solvent delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 x 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes, at -40 mV and + 320 mV. A conditioning electrode, with a potential of +40 mV, was employed before the analytical electrodes, to oxidize any possible contaminants present (Vindas, Johansen, et al., 2014).

On the day of the analysis, homogenized samples, internal standards, and standard aliquots were thawed on ice before vortexed and centrifuged at 20 000 G for 15 minutes at 4 degrees Celsius to spin down proteins and other debris. 100 µL were then transferred from the middle section of the tubes to marked HPLC-specialized vials, in addition to the mentioned standards. 70 µL of each sample were analyzed by the HPLC machine for monoaminergic neurochemistry. Samples were run in the order: STD 1 – IS 1 – up to 10 samples – STD 2 – IS 2 – up to 10 samples – STD 3 – IS 3. 9 samples from Hypothalamus were discarded as the Internal Standard peak was too high. The amplifier settings were then adjusted, to lower the peak of the internal standard. For optic tectum, some DOPAC peaks were hard to analyze as they were small. Some DOPAC peaks were therefore taken out when analyzing the chromatograms blindly in the HPLC software. The samples were measured by comparing them to standard solutions with known concentrations. The internal standard recovery was accounted for using the

chromatogram software (CSW, DataApex Ltd., Czech Republic). The sodium acetate buffer holding the Internal Standard, was different from experiment 1 to 2. The sodium acetate was based on trihydrate for experiment 1 while dehydrated sodium acetate was used for experiment 2. This difference resulted in the peak of internal standard being was found at another time point in the chromatogram for experiment 1 in relation to experiment 2. The internal standard was equal between each light regime for each experiment, but lower in one, resulting in the monoamine concentrations still being precise in relativeness to the other groups, but not completely precise in the calculation of the concentrations for experiment 1.

2.8   Quantitative PCR (qPCR)

Reverse Transcription – qPCR (RT-qPCR) is a method based on how fast the DNA-molecule is quantified, and is decides how high concentration it originally was (Fraga et al., 2008). The method is mostly used to look at gene expression by using reverse transcribed mRNA. By using reverse transcription, the quantity of active RNA can be measured (Fraga et al., 2008). Since RT-qPCR is a relatively cheap and precise method of assessing gene expression of specific nucleic acids, it is used in this study.

RNA extraction

Total mRNA from single pituitaries in 300 µL TRIzol^{TM} agent (Invitrogen, Carlsbad, U.S.A.) was extracted following a protocol optimized for Medaka pituitaries (Burow et al., 2019). Tissues were first mechanically destroyed by the beads using a homogenizer. 120 µL chloroform for each pituitary was added to separate the phases. The tubes were vortexed for 10 seconds, then incubated for three minutes at RT (room temperature). They were then centrifuged at 14 000 g for 15 minutes at 4 degrees Celsius.

Because of the acidic nature of guanidium thiocyanate (the TRIzol^{TM} agent) the RNA separates into the clear upper phase. The aqueous phase was carefully transferred into the new tubes with 1,5 µl of GlycoBlue^{TM} to visibly avoid TRIzol^{TM} contamination. 150 µL 100% isopropanol was thereafter added. The tubes were vortexed for 5 seconds and spun down before incubating for 10 min at RT. After incubation they were centrifuged at 14 000 g for 15 minutes at 4 degrees Celsius to precipitate the RNA.

Washing the RNA, the supernatant was removed, with the GlycoBlue™ colored RNA-pellet remained. The pellet was then washed with 300 μL RNase-free 75% Ethanol, before the tube was flicked a couple of times to release the pellet into the Ethanol. The tubes were then centrifuged for 10 minutes at 4 degrees Celsius at 10 000 g. The supernatant is lastly removed, and the remains dried by a block heater set at 40 degrees Celsius.

As the last step of extraction, 14 μL qPCR-water was added to the pellet. Mixing the RNA-pellet was done by homogenizing it with the water, flicking the tube, vortexing, spun down, vortexing and spun down one more time. The tube was lastly put on ice.

Spectrophotometric analysis

To measure the quality of the RNA previously extracted, a UV/Vis-spectrophotometric analysis was done the same day to avoid unnecessary freeze-thaw cycles (Biotek Epoch). The analysis was done to quantify nucleic acid of the RNA.

Before analysis of extracted RNA, the wells were cleaned with fine paper (VWR Light-Duty Tissue Wipers) and checked for proper calibration. 1 μL of either qPCR-grade water for the controls or the extracted RNA was pipetted onto the different wells. After a good read, the control wells turned green, and samples were properly analyzed. The samples should have 260/280 absorbance levels between 1.8 and 2. If the sample values subceed or exceed these values, they had been contaminated by the TRIzol™ from the phase separation, and they were thus taken out.

cDNA synthesis (20 μL reaction)

To prepare cDNA synthesis a table with sample names and RNA concentration in ng/μL was made. The concentration was then multiplied by 11 for total RNA in nanogram. The lowest total RNA was then divided by the RNA concentration for each sample. Lastly, the RNA volume was subtracted from 11 to get the amount of qPCR water that should be added.

Following the calculations, qPCR water and sample RNA was pipetted into the PCR-strip tubes so that the total volume was 11 μL in each well. 1 μL dNTP mix and 1 μL random primers were mixed for each sample, before added to each of the PCR-strip tubes. The mix was made to limit the pipetting error difference. The dNTP mix consisted of 60 μL qPCR-water and 10 μL of the

four nucleoside triphosphates that complement the nucleobases guanine, adenine, cytosine, and thymine.

After addition of the dNTP mix and the random primers, the tubes were heated at 65 degrees Celsius for 5 minutes. Once taken out, they went back on ice to prevent degradation.

Next step was preparing the master mix, consisting of 4 µL 5x First Strand Buffer, 1 µL 0.1M DTT, 1 µL RNase OUT and 1 µL SuperScript III RT. For the SuperScript III RT it was important to keep it cold, and only have it out for a brief period. The master mix was tapped gently before pipetting 7 µL per tube.

As the next step, the tubes were incubated in the PCR machine. They were incubated in RT for 5 minutes before they were inserted in the PCR machine. Secondly, they were incubated at 50 degrees Celsius for 70 minutes, before the last incubation step at 70 degrees Celsius for 15 minutes. While incubating in the PCR machine, two new copies of the tube strips were labeled, to later be aliquoted towards the end of the protocol.

After incubating in the PCR machine, equal amounts of each sample were pooled together to make a positive control. The pooled samples and the positive control were then diluted 1:5. Lastly, equal amounts were aliquoted into the two labeled sets of tubes, before they were stored at -20 degrees Celsius. The aliquoting was done to lower the number of freeze-thaw cycles, when later used for qPCR.

qPCR

qPCR was performed in duplicates using 1 µL of forward and reverse primers (Eurofins genetics) in 5 µL SYBR GREEN I Master Mix (Roche Diagnostics, Basel, Switzerland) with a 3 µL cDNA template. The total reaction volume was 10 µL. The reaction condition for the qPCR was carried out as described (Burow et al., 2019). One duplicate with qPCR-grade water as the substitute for the cDNA template was used as the negative control. Pooled cDNA from all samples was used for our positive control instead of a single cDNA template. The quantification cycle (Cq) values were run on Light Cycler 96 version 1.1.0.1320 (Roche Diagnostics) following the optimized protocol (Royan et al., 2023) and qPCR parameters were set at the following parameters 10 minutes of preincubation at 95 degrees Celsius, 42 cycles of 95 degrees Celsius for 10 seconds, 60 degrees Celsius for 10 seconds, and 72 degrees Celsius for 15 seconds. The qPCR was followed by analysis of Cq-values and melting curves with Light

Cycler 96 software (Roche diagnostics). RPL7 gave the lowest standard error (Appendix 7.5) and was not too highly expressed compared to the target genes. As our housekeeping gene, RPL7 was used to calculate the relative gene expression ($\Delta\Delta$Cq). The primers used for qPCR are shown (Table 2) below.

Table 2. Nucleotide sequence of primer pairs used for qPCR. The forward primer is noted as F and reverse primer is noted as R.

Candidate gene	Primer sequence 5' → 3'	References
cyp19a1b	F: AAGAAGATGATCCAGCAAGAG R: AGCATCAGAAGAAGTAAGAAAAGTG	(Takeuchi & Okubo, 2013)
lhb	F: CCACTGCCTTACCAAGGACC R: AGGAAGCTCAAATGTCTTGTAG	(Hildahl et al., 2012)
fshb	F: GACGGTGCTACCATGAGGAT R: TCCCCACTGCAGATCTTTTC	(Burow et al., 2019)
pomca	F: GTGGTGGTTGTCGGTGGG R: GTGAGGTCAGAGCGGCAG	(Royan et al., 2021)
tshba	F: ATGTGGAGAAGCCAGAATGC R: CTCATGTTGCTGTCCCTTGA	(Royan et al., 2021)
gh	F: TCGCTCTTTGTCTGGGAGTT R: ACATTCTGATTGGCCCTGAT	(Royan et al., 2021)
sl	F: CACCAAAGCATTACCCATCC R: ACCAGCATCAGCACAGAATG	(Royan et al., 2021)
Reference gene		
gapdh	F: CCTCCATCTTTGATGCTGGT R: ACGGTTGCTGTAGCCAAACT	(Burow et al., 2019)
rpl7	F: TGCTTTGGTGGAGAAAGCTC R: TGGCAGGCTTGAAGTTCTTT	(Burow et al., 2019)
18s	F: CCTGCGGCTTAATTTGACTC R: AACTAAGAACGGCCATGCAC	(Burow et al., 2019)

2.9   Statistics

Two-way ANOVA with Tukey's multiple comparison test was used to analyze difference between the light treatments when there were two or more experiments. The interaction effect

between the treatment groups and experiments was noted when significant, as this means the results are inconsistent across experiments. One-way ANOVA was used to study a singular experiment. Data for one-way ANOVA was checked with Levene's test, and it was followed up by either Tukey or Games-Howell depending on the homogeneity. The statistical significance was set to $P < 0.05$. To detect and remove potential outliers, the ROUT outlier test was applied for the open field test, GSI, brain morphometry, gene expression and monoamine concentrations. With one exception the outlier test was set to $Q=1\%$, as this in most cases recommended (Motulsky & Brown, 2006). For the study of monoamine concentrations, the Q was set to 5%, as it has been done this way before (Sharifi, 2022). Another reason was a high variation in data for the monoamine concentrations. Statistics were done and graphs were made using GraphPad Prism 9 (GraphPad Software, San Diego, CA, U.S.A.) For one-way ANOVA and descriptives, Jamovi was used (Version 2.3.21) (Project, 2021).

3. Results

3.1 Light measurement in the field

I first investigated the penetration of light pollution into urban waters to determine the light intensity levels the aquatic fauna is exposed to. For this, a transect through the main river of Oslo was mapped. Additionally, light pollution was measured in Oslo harbor.

A high variety of levels of light pollution were detected underwater. Even if the levels are low in some areas, light could be measured by the sensor in almost all the sampling points and at all sampling depths. In addition, we can see in specific areas that there are relatively high levels. For instance, we could measure intensity levels of 0,35 μmol m^{-2} s^{-1} at 25 cm depth in the river, which is higher than what was used as the experimental ALAN light level (0.223 μmol m^{-2} s^{-1}). Additionally, the measured value was close to what was found under a streetlight (0,5 μmol m^{-2} s^{-1}). Interestingly, there were higher levels of penetrating light detected in the harbor area than in Akerselva. These results indicate that there were either higher levels of light pollution in the harbor or lower levels in turbidity. The possible lower levels in turbidity can be as a cause of reduced organic matter and water being calmer, resulting in more effective light penetration. It is worth noting that the difference between 50 cm and 1 meter in the harbor area is minimal. Surprisingly, light levels were also measured at 5 meters of depth, signifying the penetrative success of light pollution at such depth.
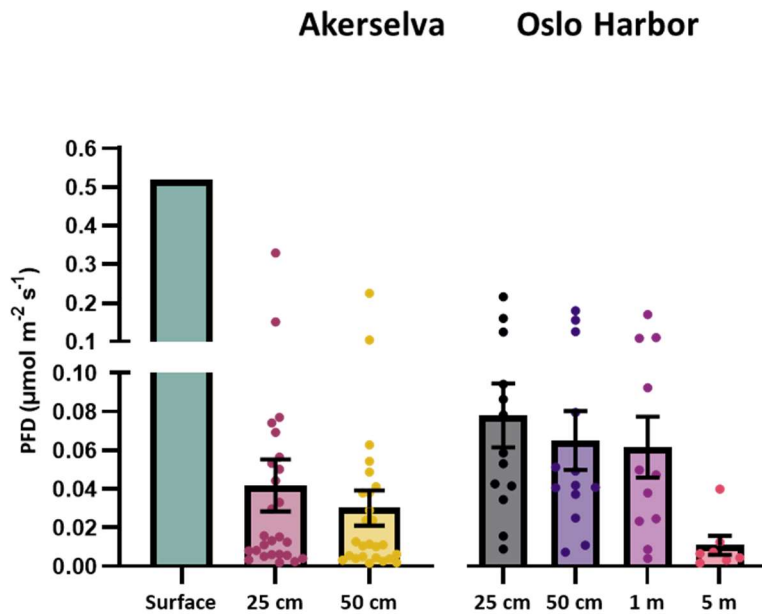
Figure 7. Light measurements in Akerselva and around Oslo Harbor. A control measurement at the surface of the road under a streetlight is also presented in the first bar. Data are presented as mean ± SEM.

I investigated the effect of different light regimes, ALAN (14/10 LD cycle with light pollution during the dark phase) and continuous light (24/0 LD) together with control conditions (14/10 LD).

3.2 Investigation of reproductive timing

Looking at the reproductive cycle of medaka (Fig. 8), a clear disruption in the timing was found. All fish from the control group spawned between 7:30 am and 11:30 am. This is natural in a laboratory setup as fish spawn relatively quickly following the light onset, which is at 8 am in our facility. In contrast, ALAN spawned before 7:30 am and until 11:30 am. Indeed, only one spawning event was observed between 11:30 am and 15:30 pm. Continuous light seems to be even more disrupting, as most of the fish did not spawn between 7:30 am and 11:30 am, opposing the natural behavior and control fish. They spawned most often between 15:30 pm and 7:30 am, and sometimes between 11:30 am and 15:30 pm. Worth mentioning, there is a much longer time between 15:30 pm and 7:30 am than the other intervals, and this might explain why there are more fish registered spawning before 7:30 am than before 11:30 am and 15:30 pm. These results suggest that the control fish group has a synchronized reproductive timing, while ALAN and continuous light have their reproductive timing desynchronized, with a higher impact of continuous light than ALAN.

28
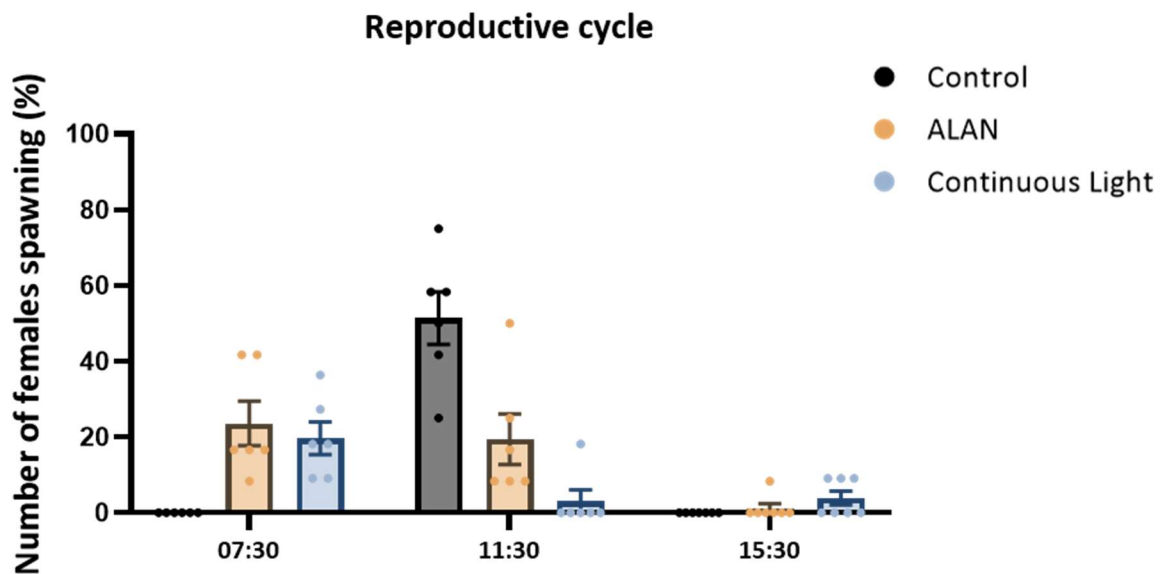
Figure 8. Number of females which spawned at three different time points over the 24-hour cycle. The n was between 6 and 7 per group per time point.

I then further investigated the number of spawned eggs and developing embryos (Table 3) as an indicator of female gamete quantity and quality. I also calculated the survival rate. There was not detected any significant difference between the groups for any of the parameters analyzed, suggesting that the light regime may not affect female gamete quantity or quality.

Table 3. Number of spawned eggs and developing embryos eggs per female. These two parameters were then used to calculate the survival rate in %. Differences between treatments were investigated with one-way ANOVA for each parameter. N was 13-23 per group.

Parameter	Control	ALAN	Continuous Light	One-way ANOVA
Spawned eggs	4.24 ± 0.458	4.87 ± 0.556	4.00 ± 0.480	$F_{2,50}$ =0.747, p=0.479
Developing embryos	3.35 ± 0.507	2.83 ± 0.370	3.00 ± 0.531	$F_{2,50}$=0.374, p=0.374
Survival rate (%)	74.6 ± 8.73	63.8 ± 7.20	74.1 ± 8.91	$F_{2,50}$ =0.621, p=0.542

3.3 Behavioral analysis

Dive test

To investigate whether the light regimes affect fish behavior, I performed a dive test by looking at the time individual fish put in a group of four spent in each zone of the water column. Using three-month-old fish (Fig. 9), fish from the continuous light group spent significantly more time

in the bottom part of the tank, than both the control and ALAN group. Control and ALAN fish spent significantly more time both in the zones middle and the top. The results suggest that the fish behavior is affected by the light regimes, and the differences are clear at 3 months.
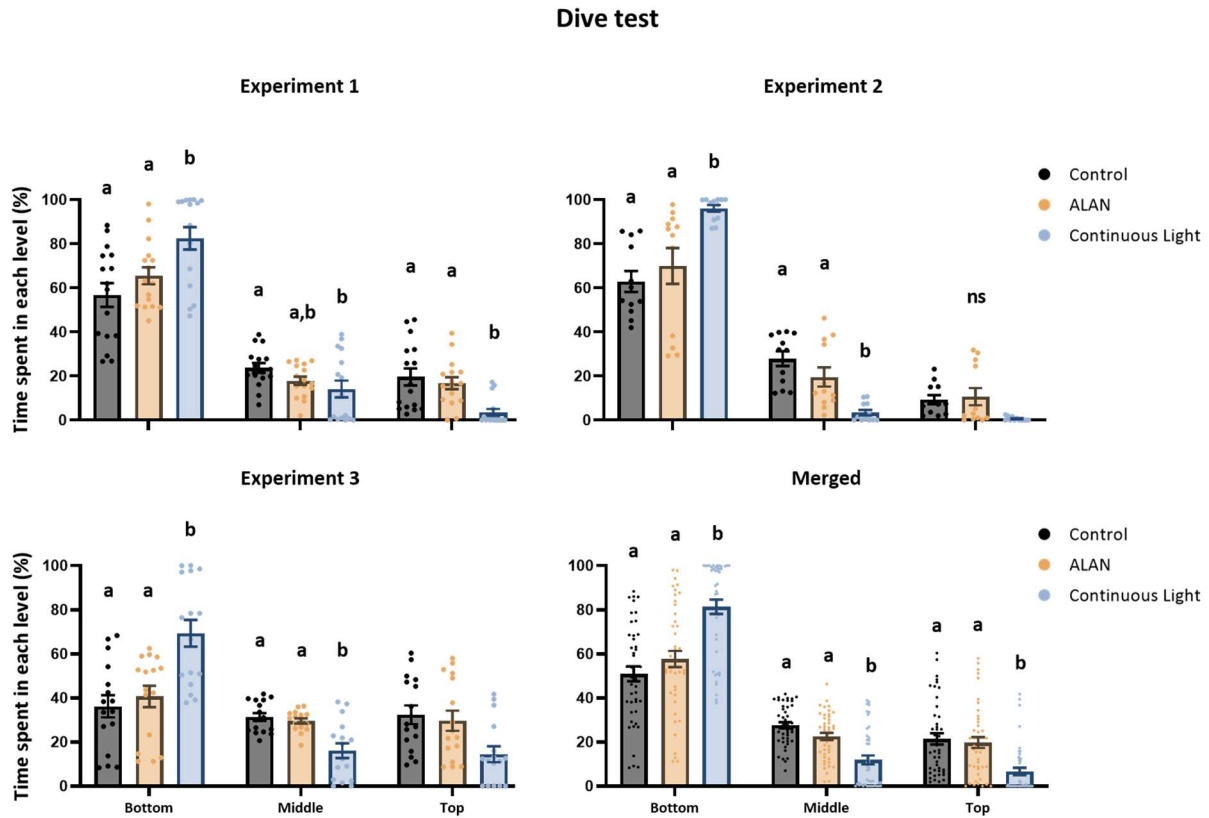
Figure 9. Dive test analyzing the time spent in three levels in the water column over 10 minutes in 3-month-old fish. Two-way ANOVA was used, analyzing one area at a time for the zones bottom ($F_{2, 123}$ = 28.14. $P < 0.0001$), middle ($F_{2,123}$, $p < 0.0001$) and top ($F_{2, 123}$, $p < 0.0001$). The data consists of three independent experiments and the n was 12 − 16 fish per group per experiment.

The dive test was repeated for six-month-old fish (Figure 16). Here, the fish showed a different behavior than for the three months old fish, with all groups spending above 80% of their time in the bottom area. In contrast to the findings for three months old fish, the control group spend significantly more time in the bottom area than the ALAN group. The continuous light group also spent less time in the bottom than the control group, but this difference was not significant.

Open field test

As the behavior was affected in the dive test, behavior was further studied by using the open field test. This test is often used to investigate signs of anxiety and differences in boldness. I analyzed three parameters; stationary status (percent of time spent moving less than 1.75 cm/s),

distanced moved (cm) and time in center (percent of time spent in the center area of the box). The control group fish was found to spend significantly less time stationary than both ALAN and continuous light (figure 10. a). "Distance moved" was significantly higher for control group fish than for both ALAN and continuous light (figure 10. b), which makes sense as these two factors most often go together. Surprisingly, even though the open field test is primarily used to look at the time spent in center (figure 10. c), as this can be seen as less anxiety-driven individuals, we found no significant difference between the groups when looking at the time spent in center ($F_{2, 68} = 0.9218$, *p=0.4027*).

Open field test
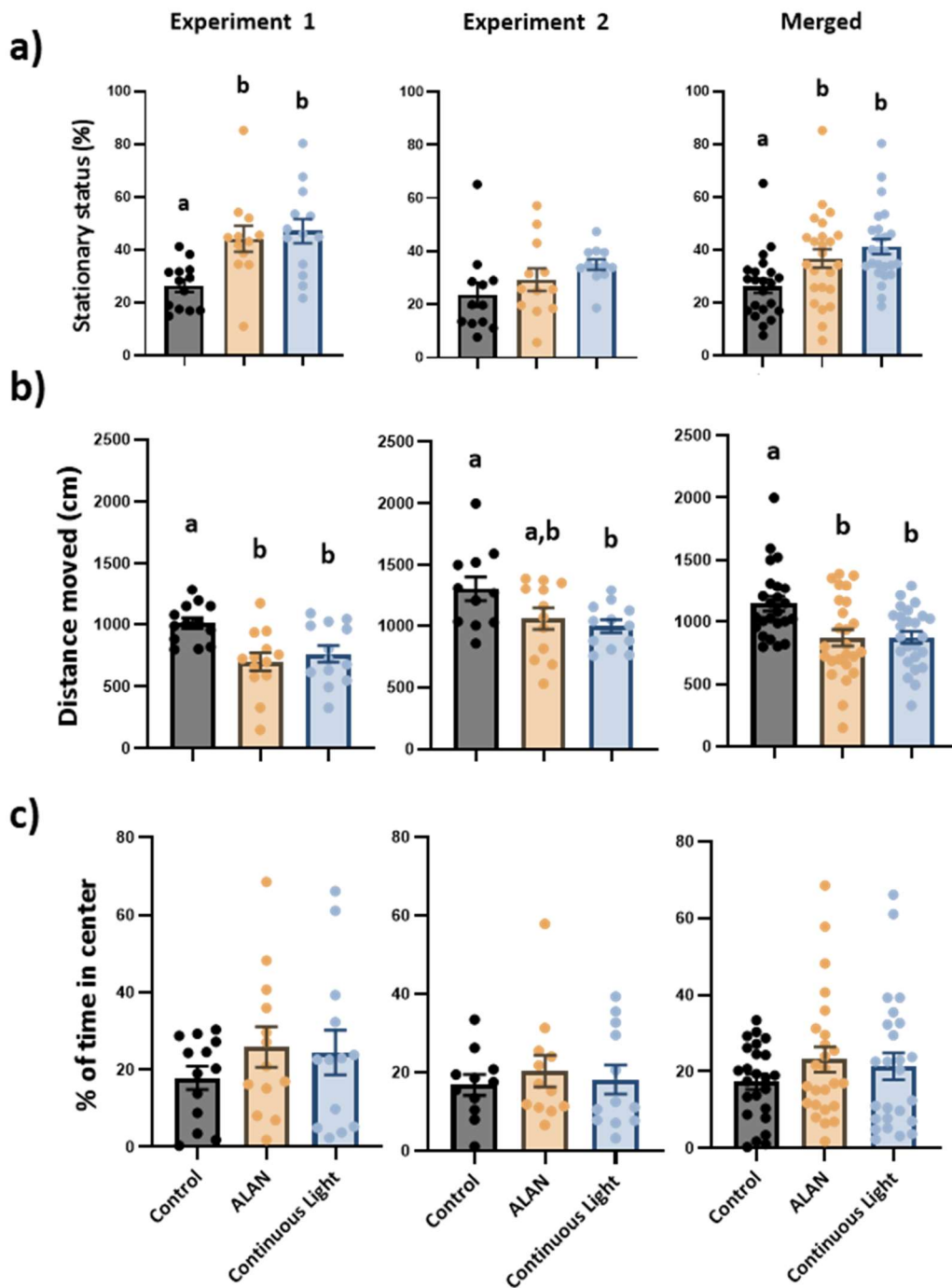
Figure 10. Open Field Test data are presented as mean ± SEM. Two-way ANOVA was used to check for light treatment differences taking both experiments into account. Significant differences are shown with different letters. The n was 11-13 for each experiment and treatment group.

The behavior of the six-month-old fish was also quantified by using the open field test (Fig. 17). Here, the behavior differed from the three-month-old fish. Continuous light was found to

spend significantly more time stationary than ALAN, with control group showing neither significance to ALAN nor continuous light. The distance moved and % of time in center did not differ between the groups. By looking at the % of time spent in center graph, we can see that there is a split in behavior for all groups, with approximately half of the fish spending most of their time in the center area, while the other half spend most of their time outside the center area.

3.4   Fish development

Gonadosomatic index (GSI)

When sampling fish, various measurements were taken, including total fish and gonadal weight, which were used to study the gonadosomatic index (GSI) as a sign of gonadal development and thus by extension reproductive activity.

Female GSI was not significantly affected by the light treatments. There was however, found a mild trend when studying the GSI for females at 3 months age (Fig. 11. a). Here we see that the control group GSI is lower than the other groups, with continuous light having the highest number of GSI.

Male GSI was not significantly affected at three months age (Fig. 11. c), but at 6 months age (Fig. 11. d), continuous light fish have a significantly higher GSI than the control group. Interestingly, the GSI of the ALAN group is higher than the control group, although not significantly so.
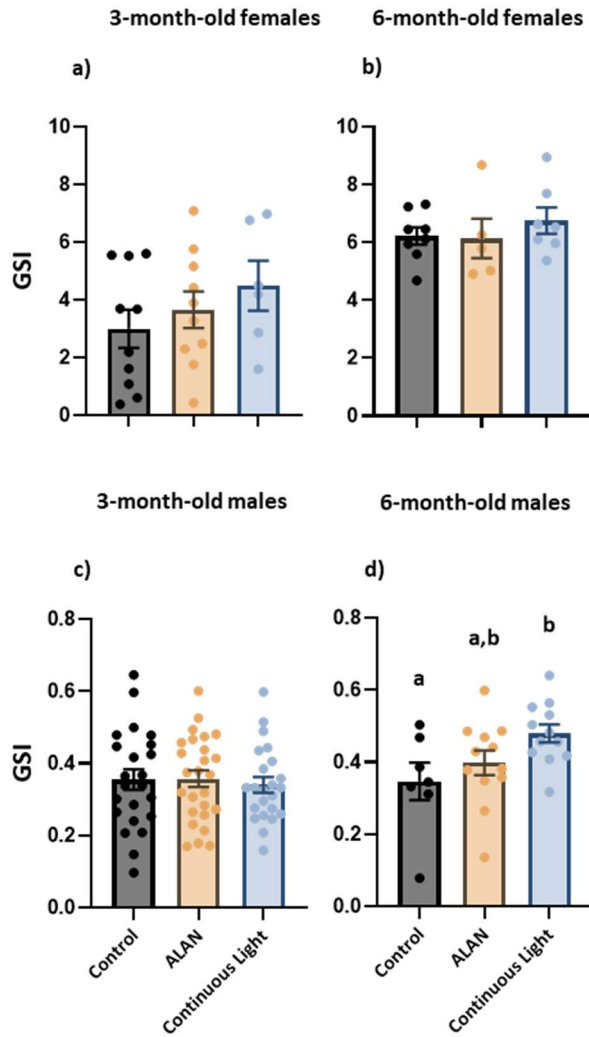
Figure 11. The GSI for the two genders at 3 month and 6-month age. Data are presented as mean ± SEM. For the three-month-old fish, two-way ANOVA was used considering the two genotypes WT and DTG. For GSI, only experiment 1 was used, as the values for experiment 2 were discarded due to unprecise measurement. For 6-month-old fish, one-way ANOVA was used. For females (Fig.11 a-b), at three months the n was 2-6 for each genotype and light treatment, and at 6 months the n was 5-8. ANOVA showed no significance for neither females at 3 months ($F_{2, 20}$ = 1.112, p=0.3483) nor 6 months of age. For males (Fig.11 c-d), the n ranged from 11-13 within each genotype at 3 months age, while the n was 7-12 at 6 months of age. ANOVA showed no significance for males at three months age ($F_{2, 66}$ = 0.1586, p=0.8537), but significance was detected at 6 months age ($F_{2, 28}$ = 3.46, p=0.045).

Weight, length, and condition factor for males

For 3-month-old male fish (Fig 12. a-c), weight and length were not affected by the light treatments. Despite this, continuous light fish were found to have a significantly higher condition factor than both ALAN and the control group.

For 6-month-old male fish (Fig. 12. d-f), not only the condition factor is significantly higher than the two other treatment groups but also the weight and size. Interestingly, ALAN seems to have higher weight, length, and condition factors in comparison to the control group, but no significant difference was detected. Taken together, these results suggest two things: six-month-old males in the continuous light regime are more affected than three-month-old males in the continuous light regime and, ALAN may influence growth but the relatively low n for six-month-old fish did not allow this reveal.

Weight, length and condition factor

3–month-old males
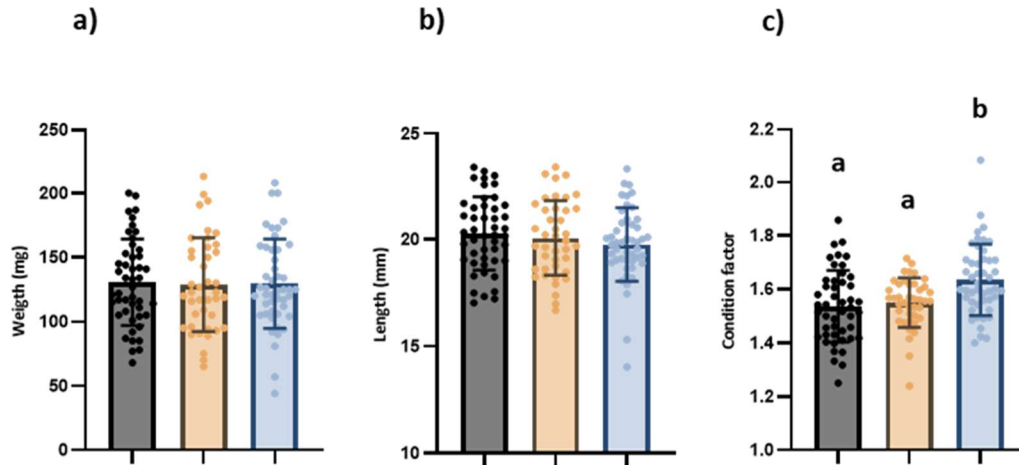
6–month-old males
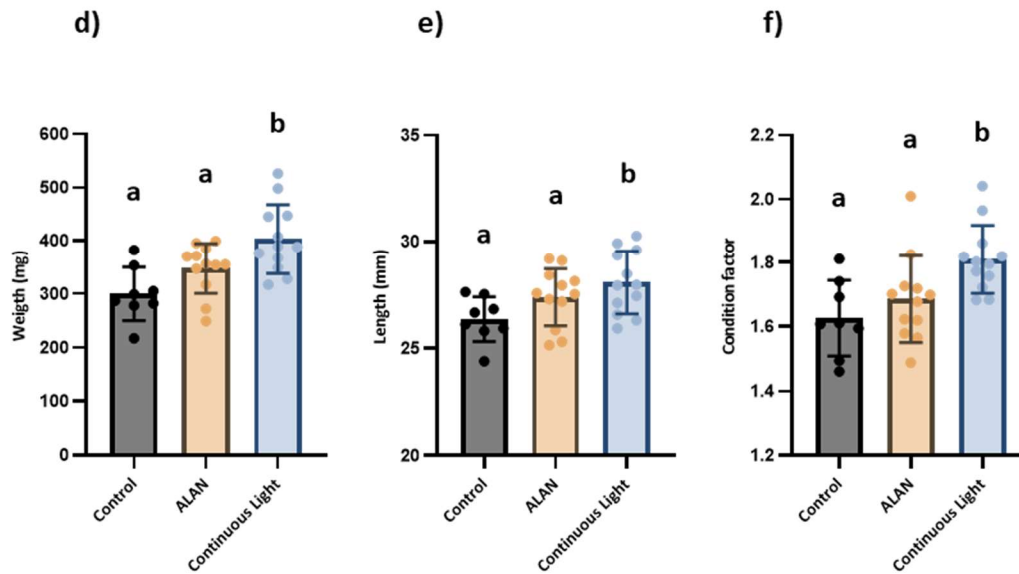
Figure 12. Weight, length, and condition factor data are shown as mean ± SD. For 3-month-old males (5.a-c) weight, length, and condition factor for 3-month-old males were merged as neither the genotype nor the experiment interacted with the effect on the light treatments. Two-way ANOVA shows that there is no significant difference between the light treatments when looking at weight (a) ($F_{2,123}=0.02507$, $p=0.9752$) and (b) length ($F_{2,123}=0.8234$, $p=0.4413$). The condition factor (c) was significantly affected ($F_{2,123}=5.792$, $p=0.0039$) by the light treatments. The n was between 6 and 16 for each treatment within each experiment separated for genotype. For 6-month-old-males (5.e-f) the N was between 8 and 12 per group. One-way ANOVA found significant light treatment effect for weight (d) ($F=8.65$, $p=0.001$), length (e) ($F=3.97$, $p=0.030$) and condition factor (f) ($F=6.22$, $p=0.006$).

The female growth parameters (Table 4) weight, length and condition factor were not significantly different for 3- and 6-month-old fish.

Table 4. Female growth parameters, including condition factor presented as mean ± SD, with one-way ANOVA used for 3-month-old females and two-way ANOVA used for 6-month-old females. For 3-month-old females the n ranged from 2-16 when divided by experiment and genotype.

Age	Parameter	Control	ALAN	Continuous Light	ANOVA	n
3 months	Weight	140 ± 6.79	135 ± 6.7	150 ± 9.44	$F_{2, 65} = 1.35$, *p=0.2664*	2-16
3 months	Length	20.6 ± 0.269	20.2 ± 0.3	20.8 ± 0.32	$F_{2, 65} = 1.602$, *p=0.2094*	2-16
3 months	Condition factor	1.58 ± 0.0274	1.6 ± 0.0237	1.63 ± 0.0365	$F_{2, 65} = 0.8583$, *p=0.4286*	2-16
6 months	Weight	312 ± 11.6	310 ± 26.2	293 ± 21.3	$F_{2, 17} = 0.325$, *p=0.727*	5-8
6 months	Length	26.5 ± 0.415	26.3 ± 0.482	25.3 ± 0.516	$F_{2, 17} = 2.00$, *p=0.166*	5-8
6 months	Condition factor	1.68 ± 0.0473	1.69 ± 0.0599	1.79 ± 0.0441	$F_{2, 17} = 1.78$, *p=0.199*	5-8

3.5 Brain morphology

Three-month-old fish

There were large experimental differences for three-month-old fish, with possible interaction effects presented.

When analyzing the females (Table 5), a tendency of difference in optic tectum length (a) between the control group and continuous light was seen, where the length for continuous light seems to be longer (p=0.0681). There was also a significant difference for the width of telencephalon (p=0.0061), but a stronger interaction effect (p=0.0006), telling us that there were differences between experiment 1 and 2 that suggest that the significance found, might be due to an experimental difference, and not due to the treatment of light. For the length of

telencephalon there was tendency of significance due to the light treatments (p=0.0787), but also an interaction effect (p=0.0888).

Table 5. Three-month-old females brain morphometry measured in µm. Data are presented as mean ± SEM.

Experiment	Measurement	Control	ALAN	Continuous Light	Two-way ANOVA		n
					Treatment	Possible Interaction	
1	a	10.9 ± 0.327	10.6 ± 0.304	11.7 ± 0.125	$F_{2,43}$ =2.999, *p=0.0604*		4-6
2	a	10.8 ± 0.356	11.3 ± 0.187	11.4 ± 0.223			8-16
1	b	18.6 ± 0.265	18.1 ± 0.399	19.6 ± 0.205	$F_{2,43}$ =1.794, p=0.1786		4-6
2	b	19.8 ± 0.328	19.9 ± 0.266	19.9 ± 0.439			8-16
1	c	11.9 ± 0.25	10.8 ± 0.229	12.9 ± 0.085	$F_{2,39}$ =5.830, **p=0.0061**	$F_{2,39}$ =8.939, **p=0.0006**	4-6
2	c	12.7 ± 0.264	12.9 ± 0.144	12.7 ± 0.304			8-12
1	d	8.6 ± 0.192	8.11 ± 0.189	9.04 ± 0.086	$F_{2,39}$ =2.715, *p=0.0787*	$F_{2,39}$ =2.578, *p=0.0888*	4-6
2	d	9.40 ± 0.252	9.08 ± 0.162	9.06 ± 0.177			8-12
1	e	4.48 ± 0.2212	3.92 ± 0.067	4.45 ± 0.1237	$F_{2,40}$ =0.9278, p=0.4038		4-5
2	e	4.48 ± 0.173	4.68 ± 0.188	4.75 ± 0.133			8-15
1	f	8.14 ± 0.211	8.28 ± 0.298	8.91 ± 0.193	$F_{2,42}$ =1.869, p=0.1668		4-6
2	f	8.75 ± 0.142	8.73 ± 0.125	8.70 ± 0.167			8-15
1	g	4.52 ± 0.106	4.53 ± 0.202	5.01 ± 0.152	$F_{2,37}$ =1.35, p=0.2718		4-6
2	g	5.13 ± 0.11	4.98 ± 0.093	4.95 ± 0.126			8-11
1	h	12.3 ± 0.135	12.2 ± 0.181	12.9 ± 0.249	$F_{2,44}$ =1.541, p=0.2256		4-6
2	h	12.9 ± 0.23	12.8 ± 0.187	13.1 ± 0.28			8-16

Analyzing the males (Table 6), the height of the telencephalon (g) was lower for ALAN than the control group and continuous light, but no significance was detected. Exploring the data, the biggest difference was found between ALAN and continuous light (p=0.057), while there was a smaller tendency of difference between the control group and ALAN (p=0.1259).

Table 6. 3-month-old males brain morphometry measured in µm. Data are presented as mean ± SEM.

Experiment	Measurement	Control	ALAN	Continuous Light	Two-way ANOVA Treatment	Possible Interaction	n
1	a	10.9 ± 0.203	11.2 ± 0.16	10.7 ± 0.233	$F_{2,66}$ =0.2482, $p=0.7809$		11-14
2		11.6 ± 0.198	11.1 ± 0.416	11.8 ± 0.236			7-16
1	b	18.9 ± 0.253	19.1 ± 0.213	18.7 ± 0.26	$F_{2,66}$ =0.8925, $p=0.4145$		11-14
2		20.2 ± 0.248	19.4 ± 0.650	20.6 ± 0.326			7-16
1	c	11.8 ± 0.374	12.2 ± 0.183	11.8 ± 0.248	$F_{2,58}$ =0.6348, $p=0.5337$	$F_{2,58}$ =4.443, $p=0.0160$	9-11
2		13.1 ± 0.181	12.2 ± 0.494	13.3 ± 0.169			7-15
1	d	8.75 ± 0.195	8.82 ± 0.146	8.43 ± 0.204	$F_{2,59}$ =0.7637, $p=0.4705$		10-11
2		9.42 ± 0.142	9.04 ± 0.303	9.31 ± 0.160			7-15
1	e	4.52 ± 0.174	4.31 ± 0.169	4.44 ± 0.159	$F_{2,60}$ =0.5931, $p=0.5558$		9-13
2		4.63 ± 0.151	4.60 ± 0.193	4.85 ± 0.179			8-13
1	f	8.36 ± 0.183	8.63 ± 0.14	8.21 ± 0.110	$F_{2,62}$ =0.05823, $p=0.9435$	$F_{2,62}$ =4.077, $p=0.0217$	10-13
2		9.00 ± 0.144	8.64 ± 0.298	9.17 ± 0.155			8-14
1	g	4.89 ± 0.21	4.76 ± 0.053	4.92 ± 0.158	$F_{2,51}$ =3.142, $p=0.0517$		8-10
2		5.24 ± 0.118	4.78 ± 0.206	5.34 ± 0.148			7-14
1	h	12.5 ± 0.213	12.4 ± 0.149	12.1 ± 0.22	$F_{2,67}$ =1.705, $p=0.1895$	$F_{2,67}$ =3.205, $p=0.0468$	11-14
2		13.2 ± 0.201	12.4 ± 0.371	13.3 ± 0.232			8-16

Six-month-old fish

The length of telencephalon (d) was found to be reduced for six-month-old females (Table 7) in ALAN and continuous light, while the telencephalon width (c) was not significantly affected.

Table 7. Six-month-old females brain morphometry measured in µm. Data are presented as mean ± SEM.

Measurement	Control	ALAN	Continuous Light	One-way ANOVA	n
a	14.0 ± 0.241	14.2 ± 0.157	13.9 ± 0.233	$F_{2,17}$ =0.438, $p=0.652$	5-8
b	24.4 ± 0.182	24.4 ± 0.196	24.4 ± 0.546	$F_{2,10.2}$ =0.0280, $p=0.972$	5-8

c	16.1 ± 0.356	15.8 ± 0.144	15.0 ± 0.375	$F_{2,15}$ =2.57, $p=0.110$	5-7
d	11.4 ± 0.171	10.6 ± 0.244	10.5 ± 0.142	$F_{2,15}$ =7.58, **$p=0.005$**	5-7
e	5.11 ± 0.229	5.58 ± 0.162	4.98 ± 0.127	$F_{2,15}$ =2.02, $p=0.167$	5-8
f	10.5 ± 0.121	10.5 ± 0.141	10.2 ± 0.251	$F_{2,9}$ =1.03, $p=0.382$	5-8
g	6.43 ± 0.245	6.16 ± 0.302	5.82 ± 0.246	$F_{2,9}$ =1.21, $p=0.343$	3-5
h	15.8 ± 0.1057	15.5 ± 0.0705	15.4 ± 0.2085	$F_{2,15}$ =1.74, $p=0.209$	4-7

For six-month-old males (Table 8), no significant difference was detected. There was a trend of difference (p=0.080) for the width of hypothalamus (h). Here the width was reduced for the control group, in relation to ALAN (p=0.107) and continuous light (p=0.114), but no significance was detected.

Table 8. 6 months old males brain morphometry measured in μm. Data are presented as mean ± SEM.

Measurement	Control	ALAN	Continuous Light	One-way ANOVA	n
a	14.3 ± 0.399	14.4 ± 0.217	14.8 ± 0.198	$F_{2,29}$ =1.13, $p=0.338$	8-12
b	24.7 ± 0.363	24.8 ± 0.237	25.4 ± 0.208	$F_{2,29}$ =2.01, $p=0.153$	8-12
c	16.5 ± 0.393	16.4 ± 0.164	16.7 ± 0.196	$F_{2,24}$ =0.517, $p=0.603$	6-12
d	11.3 ± 0.345	11.4 ± 0.134	11.4 ± 0.273	$F_{2,24}$ =0.015, $p=0.985$	6-12
e	5.43 ± 0.246	5.47 ± 0.202	5.50 ± 0.231	$F_{2,24}$ =0.0243, $p=0.976$	7-10
f	10.8 ± 0.286	10.7 ± 0.171	10.9 ± 0.120	$F_{2,26}$ =0.520, $p=0.601$	7-12

g	6.98 ± 0.264	6.77 ± 0.195	6.79 ± 0.201	$F_{2,19}$ =0.207, *p=0.815*	4-9
h	15.4 ± 0.231	16.1 ± 0.216	16.1 ± 0.219	$F_{2,27}$ =2.77, *p=0.080*	8-12

3.6 Heart morphology

As an indicator of cardiac function, I investigated several aspects of heart morphology.

Alignment of bulbus arteriosus

A good alignment of the bulbus arteriosus with the ventricle enables good blood flow. As there was no experimental nor genotype effect on the alignment of bulbus arteriosus, the three months old fish bulbus alignment is presented merged in one graph.

The bulbus alignment of three-month-old fish (Fig. 13. a) showed no significance between the light regimes. ALAN has a mild tendency of a higher bulbus alignment than the control group, but with no significance (p=0.2075). There is a higher visible spread of the data for continuous light fish than the control and ALAN group.

For six-month-old fish (Fig. 13. b), there is a significant effect on the light treatment, with continuous light having a significantly more angled bulbus than the control group (p=0.030). Although not significantly, ALAN seems to also have a more angled bulbus than the control group, suggesting a mild light treatment effect. It is worth noting that the control group had a very low n, because of a higher number of excluded heart pictures from analysis than ALAN and continuous light.
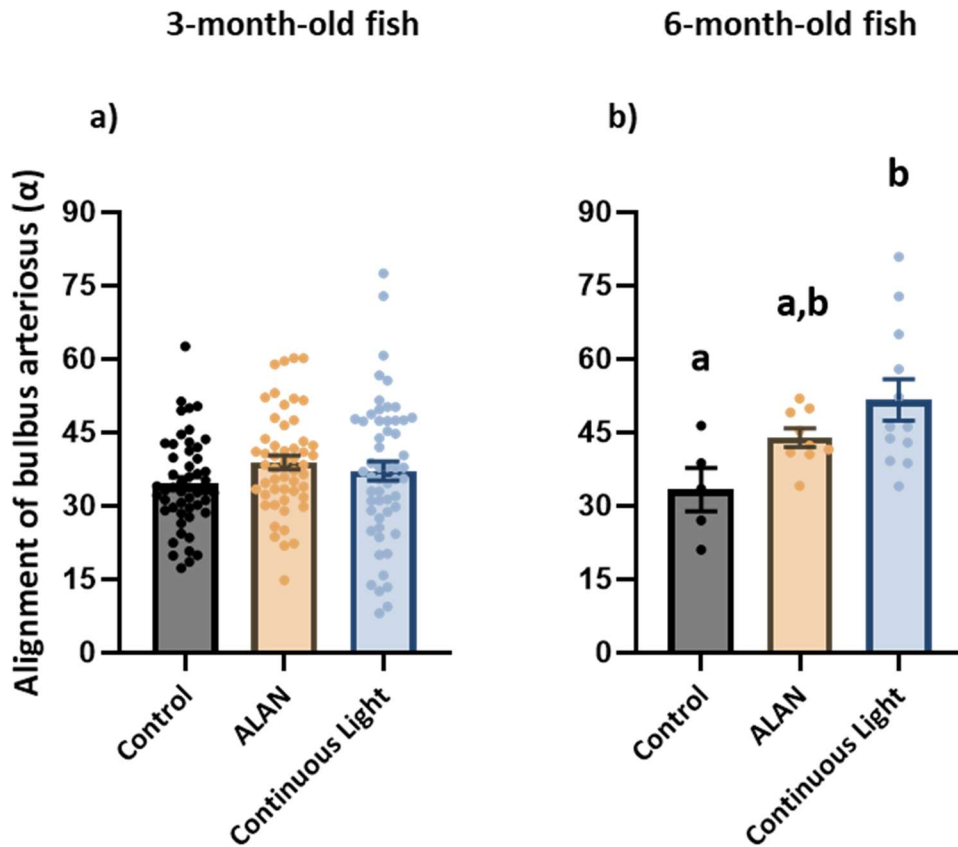
Figure 13. a) Effect of light regimes on the alignment of bulbus arteriosus in relation to the ventricle for 3 months old medaka pooled. The n was 8 to 17 for each genotype and experiment within the different light treatments. Two-way ANOVA ($F_{2,146}=1.454$, $p=0.2370$) took the genotype and experiment into accord but found no significance between the light regimes. A post-hoc test showed that the difference was largest between the control group and ALAN ($p=0.2075$). **b)** Effect of light regimes on the alignment of bulbus arteriosus in relation to the ventricle for 6 months old medaka. The n was 5-12. One-Way ANOVA ($F_{2,10.1}=4.25$, $p=0.046$), shows significance between the light regimes, with the continuous light being significantly different from the control group ($p=0.030$).

Ventricular height:width ratio

Ventricular height:width ratio is a way to measure the relative roundness of the heart, and was measured to look at reduced capability in pumping blood. As there was no experimental nor genotype effect the height:width ratio for the three-month-old fish is presented merged.

For three-month-old fish (Fig 14. a), it was found no significant light regime effect on the roundness of the ventricle. There was a slight tendency of continuous light having a higher height:width ratio than the ALAN group ($p=0.1338$), although not significant.

In six-month-old fish (Fig 14. b), continuous light hearts were found to be rounder than the other groups. Hearts from the continuous light regime were found to have a significant decrease in height:width ratio (p=0.032) in comparison to the control group, while a tendency of decrease was found when comparing continuous light to ALAN (p=0.070).

By comparing three- and six-month-old fish ventricular height:width ratio, we see a remodeling tendency as they grow older, for continuous light in comparison to ALAN. At three months age the continuous light hearts height:width ratio was higher, while at six months, the height:width ratio was almost significantly reduced.
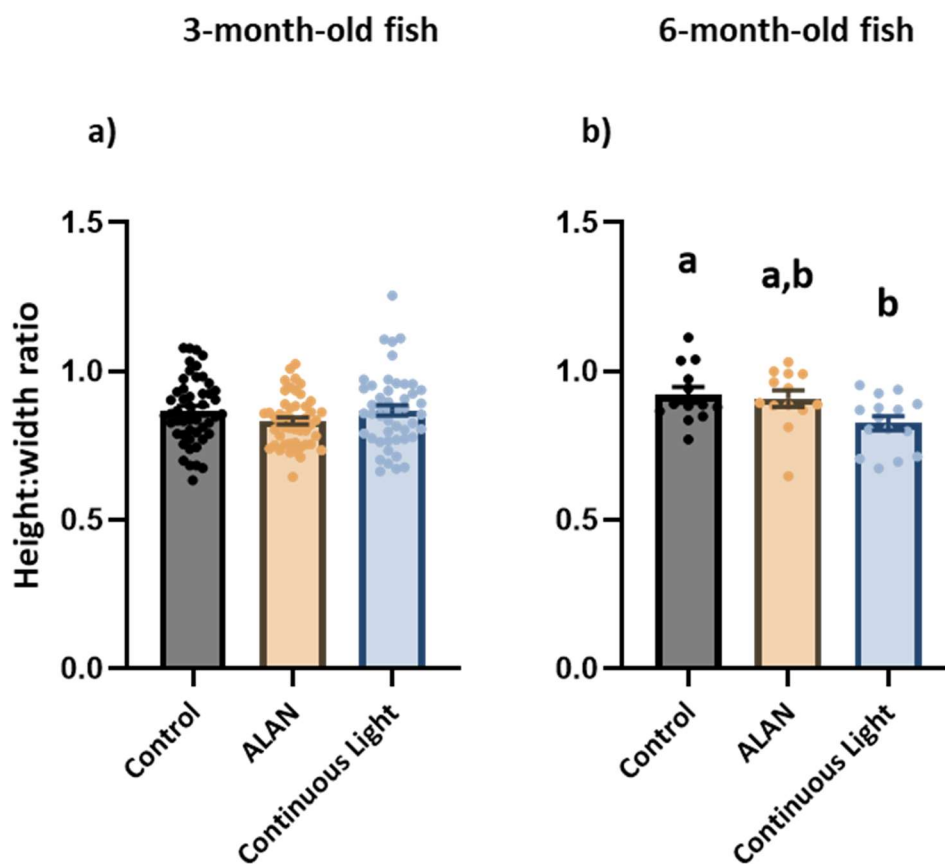
Figure 14. a) Height:width ratio of 3 months old medaka. The n ranged from 4 to 20 when for each experiment and genotype. Two-way ANOVA ($F_{2,135}$=1.875, p=0.1573) found no significant difference between the light regimes for at three-month age. **b)** Height:width ratio for 6 months old medaka. One-way ANOVA ($F_{2,38}$=4.19, p=0.023) found significant difference between the light regimes. Continuous light fish was found to have a significantly lower height:width ratio than the control group, while also having an almost significantly lower height:width ratio than ALAN (p=0.070).

3.7 Monoamine neurochemistry quantification

HPLC was run to look at possible brain chemistry difference between the light treatment groups, following the welfare research question in relation to the light regimes in aquaculture, but also wild fish. The HPLC analysis was also run to complement the behavior studied.

The descriptives and statistical analysis are presented in tables, for each brain part. The post hoc tests are presented in the appendix. In addition to the monoamine concentrations, the metabolite/parent monoamine-ratios are also presented, as they give information on the relative monoaminergic brain activity (elaborate in introduction).

In the telencephalon (Table 9), among all monoamines investigated only NE showed significant differences between the light treatment groups. The difference was investigated with a post hoc test, which found that there was a difference between the control group and ALAN, and a tendency of difference between the control group and continuous light Interestingly, a tendency of difference in 5-HT concentration was also found between ALAN and the control group.

Table 9. Monoamine concentrations (mean ± SEM) in the brain region telencephalon. Due to experimental differences from some monoamines, the data was analyzed with two-way ANOVA, detecting possible differences between the light regimes taking the two experiments into account. The outlier test was set to Q=5, and detected outliers were removed based on each treatment separated by experiment. F (a, b) is calculated, with a being the degrees of freedom, and b being the total n-1 per group. A significant p-value is marked bold. The interaction effect between experiment 1 and 2 are presented where significance is detected. This table information applies for the other brain regions.

Area	Control		ALAN		Continuous Light		Two-way ANOVA		
Telencephalon	Exp 1.	Exp 2.	Exp 1.	Exp 2.	Exp 1.	Exp 2.	Effect of experiment	Effect of light treatment	Interaction effect
5-HT	2935.75 ± 177.54	3090.43 ± 270.11	2633.7±242.89	2363.52 ± 200.554	3210.75±239.736	2405.18 ± 217.97	$F_{(1,62)} =$ 2.757, $P=0.1019$	$F_{(2,62)} =$ 2.772, $\underline{P=0.0703}$	
5-HIAA	161.78 ± 20.58	203.87 ± 35.15	181.31 ± 21.03	159.173 ± 16.27	178.69 ±20.05	142.3 ± 22.55	$F_{(1,62)} =$ 0.0826, $P=0.7748$	$F_{(2,62)} =$ 0.4422, $P=0.6447$	
5-HIAA/5-HT	0.0496 ± 0.0039	0.06454 ± 0.0099	0.0634 ± 0.0047	0.0674 ± 0.0047	0.0590 ± 0.0043	0.0559 ± 0.0051	$F_{(1,63)} =$ 1.359, $P=0.2481$	$F_{(2,63)} =$ 1.498, $P=0.2315$	
DA	113.1 ± 12.08	136.79 ± 5.24	107.67 ± 15.03	132.437 ± 13.2	106.74 ± 8.84	145.03 ± 9.21	$F_{(1,59)} =$ 7.823, $\textbf{\textit{P=0.0070}}$	$F_{(2,59)} =$ 0.1285, $P=0.8797$	
DOPAC	6.668 ± 0.873	12.43 ± 2.956	6.328 ± 1.127	9.584 ± 2.13	8.429 ± 1.787	9.699 ± 1.94	$F_{(1,60)} =$ 5.526, $\textbf{\textit{P=0.0220}}$	$F_{(2,60)} =$ 0.4512, $P=0.6390$	
DOPAC/DA	0.0728 ± 0.0111	0.0702 ± 0.0097	0.0503 ± 0.0044	0.0733 ± 0.0126	0.0622 ± 0.01	0.0651 ± 0.0129	$F_{(1,57)} =$ 0.8093, $P=0.3721$	$F_{(2,57)} =$ 0.5034, $P=0.6071$	

| NE | 3103.64 ± 224.07 | 3680.95 ± 251.046 | 2391.51 ± 271.493 | 2780.09 ± 328.548 | 2799.01 ± 194.657 | 2854.42 ± 169.252 | $F_{(1,63)} =$ 2.671, *P=0.1072* | $F_{(2,63)} =$ 5.549, *P=0.0060* | |

For hypothalamus (Table 10), like for telencephalon, 5-HT and NE concentrations showed significant differences between the control and the ALAN group. In addition, 5-HT levels were also significantly different between the control and continuous light group. The NE concentration of NE was significantly lower for the ALAN group in comparison to the control group. Continuous light seems to be lower, but no significance was detected (p=0.1212). Finally, the concentration of DA was also found to be significantly different between the control and ALAN group, while the 5-HIAA/5-HT was significantly different between ALAN and the continuous light group. Interestingly, the concentration of 5-HIAA shows a tendency of significance between the control and continuous light group.

Table 10. Monoamine concentrations (mean ± SEM) in the brain region hypothalamus.

Area	Control		ALAN		Continuous Light		Two-way ANOVA		
Hypothalamus	Exp 1.	Exp 2.	Exp 1.	Exp 2.	Exp 1.	Exp 2.	Effect of experiment	Effect of light treatment	Interaction effect
5-HT	1178.95 ± 85.23	5021.5 ± 668.133	1181.32 ± 124.00	3430.08 ± 370.401	1408.84 ± 123.95	3510.55 ± 253.20	$F_{(1,54)} =$ 166.2, *P<0.0001*	$F_{(2,54)} =$ 5.204, *P=0.0086*	$F_{(2,54)} =$ 6.881, *P=0.0022*
5-HIAA	102.72 ± 9.92	296.28 ± 43.21	94.61 ± 10.07	242.63 ± 35.11	113.04 ± 14.34	190.57 ± 22.39	$F_{(1,55)} =$ 68.64, *P<0.0001*	$F_{(2,55)} =$ 2.772, *P=0.0713*	$F_{(2,55)} =$ 4.067, *P=0.0225*
5-HIAA/5-HT	0.0783 ± 0.0025	0.0590 ± 0.0040	0.0807 ± 0.0032	0.0695 ± 0.0031	0.0746 ± 0.0030	0.0534 ± 0.0039	$F_{(1,54)} =$ 39.29, *P<0.0001*	$F_{(2,54)} =$ 5.507, *P=0.0067*	
DA	155.21 ± 16.39	662.44 ± 91.35	163.66 ± 21.42	417.83 ± 74.23	176.14 ± 21	562.46 ± 77.01	$F_{(1,54)} =$ 101.1, *P<0.0001*	$F_{(2,54)} =$ 3.274, *P=0.0455*	$F_{(2,54)} =$ 3.625, *P=0.0333*
DOPAC	15.19 ± 1.06	37.35 ± 6.98	16.68 ± 1.46	23.89 ± 3.64	18.77 ± 2.47	21.02 ± 3.71	$F_{(1,53)} =$ 17.41, *P=0.0001*	$F_{(2,53)} =$ 2.624, *P=0.0819*	$F_{(2,53)} =$ 5.567, *P=0.0064*
DOPAC/DA	0.1113 ± 0.0077	0.0606 ± 0.0133	0.1072 ± 0.0103	0.0588 ± 0.0046	0.1107 ± 0.0089	0.0369 ± 0.0056	$F_{(1,55)} =$ 54.22, *P<0.0001*	$F_{(2,55)} =$ 0.9047, *P=0.4106*	
NE	1121.69 ± 84.16	4656.72 ± 764.64	1038.8 ± 99.50	2992.69 ± 391.42	1205.83 ± 117.08	3379.27 ± 407.91	$F_{(1,54)} =$ 109.3, *P<0.0001*	$F_{(2,54)} =$ 4.381, *P=0.0172*	$F_{(2,54)} =$ 4.048, *P=0.0230*

In the optic tectum (Table 11), significant differences were found in the concentrations of DA and NE, and the 5-HIAA/5-HT ratio. A significant difference for the 5-HIAA/5-HT ratio was found between the control group and ALAN. For DA, a significant difference was detected for both ALAN and continuous light, in relation to the control group. Lastly, the NE concentration

was found to be significantly different for the control group in relation to both ALAN and continuous light.

Table 11. Monoamine concentrations (mean ± SEM) in the brain region optic tectum.

Area	Control		ALAN		Continuous Light		Two-way ANOVA		
Optic tectum	**Exp 1.**	**Exp 2.**	**Exp 1.**	**Exp 2.**	**Exp 1.**	**Exp 2.**	**Effect of experiment**	**Effect of light treatment**	**Interaction effect**
5-HT	479.70 ± 26.34	893.8 ± 23.02	445.44 ± 26.95	946.75 ± 144.82	436.51 ± 24.53	876.3 ± 66.01	$F_{(1,61)} =$ 103.5, *P<0.0001*	$F_{(2,61)} =$ 0.2917, *P=0.7480*	
5-HIAA	132.2 ± 7.54	92.31 ± 9.02	145.94 ± 11.72	104.96 ± 16.62	121.01 ± 5.47	93.14 ± 11.63	$F_{(1,65)} =$ 17.20, *P<0.0001*	$F_{(2,65)} =$ 1.504, *P=0.2298*	
5-HIAA/5-HT	0.258 ± 0.0154	0.0958 ± 0.0063	0.3133 ± 0.0155	0.1175 ± 0.008	0.2802 ± 0.0085	0.1053 ± 0.0104	$F_{(1,66)} =$ 280.1, *P<0.0001*	$F_{(2,66)} =$ 4.561, *P=0.0139*	
DA	68.91 ± 7.48	45.27 ± 4.74	54.40 ± 4.66	32.36 ± 7.1	53.10 ± 3.82	31.55 ± 4.35	$F_{(1,66)} =$ 22.88, *P<0.0001*	$F_{(2,66)} =$ 4.378, *P=0.0164*	
DOPAC	23.25 ± 3.1	7.153 ± 2.15	20.39 ± 2.06	9.209 ± 1.81	19.46 ± 1.9	4.168 ± 0.82	$F_{(1,47)} =$ 69.85, *P<0.0001*	$F_{(2,47)} =$ 1.546, *P=0.2237*	
DOPAC/DA	0.349 ± 0.0788	0.1828 ± 0.0469	0.4201 ± 0.0599	0.2686 ± 0.0456	0.3796 ± 0.0548	0.2022 ± 0.0347	$F_{(1,50)} =$ 14.11, *P=0.0005*	$F_{(2,50)} =$ 1.107, *P=0.3386*	
NE	1754.44 ± 113.24	1913.08 ± 80.99	1279.97 ± 86.66	1365.83 ± 165.63	1254.64 ± 93.76	1268.52 ± 73.65	$F_{(1,65)} =$ 1.040, *P=0.3115*	$F_{(2,65)} =$ 19.38, *P<0.0001*	

In the brain stem (Table 12), significant differences between the light condition treatments were found for the ratio of 5-HIAA/5-HT, and the concentrations of DA and NE. Despite the significant difference in the 5-HIAA/5-HT ratio, no difference was found with the post hoc. There was however a tendency in difference between ALAN when comparing to both the control and continuous light group. For NE, it was found that the control group was significantly higher for both ALAN and continuous light. For DA, the concentration was significantly different between ALAN and the control group.

Table 12. Monoamine concentrations (mean ± SEM) in the brain region hypothalamus.

Area	Control		ALAN		Continuous Light		Two-way ANOVA		
Brain stem	**Exp 1.**	**Exp 2.**	**Exp 1.**	**Exp 2.**	**Exp 1.**	**Exp 2.**	**Effect of experiment**	**Effect of light treatment**	**Interaction effect**

5-HT	685.21 ± 25.89	1800.45 ± 121.30	611.91 ± 21.92	1582.26 ± 100.28	694.47 ± 64.6	1458.11 ± 125.94	$F_{(1,62)}$ = 205.6, **P<0.0001**	$F_{(2,62)}$ = 2.390, P=0.1000	
5-HIAA	132.35 ± 10.28	143.44 ± 16.36	140.5 ±9.59	124.33 ± 10.65	120.75 ± 10.74	113.68 ± 15.11	$F_{(1,64)}$ = 0.1676, P=0.6836	$F_{(2,64)}$ = 1.488, P=0.2334	
5-HIAA/5-HT	0.1913 ± 0.0106	0.079 ± 0.0055	0.2378 ± 0.0119	0.0779 ± 0.0044	0.1986 ± 0.0115	0.0747 ± 0.0057	$F_{(1,66)}$ = 262.2, **P<0.0001**	$F_{(2,66)}$ = 3.394, **P=0.0395**	$F_{(2,66)}$ = 3.215, **P=0.0465**
DA	271.59 ± 21.19	173.62 ± 21.57	188.64 ± 16.45	158.2 ± 7.71	235.17 ± 17.14	162.94 ± 13.54	$F_{(1,64)}$ = 21.08, **P<0.0001**	$F_{(2,64)}$ = 3.863, **P=0.0261**	
DOPAC	36.24 ± 2.89	7.03 ± 1.3	28.69 ± 2.53	10.54 ± 2.39	33.1 ± 3.98	10.21 ± 2.4	$F_{(1,62)}$ = 93.33, **P<0.0001**	$F_{(2,62)}$ = 0.3401, P=0.7130	
DOPAC/DA	0.1367 ± 0.009	0.0467 ± 0.0082	0.151 ± 0.0166	0.0755 ± 0.0166	0.141 ± 0.0099	0.059 ± 0.0128	$F_{(1,63)}$ = 51.84, **P<0.0001**	$F_{(2,63)}$ = 1.223, P=0.3012	
NE	1578.31 ± 72.19	2199.99 ± 159.96	1359.02 ± 37.77	1606.37 ± 103.4	1379.81 ± 63.12	1563.55 ± 95.23	$F_{(1,61)}$ = 22.86, **P<0.0001**	$F_{(2,61)}$ = 13.78, **P<0.0001**	$F_{(2,61)}$ = 3.383, **P=0.0404**

3.8 qPCR

To study relative expression housekeeping genes must be selected. The housekeeping genes *rpl7* and *18s* both have low standard deviation in comparison to *gapdh* (Table 23). However, as it is not recommended to use a housekeeping gene with too high expression (low Cq-number), relative expression was calculated based on rpl7. For gene expression, it is worth noting the low number of females. This was because there was a skewed sex-ratio when sampling.

lhb was significantly affected by the light treatments in females (Fig. 15). Here, the continuous light expression level was found to be significantly decreased in comparison to the control group. Interestingly, we see the expression level of the ALAN group being in the middle.

For males, the continuous light group was found to have a bigger relative expression in *tshba* than the other groups (Fig. 15). However, only the ALAN group was found to have a tendency of significance when compared to the continuous light group (p=0.092).

One-way ANOVA showed significance between the groups for the expression levels of *sl*. As we can see, the control group appears to be lower than the ALAN and continuous light groups (Fig. 15). A post hoc test, however, found no significant difference of expression levels when comparing the control group to ALAN (p=0.107) and continuous light (p=0.151). The complete gene expression dataset can be found in the appendix.
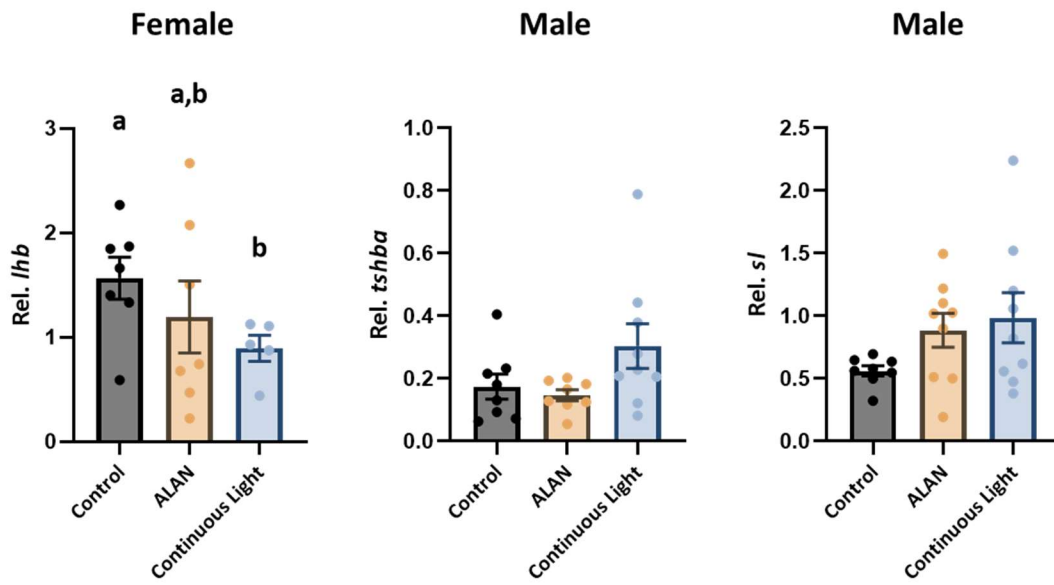
Figure 15. Relative mRNA levels to the housekeeping gene *rpl7* of *lhb, tshba* and *sl*. Data are presented as mean ± SEM.

4. Discussion

This thesis will try to identify the effects of light pollution and the use of continuous light in aquaculture on fish, by following the presented hypotheses.

4.1 Light measurement in the field

Light pollution was found to affect almost all the sampling points and depths studied. Underwater light pollution was most prevalent where the lights were facing the water (e.g. under bridges). The highest light pollution levels were however detected in the harbor area.

Interestingly, a high difference between the main river and the harbor in underwater light pollution was seen. To my knowledge this is the first study that has detected a difference in underwater light pollution between urban rivers and coastal waters. Studies have found that differences in penetrative light vary because of changes in turbidity, rather than light reaching the water surface. The water turbidity is found to mainly originate from circulating inorganic matter, which mostly cause turbidity when stirred up and detached from the sediment (Chandler, 1942). This may be an explanation for the comparatively lower light pollution values in the river. It is also worth mentioning that another study found that instead of inorganic matter - phytoplankton had the highest effect of underwater light extinction (Cristofor et al., 1994), suggesting that light pollution will be lowered in periods of algal blooms. This study points towards the fact that water bodies are varied depending on the type and location.

Light pollution was worryingly also measured at one point at 5 meters depth. Because of this, light pollution not only affects aquatic life under the surface, but also deeper in the water column. This study especially fills a gap in how rivers are affected by light pollution (Jechow & Hölker, 2019).

4.2 Investigation of reproductive timing

The reproductive cycle was found to be desynchronized for ALAN and continuous light, with a higher effect of desynchronization for the continuous light group. As light pollution has been shown to disrupt the natural melatonin balance (Brüning et al., 2015), it has been proposed that light pollution can indeed disturb biological rhythms. In the wild, medaka are breeding seasonally, with no spawning during the winter period (Chan, 1976). In a laboratory setup, however, medaka can spawn daily (Hamilton et al., 1969; Kinoshita et al., 2009), as the light and temperature can be adjusted. In addition to having a circadian rhythm conditioned by light

regimes, medaka are hypothesized to be dependent on visual cues to breed, as they need visual cues to habituate in a new environment (Matsunaga & Watanabe, 2010). This study proves that light pollution and continuous light disturbs the spawning rhythms for medaka and should be replicated in the wild. Whether the desynchronized spawning is due to the addition of light and thus allowing the medaka to see, or the disruption of their circadian clock should be assessed in the future.

4.3   Behavior

Dive test

For three-month-old fish, fish from the continuous light regime spent significantly more time in the bottom area of the tanks in comparison to both control and ALAN fish. It is worth noting that, although not significantly different, for each separate experiment, the ALAN fish spend more time in the bottom area of the tanks.

For six-month-old fish, the behavior was altered, with all fish spending more than 80% of their time in the bottom zone. It is not uncommon that behavior changes as fish gets older, but it might also be due to individual variation. As six-month-old fish were only tested once, the variation might dominate the potential light treatment effect. It is also worth mentioning that the fish tend to school as they were released in the Dive Test as a group, making the individual points tested less statistically powerful. The emphasis will therefore be put on the younger fish in this study.

Towards the top of the water column there is a higher predation risk, but also normally more food for fish. Because of this, it is thought that there is a trade-off between predation risk and successful feeding when nearing the top of the water column (Oppedal et al., 2001). These results fit into one study on 1 month old Atlantic salmon fry, as they were found to use water column differently. What was found was that the Atlantic salmon from the control group and low continuous light was distributed higher in the water column than the high continuous light group (Stefansson et al., 1990). There was a graphical difference from their control group and continuous low light, but this difference was not significant, same as my study. That the two different fish species show the same behavior is interesting. Whether the reason for the altered

In compliance with the former study (Stefansson et al., 1990), my study show that fish have a dark nighttime seem to be more active at daytime, spending more time towards the top of the water column in comparison to the fish exposed to continuous light. Whether the reason for this

altered behavior is anxiety or a desynchronized circadian rhythm linked to the photoperiod should be further assessed.

Open field test

For three-month-old-fish, the open field test found no difference between the light treatment groups when assessing the time spent in the center, which is the main goal of the open field test, suggesting that anxiety levels are not significantly altered for the fish exposed to altered light regimes. Despite this, the control group fish were found to swim a longer distance and spend less of their time stationary, in comparison to both ALAN and continuous light. In a the Open Field Test swimming is considered to be an exploratory behavior, and is found to be inversely correlated with the stationary status (Warren & Callaghan, 1976), as also detected by this study. The younger fish in this experiment were found to have their explorative behavior reduced. If this is also true for wild fish, should be tested, and if found true it could have large ecological implications. As the time spent in the middle zone was not different between the groups, it is not possible to know whether the fish in the altered light regimes are more anxious. There was also a change in behavior for the ALAN group at six months, where they spent significantly less time stationary than the continuous light group. It can therefore be hypothesized that artificial light might impair natural behavior at a younger age, but that the impaired behavior is gone when the fish gets older. The reason for this should be further investigated. A reason could be the drop in light after four months age, as the ALAN levels for this behavior might lay somewhere between 0.223 and 0.126 μmol m^{-2} s^{-1} as the behavior was changed. Another reason for the change in behavior could also be that the fish found a way to cope with light pollution levels. Still, continuous light fish showed signs of impaired behavior at both ages tested, which could have welfare implications.

4.4   Fish development

GSI and growth

The GSI showed a trend for both three-month-old females and six-month-old males, where the control group had a lower GSI in relation to the other light regimes, with continuous light having the highest. This trend was not significant for the three-month-old females, possibly due to a relatively low number of n for each genotype and light treatment (2-5), and if repeated significance could be detected. For six-month-old males, significance was found between

continuous light and the control group. The light regimes effect on gonadal development for males should be further studied for six-month-old males, because of this finding. In another study medaka's response on light pollution (Closs et al., 2023), light pollution was found to increase the fertilization success of dominant males, and thus lowering the reproductive success of subordinate males. The GSI, however, was not significantly different between fish affected by light pollution at night and the control group. In the experiment on male medaka reproductive success, fish were exposed to the different light regimes only for two weeks, while my fish were exposed for the light regimes since the larvae spawned. Because of this, it makes sense that the GSI for six-month-old males are more affected.

For females specifically, GSI is found to increase in the hours leading towards ovulation (Yoneda et al., 2013), and the tendency of difference in GSI for the three-month-old females can be argued to be because of the desynchronized spawning detected. As medaka spawn daily in our laboratory setup, and the data consist of fish sampled at midnight and from 8 to 12, continuous light fish might have a higher GSI because of the coming ovulation.

My study found that males in the continuous light regime have a higher condition factor at both three- and six-months age. Additionally, continuous light has a higher weight and length than the control group and ALAN at 6 months age. It is also worth mentioning that the ALAN group had a graphically visible higher weight, length, and condition factor for six months old fish, although not significantly so. Extended light regimes are known to be used to increase growth (Berg et al., 1992) in aquaculture. In our setup, fish were only fed when the light was on for all light regimes. This tells us that the extended light regime increased the feed conversion in the continuous light regime. This is in compliance with studies on green sunfish (*Lepomis cyanellus*) (Gross et al., 1965) and rainbow trout (*Oncorhynchus mykiss*) (Mäkinen & Ruohonen, 1992). These studies showed that the feed conversion rate was more successful for extended photoperiods. Growth hormone (GH) levels has been found to be increased in the freshwater phase for Atlantic salmon (*Salmo salar*) reared in a continuous light regime, paired with increased growth (Stefansson et al., 1991). The fact that extended light regimes promote the production of GH and thus give an increase in both growth and feed conversion, could explain the results found by this study.

Three-month-old-females were not affected by the extended light regime and might point towards a sex difference on the growth effects of extended light regimes. For six-month-old females, the continuous light seems to give a higher condition factor. The possible difference was not found significant and should be further investigated in further studies.

Brain morphology

Medaka have been found to have differences in size of brain parts based on genotype. It is also stated that there is high probability that brain size can be affected by environmental factors, such as altering the light dark cycle (Ishikawa et al., 1999).

For three-month-old females brain morphology was almost significantly changed for the optic tectum, with continuous light having an almost significantly longer optic tectum than the control group. The main function of the optic tectum is to process sensory, and mainly visual, inputs (Nguyen et al., 1999). As these fish were exposed to light continuously, this might be the cause for the long optic tectum tendency.

For three-month-old males, ALAN had lower telencephalon height than the control and continuous light groups, but no significance were detected. Surprisingly, the biggest difference was found to be between ALAN and continuous light. Telencephalon is a brain part that is essential for stress-regulation, but also for emotional learning and decision-making (Kotrschal et al., 1998). As six-month-old females in the ALAN and continuous light groups were found to have a shorter telencephalon length than the control group, these groups might be more affected by stress, or at least have difficulty by coping with it. Though there was treatment effect on the three-month old females, the data were inconsistent from experiment 1 to 2, making these results unreliable. For three-month-old males, the ALAN group had a lower telencephalon height than the control and continuous light groups. The continuous light fish did not have differing telencephalon height from the control group. As the telencephalon was affected by both males and females, the light treatment effect on telencephalon should be further investigated.

Heart morphology

Studying the alignment of the bulbus arteriosus in relation to the ventricle, found a higher degree in bulbus alignment for ALAN and continuous light than the control group. For three-month-old fish, ALAN and continuous light both experienced an increase in the bulbus alignment, although the difference was not significant. ALAN was found to have the highest difference from the control group with a p-value of 0.070 signifying a tendency of higher degree of bulbus alignment.

For 6-month-old fish, there was a significantly higher degree of bulbus alignment for the continuous light group, suggesting that over longer time, the light has a stronger effect on the morphologically development of the heart. ALAN also has a higher mean in bulbus alignment than the control group, although not significant (p=0.155). It is worth noting that the n for the control group was much lower than the continuous light group. When analyzing the pictures, hearts with an enlarged bulbus were hard to analyze and therefore discarded from analysis. First off, this makes it hard to be sure about the data for the 6-month-old bulbus alignment. It may also say something about the hardness of the bulbus arteriosus, as the continuous light hearts were easier to dissect, possibly due to a change in tissue development. Studies that follow up this, should assess the light treatment effect of composition of the bulbus arteriosus and the ventricle.

For three months old fish, no difference in the height:width ratio was found, possibly due to a detected experimental difference. For 6-month-old fish, however, continuous light hearts were found to have significantly lower height:width ratio than the control fish hearts, while ALAN had a tendency of significantly lower height:width ratio (p=0.070) than the control group. The height:width ratio was also analyzed, to look at the ventricular roundness. In fish, the ventricle is pyramidical, and thereby supplies greater ventricular pump, which results in better blood flow (Sanchez-Quintana et al., 1995). A rounder ventricle could therefore have a negative impact on the blood flow for fish exposed to light pollution and continuous light.

The bulbus alignment and roundness of the hearts have previously been studied, comparing farmed and wild rainbow trout and Atlantic salmon (Brijs et al., 2020; Frisk et al., 2020; Poppe et al., 2003). The farmed salmonids have a higher degree of bulbus alignment and an increased roundness than the wild salmonids. In addition, the same tendencies have been found when comparing slow and fast-growing smolts, where fast-growing smolts have a higher degree of bulbus alignment and increased roundness than slow-growing smolts. This study is however the first to study the light factor separately, with continuous light affecting the hearts in the older fish, while a tendency is seen for younger fish. The increase in roundness was not seen until 6 months, suggesting that the changed bulbus alignment starts to form earlier. In this study it is proposed that the misalignment of the bulbus results from the altered heart shape, but my findings contradict this, as the misalignment of the bulbus appeared earlier than the increased roundness. It is also highly possible that there are species-differences in the alteration of the heart, as this is the first study on the morphology of medaka hearts.

Farmed fish are raised with the goal of rapid growth, and the farming conditions are harsh, as the fish are raised with extended light regimes, increased temperature, increased feeding and are subjected to selective breeding with the goals of rapid growth, nice flesh color and late sexual maturation (Poppe et al., 2003). By looking on only light as a separated factor, it is interesting to see its prevalent effects on heart development.

4.5   Monoamine quantification in the brain

An increase in serotonergic and dopaminergic activity is linked to stress, and gilthead seabream (*Sparus aurata*) exposed to chronic stress at an early life stage was found to have both a higher ratio of 5-HIAA/5-HT and DOPAC/DA (Vindas et al., 2018).

Serotonergic activity

In addition to serotonin regulation being important to biological reactions, for example energy regulation and neural plasticity (Andrews et al., 2015; Lanfumey et al., 2008), sustained serotonergic activity in the brain is associated with chronic stress, which can induce pathologies, such as depression-like states. To investigate the research question, that fish exposed to artificial light treatments are exposed to chronic stress, after the findings in the behavioral data presented, sustained serotonergic activity is best measured by either an increase in 5-HT's metabolite 5-HIAA or the 5-HIAA/5-HT ratio (Shannon et al., 1986).

As the 5-HIAA/5-HT ratio was found to be higher for the ALAN-group in the brain stem, hypothalamus, and the optic tectum. For the areas mentioned, ALAN had at least a higher 5-HIAA/5-HT ratio than one of the other groups. This suggests that ALAN had an increased serotonergic activity than the other groups, although not significant towards both in all brain area.

These findings suggest that the ALAN group are indeed chronically stressed. This is surprising, as the continuous light fish are raised in a more extreme light regime. The reason for this effect is unsure and should be further studied. That ALAN are chronically stressed is an interesting hypothesis, although radical one, as an earlier study on perch (Brüning et al., 2015) did not find any light pollution effect on the stress hormone cortisol. Our finding on the serotonergic activity in effect to light pollution should be assessed in a further study.

Dopaminergic activity

For the dopaminergic activity in fish, DOPAC is proven to be an important metabolite of DA, but this is not the most important metabolite of DA for all fish (Winberg & Nilsson, 1993). For medaka (*Oryzias latipes*) however, both DOPAC and DA have been found to be lowered in fish exposed to a chemical neurotoxin in the brain (Matsui et al., 2009). This tells us that a decrease in DOPAC and DA levels in the medaka brain can be used to detect a decrease in dopaminergic activity after exposure the neurotoxins. In addition, neurotoxin study proved that DOPAC is an important DA metabolite in medaka. In another study, Atlantic salmon exposed to acute stress, had a higher DOPAC/DA ratio, while the concentration of DA was lowered, though not significantly so (Vindas, Sørensen, et al., 2014).

In this study the chromatogram peaks for DOPAC were very low, due to a much lower concentration than DA, resulting in unprecise results for DOPAC. The unprecise measurements of DOPAC, can have resulted in masking any possible treatment differences and thus explaining that there was found significant differences for the concentration of DOPAC and the DOPAC/DA ratio. To interpret the DOPAC/DA data with high confidence, DOPAC should be properly measured in coming experiments.

For the brain regions hypothalamus, optic tectum and the brain stem, the DA concentration was significantly lowered for ALAN in comparison to the control group. The continuous light DA concentration was affected in fewer brain areas than ALAN, by having only a lower a significantly lower DA concentration than the control group in the optic tectum.

Based on the lowered DA concentrations for the ALAN group, it is tempting to hypothesize that the ALAN group have their dopaminergic activity increased. A dopaminergic activity increase can be a compensatory coping mechanism to the possible stress the fish were exposed to (Fraser et al., 2015). Fraser et al. found that an increased dopaminergic activity could minimize behavioral inhibition, which could explain that there was not that big difference seen for ALAN in the behavioral tests done. This would indeed be an interesting finding and should be further studied. The fact that the continuous light group only had their dopaminergic activity increased in the optic tectum, could explain that fish in both altered light regimes are exposed to stress, but that the ALAN group found a better way than the continuous light of coping with it.

Noradrenergic activity

High levels of NE are associated with increased arousal in novel stressful situations (Aston-Jones et al., 1991). Contrary to serotonergic and dopaminergic activity, the noradrenergic activity is found to be increased when the metabolite, monoamine and the metabolite/monoamine ratio increases (Vindas et al., 2016). for NE specifically, it has been found that acute stress exposed animals, have a higher concentration of NE (Morilak et al., 2005). Because of this, a high NE level alone tells us that the noradrenergic activity is increased.

As the control fish had significantly higher levels of NE than the ALAN group for all brain regions tested, this suggests that the ALAN group are more acutely stressed. Continuous light fish also had lower concentrations of NE than the control group, although only significantly in the optic tectum and brain stem. For telencephalon, there was a tendency of significance, while in the hypothalamus the p-value was further from significance (p=0.1212). As NE is responding to acute and not chronic stress, this suggests that time of sampling affected the groups differently. As the sampling took place at night, the control fish were taken out from the dark cabinet, and exposed to light as the other groups were just before the sampling. The other groups, however, were used to some light from before, making the sudden transition less extreme. In addition to the sudden exposure to light and therefore possible acute stress, this might also support the earlier study, in which perch exposed to light pollution were found to have their natural sleep cycle disrupted, as their sleep hormone (melatonin) was significantly reduced (Brüning et al., 2015).

Lastly, it is important to take into account, that if NE's metabolite, MHPG, was also analyzed the noradrenergic activity would be assessed with higher confidence (Øverli et al., 2005).

qPCR

lhb was significantly affected by the light treatments in females. Here, the continuous light expression level was found to be significantly decreased in comparison to the control group. Additionally, we see that the ALAN group also has a lower relative expression than the control group, but this difference is not near significant levels.

The luteinizing hormone (LH) is controlled by *lhb* and is known to control gonadal development and maturation together with the follicle-stimulating hormone (FSH) in vertebrates (Weltzien et al., 2004). The higher expression of the *lhb* gene in the control group than the ALAN group

could mean that the three-month-old females have reached a more final stage in the maturation. Although not all females were checked for spawning at this stage, the sampling at 3-months, generally took place a couple weeks after the first fish started to spawn, meaning they were mature. In further studies, it should be checked if continuous light prevent early maturation in medaka, as it is used to delay the spawning in aquaculture (e.g. Atlantic cod and salmon) (Björnsson et al., 1994; Taranger et al., 2006). Furthermore, it would be interesting to see if ALAN delay maturation in fish, as we saw a tendency of lower *lhb* in medaka, although not significantly so.

tshba was graphically lower for males in ALAN and control fish than the continuous light group. This was not significant however, with control showing no significance. ALAN on the other side showed a statistical tendency towards being lower than the continuous light fish.

The thyroid stimulating hormone (TSH) is known to contribute to growth and development, metamorphosis, reproduction, osmoregulation, as well as feeding and nutrient metabolism in fish (Deal & Volkoff, 2020), and an upregulation of its corresponding pituitary gene thus regulate these processes. However, a new study shows that Atlantic salmon *tshba* is thought to only be a pseudogene, meaning that it has lost its former ability to produce functional proteins. Another paralog of the TSH-gene, *tshbb*, has on the other hand been found to the active gene (Fleming et al., 2019), and should in further studies be looked at in addition to understand a more complete picture when it comes to the production of the Thyroid-stimulating hormone (Tsh). It is still interesting that continuous light fish have a higher expression of *tshba* than the ALAN group, although not significant.

For medaka, expression of *sl* (somatolactin-gene) was found to be decreased for adult in relation to juvenile fish (Royan et al., 2021). As there was a small tendency towards males from the control group having a lower expression level of *sl* than the ALAN and continuous light, this could signify that the control fish have indeed reached a more mature stage than the fish in the altered light regimes. This interpretation is, however, a bit radical, as the *lhb* expression levels were only found to be changed for females and the sl expression levels were only found to be changed for the males. The study on medaka (Royan et al., 2021), suggested that another reason for the different sl expression levels was that the reason for the different sl expression levels was due to different stages of gonadal development. Because of this, the results sl expression levels might be instead different, due to the desynchronized spawning timing found.

5.   Conclusions

This study shows that light pollution can penetrate water efficiently in some places.

For the fish studied, it is proven that light pollution and continuous light affect fish in several ways. The reproductive cycle is found to be desynchronized, in addition to the behavior altered for the artificial light regimes. Desynchronization of reproductive timing can have severe effects on wild fish populations, as it can change the well-adapted basis for ecological systems. Furthermore, altered behavior was found for both altered light regimes. Though the behavioral effects were milder for ALAN fish, the reason for the alteration is not known, and must be investigated.

Growth and gonadal development showed signs of being positively affected by the altered light regimes. The continuous light regime clearly affected heart morphology, indicating that an altered light regime is likely responsible for poor welfare. The brain morphology was also found to be altered for some measurements of the telencephalon, which should be further studied. The brain chemistry was clearly affected by the light regimes, as NE showed a clear difference between the groups. Additionally, the serotonergic activity was most clearly increased for ALAN suggesting exposure to chronic stress. In further studies the dopaminergic and noradrenergic system should be investigated with the inclusion of their respective metabolites, to give more precise information. Lastly, the effect of the genes studied was only found for *lhb*, which was decreased in the continuous light regime.

The effects of altered light regimes were found to be various and potentially severe. The findings for the ALAN group may have large-scale ecological consequences. Continuous light was found to affect the same way as ALAN, apart from neurochemistry only stronger, and for a higher variety of parameters, with likely welfare implications. Even if further studies should investigate my findings on light pollution, there are enough studies in addition to this one to say that light pollution indeed affects the ecosystem, both negatively and positively. As it has rapidly increased, it is hard to know how the wildlife will adapt. Unnecessary light pollution should therefore be reduced, to lessen the impact on our planet.

6.   References

Ager-Wick, E., Hodne, K., Fontaine, R., von Krogh, K., Haug, T. M., & Weltzien, F.-A. (2018). Preparation of a high-quality primary cell culture from fish pituitaries. *JoVE (Journal of Visualized Experiments)*(138), e58159.

Akvakulturdriftsforskriften. (2008). *Forskrift om drift av akvakulturanlegg*. Retrieved 13.01.2023 from https://lovdata.no/forskrift/2008-06-17-822

Andrews, P. W., Bharwani, A., Lee, K. R., Fox, M., & Thomson Jr, J. A. (2015). Is serotonin an upper or a downer? The evolution of the serotonergic system and its role in depression and the antidepressant response. *Neuroscience & Biobehavioral Reviews*, *51*, 164-188.

Aston-Jones, G., Chiang, C., & Alexinsky, T. (1991). Discharge of noradrenergic locus coeruleus neurons in behaving rats and monkeys suggests a role in vigilance. *Progress in brain research*, *88*, 501-520.

Berg, A., Hansen, T., & Stefansson, S. (1992). First feeding of Atlantic salmon (Salmo salar L.) under different photoperiods. *Journal of Applied Ichthyology*, *8*(1-4), 251-256.

Bessei, W. (2006). Welfare of broilers: a review. *World's Poultry Science Journal*, *62*(3), 455-466.

Björnsson, B. T., Hemre, G.-I., Bjørnevik, M., & Hansen, T. (2000). Photoperiod regulation of plasma growth hormone levels during induced smoltification of underyearling Atlantic salmon. *General and Comparative Endocrinology*, *119*(1), 17-25.

Björnsson, B. T., Taranger, G. L., Hansen, T., Stefansson, S. O., & Haux, C. (1994). The interrelation between photoperiod, growth hormone, and sexual maturation of adult Atlantic salmon (Salmo salar). *General and Comparative Endocrinology*, *93*(1), 70-81.

Braithwaite, V., & Ebbesson, L. (2014). Pain and stress responses in farmed fish. *Rev Sci Tech*, *33*(1), 245-253.

Brijs, J., Hjelmstedt, P., Berg, C., Johansen, I. B., Sundh, H., Roques, J. A., Ekström, A., Sandblom, E., Sundell, K., & Olsson, C. (2020). Prevalence and severity of cardiac abnormalities and arteriosclerosis in farmed rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *526*, 735417.

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. *Science of the Total Environment*, *511*, 516-522.

Burow, S., Fontaine, R., von Krogh, K., Mayer, I., Nourizadeh-Lillabadi, R., Hollander-Cohen, L., Cohen, Y., Shpilman, M., Levavi-Sivan, B., & Weltzien, F.-A. (2019). Medaka follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels. *General and Comparative Endocrinology*, *272*, 93-108.

Buys, N., Buyse, J., Hassanzadeh-Ladmakhi, M., & Decuypere, E. (1998). Intermittent lighting reduces the incidence of ascites in broilers: an interaction with protein content of feed on performance and the endocrine system. *Poultry science*, *77*(1), 54-61.

Chan, K. K.-S. (1976). A photosensitive daily rhythm in the female medaka, Oryzias latipes. *Canadian Journal of Zoology*, *54*(6), 852-856.

Chandler, D. C. (1942). Limnological studies of western Lake Erie: II. Light penetration and its relation to turbidity. *Ecology*, *23*(1), 41-52.

Closs, L. E., Royan, M. R., Sayyari, A., Mayer, I., Weltzien, F.-A., Baker, D. M., & Fontaine, R. (2023). Impact of light pollution at night on male reproductive success in Japanese medaka (Oryzias latipes). *bioRxiv*, 2023.2004. 2017.536935.

Cristofor, S., Vadineanu, A., Ignat, G., & Ciubuc, C. (1994). Factors affecting light penetration in shallow lakes. *Hydrobiologia*, *275*, 493-498.

Cuesta, I. H., Lahiri, K., Lopez-Olmeda, J. F., Loosli, F., Foulkes, N. S., & Vallone, D. (2014). Differential maturation of rhythmic clock gene expression during early development in medaka (Oryzias latipes). *Chronobiology international*, *31*(4), 468-478.

Davidson, W. S. (2013). Understanding salmonid biology from the Atlantic salmon genome. *Genome*, *56*(10), 548-550.

Davies, T. W., Duffy, J. P., Bennie, J., & Gaston, K. J. (2014). The nature, extent, and ecological implications of marine light pollution. *Frontiers in Ecology and the Environment*, *12*(6), 347-355.

Deal, C. K., & Volkoff, H. (2020). The role of the thyroid axis in fish. *Frontiers in Endocrinology*, *11*, 596585.

Duriscoe, D. M., Luginbuhl, C. B., & Moore, C. A. (2007). Measuring night-sky brightness with a wide-field CCD camera. *Publications of the Astronomical Society of the Pacific*, *119*(852), 192.

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

Falcón, J., & Zohar, Y. (2018). Photoperiodism in fish.

Fleming, M. S., Maugars, G., Lafont, A.-G., Rancon, J., Fontaine, R., Nourizadeh-Lillabadi, R., Weltzien, F.-A., Yebra-Pimentel, E. S., Dirks, R., & McCormick, S. D. (2019). Functional divergence of thyrotropin beta-subunit paralogs gives new insights into salmon smoltification metamorphosis. *Scientific reports*, *9*(1), 4561.

Food and Agriculture Organization of the United Nations. (2022). *The State of World Fisheries and Aquaculture 2022: Towards Blue Transformation*. FOOD & AGRICULTURE ORG. https://books.google.it/books?id=9yN_EAAAQBAJ

Forskrift om hold av høns og kalkun. (2001). *Landbruks- og matdepartementet*. Retrieved 13.01.2023 from https://lovdata.no/forskrift/2001-12-12-1494

Fraga, D., Meulia, T., & Fenster, S. (2008). Real-time PCR. *Current protocols essential laboratory techniques*(1), 10.13. 11-10.13. 34.

Frank, K. D., Rich, C., & Longcore, T. (2006). Effects of artificial night lighting on moths. *Ecological consequences of artificial night lighting*, *13*, 305-344.

Fraser, T. W. K., Vindas, M. A., Fjelldal, P. G., Winberg, S., Thörnqvist, P.-O., Øverli, Ø., Skjæraasen, J.-E., Hansen, T. J., & Mayer, I. (2015). Increased reactivity and monoamine dysregulation following stress in triploid Atlantic salmon (Salmo salar). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *185*, 125-131.

Frisk, M., Høyland, M., Zhang, L., Vindas, M. A., Øverli, Ø., & Johansen, I. B. (2020). Intensive smolt production is associated with deviating cardiac morphology in Atlantic salmon (Salmo salar L.). *Aquaculture*, *529*, 735615.

Gross, W., Roelofs, E., & Fromm, P. (1965). Influence of photoperiod on growth of green sunfish, Lepomis cyanellus. *Journal of the Fisheries Board of Canada*, *22*(6), 1379-1386.

Grubisic, M., Haim, A., Bhusal, P., Dominoni, D. M., Gabriel, K. M., Jechow, A., Kupprat, F., Lerner, A., Marchant, P., & Riley, W. (2019). Light pollution, circadian photoreception, and melatonin in vertebrates. *Sustainability*, *11*(22), 6400.

Hamilton, J. B., Walter, R. O., Daniel, R. M., & Mestler, G. E. (1969). Competition for mating between ordinary and supermale Japanese medaka fish. *Animal Behaviour*, *17*, 168-176.

Hansen, T., Stefansson, S., & Taranger, G. (1992). Growth and sexual maturation in Atlantic salmon, Salmon, salar L., reared in sea cages at two different light regimes. *Aquaculture Research*, *23*(3), 275-280.

Hassanzadeh, M., Bozorgmerifard, M., Akbari, A., Buyse, J., & Decuypere, E. (2000). Effect of intermittent lighting schedules during the natural scotoperiod on T3-induced ascites in broiler chickens. *Avian Pathology*, *29*(5), 433-439.

Hesthagen, T., Wienerroither, R., Bjelland, O., Byrkjedal, I., Fiske, P., Lynghammar, A., Nedreaas, K., & Straube, N. (2021). *Fisker: Vurdering av laks Salmo salar for Norge. Rødlista for arter 2021. Artsdatabanken.* https://artsdatabanken.no/lister/rodlisteforarter/2021/8149

Hildahl, J., Sandvik, G. K., Lifjeld, R., Hodne, K., Nagahama, Y., Haug, T. M., Okubo, K., & Weltzien, F. A. (2012). Developmental tracing of luteinizing hormone β-subunit gene expression using green fluorescent protein transgenic medaka (Oryzias latipes) reveals a putative novel developmental function. *Developmental Dynamics*, *241*(11), 1665-1677.

Höglund, E., Folkedal, O., Aerts, J., Hvas, M., Øverli, Ø., Jensen, A.-M., Vindas, M. A., Nilsson, J., & Kristiansen, T. (2023). Welfare effects of environmental hypercapnia quantified by indicators

based on morphology and allostatic load in Atlantic salmon (Salmo salar). *Aquaculture*, 739512.

Icardo, J. M., Imbrogno, S., Gattuso, A., Colvee, E., & Tota, B. (2005). The heart of Sparus auratus: a reappraisal of cardiac functional morphology in teleosts. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *303*(8), 665-675.

Ishikawa, Y., Yoshimoto, M., Yamamoto, N., & Ito, H. (1999). Different brain morphologies from different genotypes in a single teleost species, the medaka (Oryzias latipes). *Brain, behavior and evolution*, *53*(1), 2-9.

Jechow, A., & Hölker, F. (2019). How dark is a river? Artificial light at night in aquatic systems and the need for comprehensive night-time light measurements. *Wiley Interdisciplinary Reviews: Water*, *6*(6), e1388.

Julian, R. J. (1993). Ascites in poultry. *Avian Pathology*, *22*(3), 419-454.

Kinoshita, M., Murata, K., Naruse, K., & Tanaka, M. (2009). *Medaka: biology, management, and experimental protocols*. John Wiley & Sons.

Kirchmaier, S., Naruse, K., Wittbrodt, J., & Loosli, F. (2015). The genomic and genetic toolbox of the teleost medaka (Oryzias latipes). *Genetics*, *199*(4), 905-918.

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

Kotrschal, K., Van Staaden, M., & Huber, R. (1998). Fish brains: evolution and anvironmental relationships. *Reviews in Fish Biology and Fisheries*, *8*, 373-408.

Kuechly, H. U., Kyba, C. C., 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 Sensing of Environment*, *126*, 39-50.

Kummu, M., De Moel, H., Ward, P. J., & Varis, O. (2011). How close do we live to water? A global analysis of population distance to freshwater bodies. *PLoS One*, *6*(6), e20578.

Lanfumey, L., Mongeau, R., Cohen-Salmon, C., & Hamon, M. (2008). Corticosteroid–serotonin interactions in the neurobiological mechanisms of stress-related disorders. *Neuroscience & Biobehavioral Reviews*, *32*(6), 1174-1184.

Lee, J. S., Britt, L. L., Cook, M. A., Wade, T. H., Berejikian, B. A., & Goetz, F. W. (2017). Effect of light intensity and feed density on feeding behaviour, growth and survival of larval sablefish Anoplopoma fimbria. *Aquaculture Research*, *48*(8), 4438-4448.

Longcore, T., & Rich, C. (2004). Ecological light pollution. *Frontiers in Ecology and the Environment*, *2*(4), 191-198.

Luchiari, A. C., De Morais Freire, F. A., Koskela, J., & Pirhonen, J. (2006). Light intensity preference of juvenile pikeperch Sander lucioperca (L.). *Aquaculture Research*, *37*(15), 1572-1577.

Matsui, H., Taniguchi, Y., Inoue, H., Uemura, K., Takeda, S., & Takahashi, R. (2009). A chemical neurotoxin, MPTP induces Parkinson's disease like phenotype, movement disorders and persistent loss of dopamine neurons in medaka fish. *Neuroscience research*, *65*(3), 263-271.

Matsunaga, W., & Watanabe, E. (2010). Habituation of medaka (Oryzias latipes) demonstrated by open-field testing. *Behavioural processes*, *85*(2), 142-150.

Moland, E., Synnes, A.-E., Naustvoll, L. J., Freitas, C., Norderhaug, K. M., Thormar, J., Biuw, M., Jorde, P. E., Knutsen, H., & Dahle, G. (2021). Krafttak for kysttorsken-Kunnskap for stedstilpasset gjenoppbygging av bestander, naturtyper og økosystem i Færder-og Ytre Hvaler nasjonalparker. *Rapport fra havforskningen*.

Morilak, D. A., Barrera, G., Echevarria, D. J., Garcia, A. S., Hernandez, A., Ma, S., & Petre, C. O. (2005). Role of brain norepinephrine in the behavioral response to stress. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *29*(8), 1214-1224.

Motulsky, H. J., & Brown, R. E. (2006). Detecting outliers when fitting data with nonlinear regression–a new method based on robust nonlinear regression and the false discovery rate. *BMC bioinformatics*, *7*(1), 1-20.

Mäkinen, T., & Ruohonen, K. (1992). Effect of delayed photoperiod on the growth of a Finnish rainbow trout (Oncorlynchus mykiss Walbaum) stock. *Journal of Applied Ichthyology*, *8*(1-4), 40-50.

Nguyen, V., Deschet, K., Henrich, T., Godet, E., Joly, J. S., Wittbrodt, J., Chourrout, D., & Bourrat, F. (1999). Morphogenesis of the optic tectum in the medaka (Oryzias latipes): a morphological and molecular study, with special emphasis on cell proliferation. *Journal of Comparative Neurology*, *413*(3), 385-404.

Nightingale, B., Longcore, T., & Simenstad, C. A. (2006). Artificial night lighting and fishes. *Ecological consequences of artificial night lighting*, 257-276.

NVE. (2023). *NVE Elvenettverk*. https://atlas.nve.no/

Oppedal, F., Juell, J. E., Tarranger, G., & Hansen, T. (2001). Artificial light and season affects vertical distribution and swimming behaviour of post-smolt Atlantic salmon in sea cages. *Journal of fish biology*, *58*(6), 1570-1584.

Owens, A. C., Cochard, P., Durrant, J., Farnworth, B., Perkin, E. K., & Seymoure, B. (2020). Light pollution is a driver of insect declines. *Biological Conservation*, *241*, 108259.

Petit, G., Beauchaud, M., Attia, J., & Buisson, B. (2003). Food intake and growth of largemouth bass (Micropterus salmoides) held under alternated light/dark cycle (12L: 12D) or exposed to continuous light. *Aquaculture*, *228*(1-4), 397-401.

Poppe, T. T., Johansen, R., Gunnes, G., & Tørud, B. (2003). Heart morphology in wild and farmed Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss. *Diseases of Aquatic Organisms*, *57*(1-2), 103-108.

Project, J. (2021). Jamovi (Version 2.3. 18)[Computer Software].

Rodríguez, A., Burgan, G., Dann, P., Jessop, R., Negro, J. J., & Chiaradia, A. (2014). Fatal attraction of short-tailed shearwaters to artificial lights. *PLoS One*, *9*(10), e110114.

Rodríguez, A., Dann, P., & Chiaradia, A. (2017). Reducing light-induced mortality of seabirds: high pressure sodium lights decrease the fatal attraction of shearwaters. *Journal for Nature Conservation*, *39*, 68-72.

Royan, M. R., Kanda, S., Kayo, D., Song, W., Ge, W., Weltzien, F.-A., & Fontaine, R. (2020). Gonadectomy and blood sampling procedures in the small size teleost model japanese medaka (Oryzias latipes). *JoVE (Journal of Visualized Experiments)*(166), e62006.

Royan, M. R., Kayo, D., Weltzien, F.-A., & Fontaine, R. (2023). Sexually dimorphic regulation of gonadotrope cell hyperplasia in medaka pituitary via mitosis and transdifferentiation. *Endocrinology*, *164*(4), bqad030.

Royan, M. R., Siddique, K., Csucs, G., Puchades, M. A., Nourizadeh-Lillabadi, R., Bjaalie, J. G., Henkel, C. V., Weltzien, F.-A., & Fontaine, R. (2021). 3D atlas of the pituitary gland of the model fish medaka (Oryzias latipes). *Frontiers in Endocrinology*, *12*, 719843.

Rutten, M., Leterrier, C., Constantin, P., Reiter, K., & Bessei, W. (2002). Bone development and activity in chickens in response to reduced weight-load on legs. *Animal Research*, *51*(4), 327-336.

Sanchez-Quintana, D., Garcia-Martinez, V., Climent, V., & Hurle, J. M. (1995). Morphological analysis of the fish heart ventricle: myocardial and connective tissue architecture in teleost species. *Annals of Anatomy-Anatomischer Anzeiger*, *177*(3), 267-274.

Sande, R. D., & Poppe, T. T. (1995). Diagnostic ultrasound examination and echocardiography in Atlantic salmon (Salmo salar). *Veterinary Radiology & Ultrasound*, *36*(6), 551-558.

Shannon, N., Gunnet, J., & Moore, K. (1986). A comparison of biochemical indices of 5-hydroxytryptaminergic neuronal activity following electrical stimulation of the dorsal raphe nucleus. *Journal of neurochemistry*, *47*(3), 958-965.

Sharifi, A. (2022). *The Biological Background of Depression-like Syndrome*

Stefansson, S., Nortvedt, R., Hansen, T., & Taranger, G. (1990). First feeding of Atlantic salmon, Salmo salar L., under different photoperiods and light intensities. *Aquaculture Research*, *21*(4), 435-441.

Stefansson, S. O., Bjömsson, B. T., Hansen, T., Haux, C., Taranger, G. L., & Saunders, R. L. (1991). Growth, parr–smolt transformation, and changes in growth hormone of Atlantic salmon (Salmo salar) reared under different photoperiods. *Canadian Journal of Fisheries and Aquatic Sciences*, *48*(11), 2100-2108.

Steindal, I. A., & Whitmore, D. (2019). Circadian clocks in fish—what have we learned so far? *Biology*, *8*(1), 17.

Takeuchi, A., & Okubo, K. (2013). Post-proliferative immature radial glial cells female-specifically express aromatase in the medaka optic tectum. *PLoS One*, *8*(9), e73663.

Taranger, G., Aardal, L., Hansen, T., & Kjesbu, O. (2006). Continuous light delays sexual maturation and increases growth of Atlantic cod (Gadus morhua L.) in sea cages. *ICES Journal of Marine Science*, *63*(2), 365-375.

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

Tidau, S., Smyth, T., McKee, D., Wiedenmann, J., D'Angelo, C., Wilcockson, D., Ellison, A., Grimmer, A. J., Jenkins, S. R., & Widdicombe, S. (2021). Marine artificial light at night: An empirical and technical guide. *Methods in Ecology and Evolution*, *12*(9), 1588-1601.

Tilton, S. C., Foran, C. M., & Benson, W. H. (2005). Relationship between ethinylestradiol-mediated changes in endocrine function and reproductive impairment in Japanese medaka (Oryzias latipes). *Environmental Toxicology and Chemistry: An International Journal*, *24*(2), 352-359.

Vindas, M. A., Fokos, S., Pavlidis, M., Höglund, E., Dionysopoulou, S., Ebbesson, L. O., Papandroulakis, N., & Dermon, C. R. (2018). Early life stress induces long-term changes in limbic areas of a teleost fish: the role of catecholamine systems in stress coping. *Scientific reports*, *8*(1), 1-12.

Vindas, M. A., Johansen, I. B., Vela-Avitua, S., Nørstrud, K. S., Aalgaard, M., Braastad, B. O., Höglund, E., & Øverli, Ø. (2014). Frustrative reward omission increases aggressive behaviour of inferior fighters. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1784), 20140300.

Vindas, M. A., Madaro, A., Fraser, T. W., Höglund, E., Olsen, R. E., Øverli, Ø., & Kristiansen, T. S. (2016). Coping with a changing environment: the effects of early life stress. *Royal Society Open Science*, *3*(10), 160382.

Vindas, M. A., Sørensen, C., Johansen, I. B., Folkedal, O., Höglund, E., Khan, U. W., Stien, L. H., Kristiansen, T. S., Braastad, B. O., & Øverli, Ø. (2014). Coping with unpredictability: dopaminergic and neurotrophic responses to omission of expected reward in Atlantic salmon (Salmo salar L.). *PLoS One*, *9*(1), e85543.

Vitaterna, M. H., Takahashi, J. S., & Turek, F. W. (2001). Overview of circadian rhythms. *Alcohol research & health*, *25*(2), 85.

Volpato, G. L., Gonçalves-de-Freitas, E., & Fernandes-de-Castilho, M. (2007). Insights into the concept of fish welfare. *Diseases of Aquatic Organisms*, *75*(2), 165-171.

Warren, E., & Callaghan, S. (1976). The response of male guppies (Poecilia reticutata, Peters) to repeated exposure to an open field. *Behavioral Biology*, *18*(4), 499-513.

Weltzien, F.-A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K., & Norberg, B. (2004). The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *137*(3), 447-477.

Winberg, S., & Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *106*(3), 597-614.

Wittbrodt, J., Shima, A., & Schartl, M. (2002). Medaka—a model organism from the far East. *Nature Reviews Genetics*, *3*(1), 53-64.

Yamauchi, A. (1980). Fine structure of the fish heart. *Comparative anatomy and development*, 119-148.

Yoneda, M., Kitano, H., Selvaraj, S., Matsuyama, M., & Shimizu, A. (2013). Dynamics of gonadosomatic index of fish with indeterminate fecundity between subsequent egg batches: application to Japanese anchovy Engraulis japonicus under captive conditions. *Marine Biology*, *160*, 2733-2741.

Øverli, Ø., Winberg, S., & Pottinger, T. G. (2005). Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout—a review. *Integrative and comparative biology*, *45*(3), 463-474.

7. Appendices

7.1 Supplementary graphs
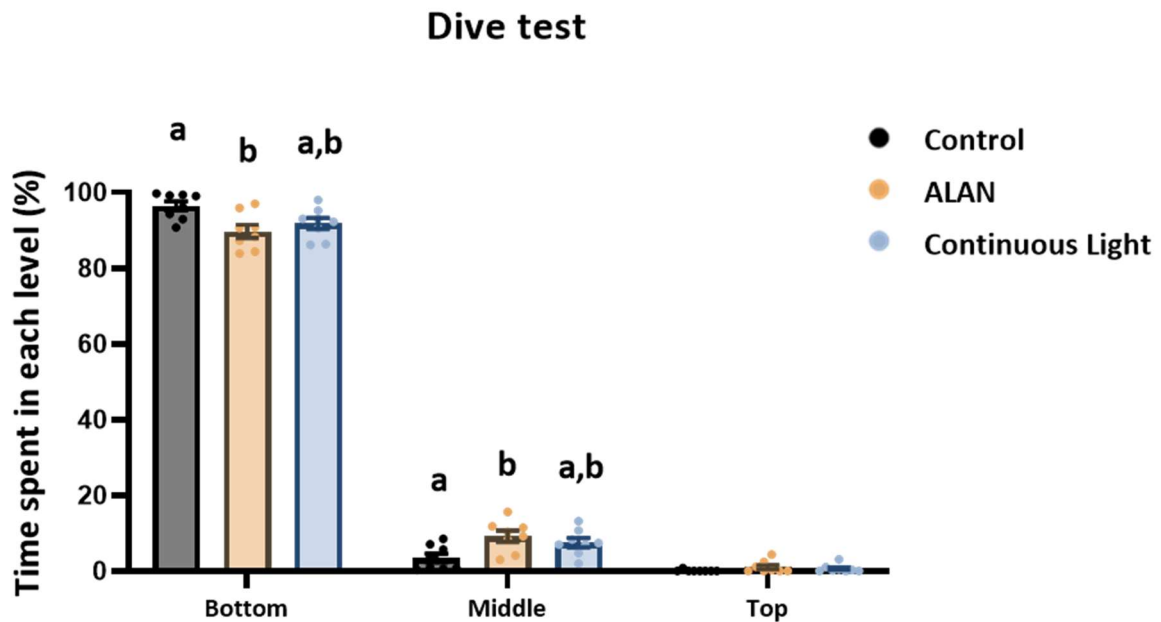
Dive test

Figure 16. Dive test for 6-month-old fish with data presented as mean ± SEM, with n being 8 for each light treatment. Light treatment differences were checked for time spent in the bottom ($F_{2, 21}$ = 5.42, p=0.013), middle ($F_{2, 21}$ = 5.32, p=0.013) and top (Kruskal-Wallis, p=0.1491) areas.
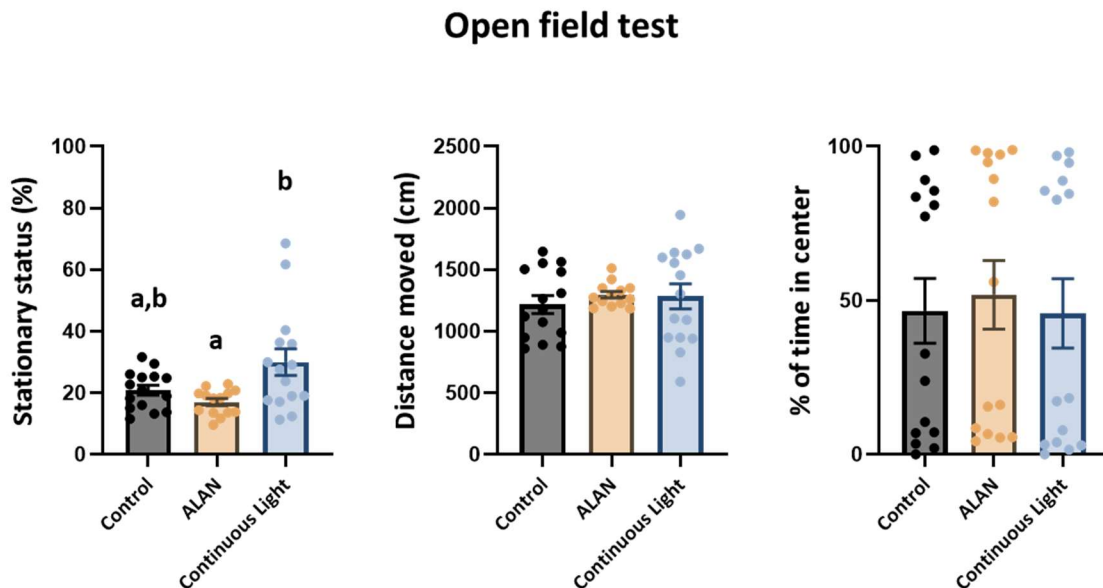
Open field test

Figure 17. Open field test for 6-month-old fish, with data presented as mean ± SEM, with n being 13-15 for each group of light treatment. Light treatment differences were checked for stationary status (Kruskal-Wallis, p=0.0376), distance moved ($F_{2,21.9}$=0.517, p=0.603) and % of time spent in center ($F_{2,42}$=0.0897, p=0.914).

7.2 Supplementary tables

Table 13. Relative expression of pituitary hormone genes. RPL-7 is used as the housekeeping gene.

Relative expression	Sex	Control	ALAN	Continuous Light	One-way ANOVA	n
Cyp19a1b	Female	0.933 ± 0.085	0.823 ± 0.179	0.765 ± 0.1	$F_{2,\ 16}$=0.378, *p=0.691*	5-7
	Male	0.918 ± 0.142	0.803 ± 0.141	0.732 ± 0.06	$F_{2,\ 24}$=0.607, *p=0.553*	9
LHb	Female	1.566 ± 0.201	1.193 ± 0.345	0.894 ± 0.124	$F_{2,\ 10.2}$ =3.84, *p=0.057*	5-7
	Male	0.591 ± 0.074	0.715 ± 0.147	0.504 ± 0.088	$F_{2,\ 24}$=0.975, *p=0.392*	9
Fshb	Female	1.034 ± 0.161	0.744 ± 0.136	1.019 ± 0.120	$F_{2,\ 16}$=1.33, *p=0.293*	5-7
	Male	1.07 ± 0.161	1.41 ± 0.265	1.26 ± 0.200	$F_{2,\ 23}$=0.658, *p=0.527*	8-9
POMCA	Female	0.658 ± 0.036	0.639 ± 0.117	0.657 ± 0.073	$F_{2,\ 16}$=0.0167, *p=0.983*	5-7
	Male	1.55 ± 0.251	2.38 ± 0.463	1.46 ± 0.275	$F_{2,\ 22}$=2.11, *p=0.145*	8-9
Tshba	Female	0.497 ± 0.093	0.976 ± 0.296	0.699 ± 0.075	$F_{2,\ 10.2}$=1.99, *p=0.187*	5-7
	Male	0.175 ± 0.040	0.147 ± 0.018	0.304 ± 0.071	$F_{2,\ 22}$=2.83, *p=0.081*	8-9
GH	Female	0.995 ± 0.140	0.752 ± 0.134	0.677 ± 0.120	$F_{2,\ 16}$=1.49, *p=0.254*	5-7
	Male	0.751 ± 0.150	0.676 ± 0.119	0.670 ± 0.107	$F_{2,\ 24}$=0.127, *p=0.881*	9
SL	Female	0.826 ± 0.099	0.752 ± 0.129	1.114 ± 0.249	$F_{2,\ 16}$=1.40, *p=0.276*	5-7
	Male	0.562 ± 0.041	0.887 ± 0.136	0.987 ± 0.200	$F_{2,\ 11.9}$=4.28, **p=0.040**	8-9

7.3   Outliers

Table 14. List of outliers taken out in the Open field test by ROUT outlier test (Q=1%). Outliers were not taken out for reproductive cycle study and the dive test as we wanted to keep the variation.

	Behavior		
Age	**Experiment**	**Light treatment**	**Number of outliers**
3 months	Stationary status (%)	Control	0
		ALAN	1
		Continuous Light	0
3 months	Movement (cm)	Control	0
		ALAN	0
		Continuous Light	0
3 months	% of time in center	Control	0
		ALAN	0
		Continuous Light	0
6 months	Stationary status (%)	Control	0
		ALAN	1
		Continuous Light	0
6 months	Movement (cm)	Control	0
		ALAN	2
		Continuous Light	0
6 months	% of time in center	Control	0
		ALAN	0
		Continuous Light	0

Table 15. For the gonadosomatic index outliers were detected and removed by ROUT at 1% for both 3- and 6-months old fish.

GSI			
Sex	Experiment	Age	Outliers removed
Female	WT	3 months	0
	DTG		0

Male	WT		1
	DTG		0
Female	WT	6 months	0
Male			1

Table 16. Removed data outliers from the monoamine neurochemistry data of serotonin (5-HT), 5-hydroxy indole acetic acid (5-HIAA), 5-HIAA/5-HT, Dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), DOPAC/DA and Norepinephrine (NE) in telencephalon, hypothalamus, optic tectum, and brain stem. Data outliers were determined and removed by the ROUT test, with Q set at 5 %.

Monoamine data experiment 1 and 2		
Brain regions	**Monoamines**	**Number of outliers**
Telencephalon	5-HT	2
	5-HIAA	2
	5-HIAA/5-HT	1
	DA	3
	DOPAC	3
	DOPAC/DA	4
	NE	1
Hypothalamus	5-HT	2
	5-HIAA	1
	5-HIAA/5-HT	2
	DA	2
	DOPAC	3
	DOPAC/DA	1
	NE	2
Optic tectum	5-HT	6
	5-HIAA	2
	5-HIAA/5-HT	1
	DA	1
	DOPAC	3
	DOPAC/DA	0

	NE	2
Brain stem	5-HT	5
	5-HIAA	3
	5-HIAA/5-HT	1
	DA	3
	DOPAC	5
	DOPAC/DA	4
	NE	6

Table 17. Removed outliers for qPCR data. Due to the low number of n, experiment 1 and 2 were merged and studied by using one-way ANOVA. Q was set to 1%.

qPCR		
Gene	**Sex**	**Number of outliers**
Cyp19a1b	Female	0
	Male	0
LHb	Female	0
	Male	0
Fshb	Female	0
	Male	1
POMCA	Female	0
	Male	2
Tshba	Female	0
	Male	2
GH	Female	0
	Male	0
SL	Female	0
	Male	1

7.4   Post hoc tests

Table 18. Post-hoc tests following ANOVA for brain morphometric measurements are presented.

Post-hoc test for Brain measurements				
Group	Experiment	Measurement	Level	P-value
6-month-old females		d	Control vs ALAN	**0.031**
			Control vs Continuous Light	**0.006**
			ALAN vs Continuous Light	0.829
6-month-old males		h	Control vs ALAN	0.107
			Control vs Continuous Light	0.114
			ALAN vs Continuous Light	0.998
3-month-old females	1	c	Control vs ALAN	**0.0381**
			Control vs Continuous Light	<u>0.0683</u>
			ALAN vs Continuous Light	**0.0002**
	2		Control vs ALAN	0.8036
			Control vs Continuous Light	0.9987
			ALAN vs Continuous Light	0.7485
3-month-old females	1	d	Control vs ALAN	0.3266
			Control vs Continuous Light	0.4312
			ALAN vs Continuous Light	**0.0421**
	2		Control vs ALAN	0.4271

			Control vs Continuous Light	0.4121
			ALAN vs Continuous Light	0.9963
3-month-old females	Merged	a	Control vs ALAN	0.9309
			Control vs Continuous Light	<u>0.0681</u>
			ALAN vs Continuous Light	0.1244
3-month-old males	Merged	g	Control vs ALAN	0.1259
			Control vs Continuous Light	0.8947
			ALAN vs Continuous Light	<u>0.057</u>

Table 19. shows the p-values for Tukey multiple comparison's test comparisons. Significant values are marked in bold, and tendency for significance are marked with underline.

Telencephalon monoamines	Level	p-Value
5-HT	Control vs ALAN	<u>0.0579</u>
	Control vs Continuous Light	0.6479
	ALAN vs Continuous Light	0.3731
5-HIAA	Control vs ALAN	0.8408
	Control vs Continuous Light	0.6228
	ALAN vs Continuous Light	0.9111
5-HIAA/5-HT	Control vs ALAN	0.2838
	Control vs Continuous Light	0.9975
	ALAN vs Continuous Light	0.3311
DA	Control vs ALAN	0.9174
	Control vs Continuous Light	0.9971
	ALAN vs Continuous Light	0.8889
DOPAC	Control vs ALAN	0.6291
	Control vs Continuous Light	0.9627
	ALAN vs Continuous Light	0.8094
DOPAC/DA	Control vs ALAN	0.6109
	Control vs Continuous Light	0.7445
	ALAN vs Continuous Light	0.9843
NE	Control vs ALAN	**0.0050**
	Control vs Continuous Light	<u>0.0843</u>
	ALAN vs Continuous Light	0.6198

Table 20 shows the p-values for Tukey multiple comparison's test comparisons. Significant values are marked in bold, and tendency for significance are marked with underline.

Hypothalamus monoamines	Level	p-Value
5-HT	Control vs ALAN	**0.0102**
	Control vs Continuous Light	**0.0424**
	ALAN vs Continuous Light	0.8227
5-HIAA	Control vs ALAN	0.3058
	Control vs Continuous Light	<u>0.0607</u>
	ALAN vs Continuous Light	0.6958
5-HIAA/5-HT	Control vs ALAN	0.1499
	Control vs Continuous Light	0.3540
	ALAN vs Continuous Light	**0.0047**
DA	Control vs ALAN	**0.0393**
	Control vs Continuous Light	0.6726
	ALAN vs Continuous Light	0.2170
DOPAC	Control vs ALAN	0.1450
	Control vs Continuous Light	0.1071
	ALAN vs Continuous Light	0.9910
DOPAC/DA	Control vs ALAN	0.9523
	Control vs Continuous Light	0.4097
	ALAN vs Continuous Light	0.6022
NE	Control vs ALAN	**0.0149**
	Control vs Continuous Light	0.1212
	ALAN vs Continuous Light	0.6243

Table 21 shows the p-values for Tukey multiple comparison's test comparisons. Significant values are marked in bold, and tendency for significance are marked with underline.

Optic tectum monoamines	Level	p-Value
5-HT	Control vs ALAN	0.9840
	Control vs Continuous Light	0.8405
	ALAN vs Continuous Light	0.7483
5-HIAA	Control vs ALAN	0.4329
	Control vs Continuous Light	0.8760
	ALAN vs Continuous Light	0.2205
5-HIAA/5-HT	Control vs ALAN	**0.0102**
	Control vs Continuous Light	0.4427
	ALAN vs Continuous Light	0.2071
DA	Control vs ALAN	**0.0477**
	Control vs Continuous Light	**0.0290**
	ALAN vs Continuous Light	0.9825
DOPAC	Control vs ALAN	0.9796
	Control vs Continuous Light	0.2561
	ALAN vs Continuous Light	0.3243
DOPAC/DA	Control vs ALAN	0.3236
	Control vs Continuous Light	0.8892
	ALAN vs Continuous Light	0.5779
NE	Control vs ALAN	**<0.0001**
	Control vs Continuous Light	**<0.0001**
	ALAN vs Continuous Light	0.8316

Table 22. shows the p-values for Tukey multiple comparison's test comparisons. Significant values are marked in bold, and tendency for significance are marked with underline.

Brain stem monoamines	Level	p-Value
5-HT	Control vs ALAN	0.1772
	Control vs Continuous Light	0.1194
	ALAN vs Continuous Light	0.9629
5-HIAA	Control vs ALAN	0.8889
	Control vs Continuous Light	0.2267
	ALAN vs Continuous Light	0.4194
5-HIAA/5-HT	Control vs ALAN	<u>0.0662</u>
	Control vs Continuous Light	0.9881
	ALAN vs Continuous Light	<u>0.0840</u>
DA	Control vs ALAN	**0.0195**
	Control vs Continuous Light	0.3998
	ALAN vs Continuous Light	0.3226
DOPAC	Control vs ALAN	0.7735
	Control vs Continuous Light	>0.9999
	ALAN vs Continuous Light	0.7565
DOPAC/DA	Control vs ALAN	0.2849
	Control vs Continuous Light	0.8349
	ALAN vs Continuous Light	0.5942
NE	Control vs ALAN	**<0.0001**
	Control vs Continuous Light	**<0.0001**
	ALAN vs Continuous Light	0.9916

7.5   Cq-values for housekeeping genes.

Table 23. Cq-values with standard deviation from qPCR on pituitary from 3-month-old fish. The lowest merged SD is marked bold.

Housekeeping genes	*gapdh*		*rpl7*		*18s*	
	Mean	SD	Mean	SD	Mean	SD
Merged	28.23	1.38	24.75	**0.63**	13.9	0.76
Control	28.27	1.41	24.83	0.48	13.9	0.69
ALAN	28.26	1.36	24.63	0.82	13.83	1
Continuous Light	28.15	1.49	24.80	0.56	14	0.53