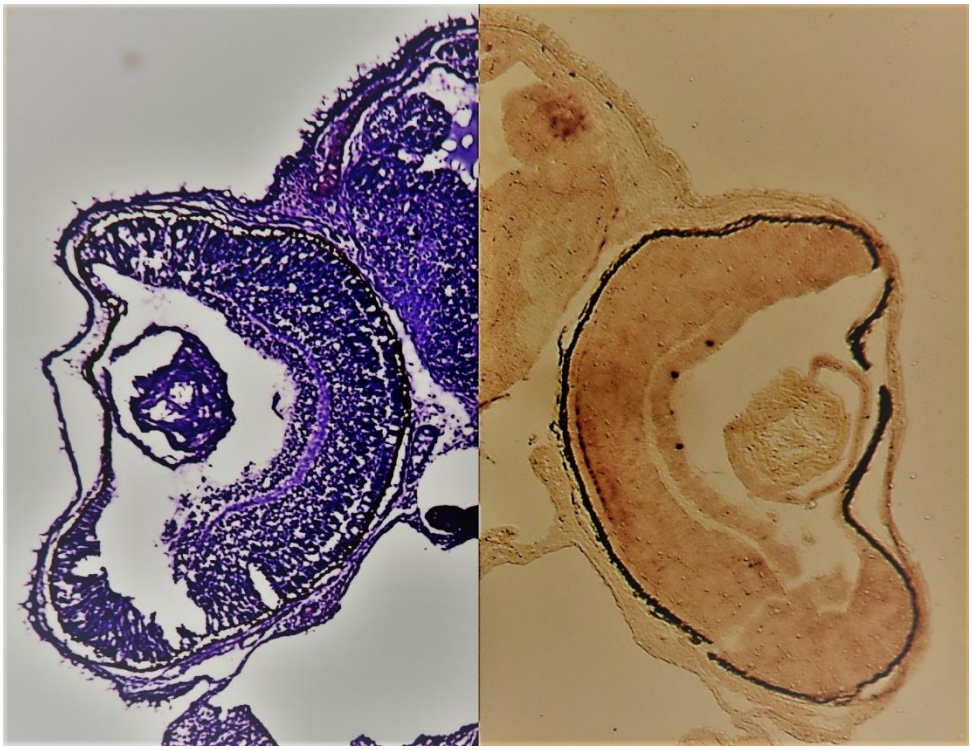
Functional analysis of the nonvisual opsins melanopsin and vertebrate ancient opsin (VA opsin) in Atlantic salmon (*Salmo salar*)



Thesis submitted in partial fulfilment of the requirements for the degree

Master of Science in Developmental Biology and Physiology

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Dedication

For those who believed in me, My Parent,

For those who were always besides, My Brothers,

For the one I have loved without limits, My Wife, and for my exceptional mother-in-law

For my unborn baby, Barra

For the carer and compassionates, My Friends,

For all who believe in their chances, never abandon their goals, the achievers.

I dedicate this.

Contents

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Abstract

The role of nonvisual photoreceptors is yet to be elucidated regarding the link to biological function. This study aims to characterise the expression pattern of melanopsin and vertebrate ancient opsin during early developmental stages of Atlantic salmon, and to relate the function of these genes to hatching.

Fertilised salmon's eggs were subjected to different light qualities using LED technology where intensity and spectrum was manipulated. Two light regimes, 24 hours of continuous light (LL) and 14 hours of light:10 hours of darkness (LD) of white light of different intensities, high, medium, and low, beside different light spectrum of the same intensity, deep red, amber, green, blue, royal blue, and ultra violet were used. Continuous dark was used as a control. Eggs were monitored during the study period and hatched eggs recorded. *In situ* hybridization technique was used to characterise the expression of two nonvisual opsin, the vertebrate ancient opsin (VA) and melanopsin.

The results from hatching experiment show that, while continues white light and LD cycles of white light of the medium intensity increase the hatching period (span), LD cycles of the low intensity white light decease it significantly. However, the time to 50% of hatching is significantly increased by LD cycles of green light blue light, and low intensity of white light.

The results from the expression experiments has shown that both melanopsin and VA opsin are expressed in the brain of salmon during the early developmental stages. Both were found in the left habenula, thalamus, hindbrain and spinal cord. Moreover, they have been found to be co-localised in several regions in the brain. Furthermore, regional specific neural activation was found in the habenula and hindbrain, where melanopsin and VA opsin are co-localized, upon light stimulation. This indicate direct photoreception in these brain regions already around hatching.

The result indicates that, apparently, the hatching process in salmon may be affected by light to some degree, but there is no strong inhibition of hatching by light such reported for Atlantic halibut. Other factors like the temperature and low levels of oxygen might be other environmental cues that are used by Atlantic salmon to regulate the time of hatching. The nonvisual system is clearly developed and functional prior to hatching and may be part of the regulation of hatching. The specific nonvisual hindbrain cluster found to regulate hatching in Atlantic halibut are not apparent in salmon. Our data clearly shows the important of nonvisual

photoreception in the brain at early developmental stages of fish, prior to development of functional eyes. There seems to be species-specific patterning of the nonvisual photoreceptors in the brain, which indicates species specific tailoring of biological function.

1 Introduction

1.1 Light in animal's life

The main natural light source is sunlight, nevertheless, the other secondary sources such as moonlight, starlight, luminescent organisms etc. might be considered in specific circumstances. Light is essential to life for almost all living organisms (Boeuf and Le Bail, 1999).

Its irrefutable that, all creatures and different types of life rely on upon light and additionally its distinctive properties e.g. periodicity, power, and spectral properties to regulate their conduct and physiology, furthermore, the light modifies with the sun oriented cycle therefore make the creatures adjust to the photic changes (Boeuf and Le Bail, 1999). While the vertebrate eye is in charge of picture framing vision, and the retina can recognize spatial and ghostly contrasts of light, the nonvisual photoreception supplies creatures with estimations of irradiance and nondirectional photoreception (Davies et al., 2010; Peirson et al., 2009).

Circadian rhythms speak to organic cycles that have a time of about a day length and numerous physio-behavioural varieties rely on upon this, for example, body temperature, hormonal change, heart rate, rest, and intellectual execution (Berson, 2003; Foster, 2002). On the other hand, this biological daily clock should be harmonized with the solar day (dawn and dusk), and it seems to provide a good indicator for the changing in intensity and spectral of light (Roenneberg and Foster, 1997).

In fishes, where many species have been studied, early development, growth, and sexual maturation and reproduction are dependent on the seasonal changes, the day interval is considered as a vital hint for timing the seasonal events (Bromage et al., 2001; Villamizar et al., 2011). Furthermore, light properties (intensity, quality, and periodicity) can be extremely variable, as in regard to fish, in their aquatic environment light shows interesting dynamicity as the altitude, latitude, and water depth influencing the properties of light significantly (Villamizar et al., 2011). Consequently, the majority of living organisms there have light sensitive receptors to detect the solar rhythms (Davies et al., 2010; Peirson et al., 2009) and should be considered when studying the light-dependant biological processes (Boeuf and Le Bail, 1999).

1.2 Nonvisual opsins

A wide range of opsins have been detected up to date in vertebrates (Davies et al., 2010). They play a major role in photoreception and vertebrates use these opsins/vitamin A-based photoreceptor, which consist of an opsin protein bound to vitamin-A chromophore (Peirson et al., 2009). These 7 transmembrane opsins belong to the superfamily of guanine nucleotide binding protein (G protein) coupled receptors (GPCR) which function through the activation of a G protein and activation of an effector enzyme (Bockaert and Pin, 1999). Functionally, opsins have been identified as visual and nonvisual (Davies et al., 2010). The later received an extensive interest regarding their important role in photo-entrainment (Davies et al., 2010). Among these, melanopsin (*opn4*) and vertebrate ancient opsin (VA) will be under focus of the current study.

1.2.1 Melanopsin (*opn4*)

A resemblance of opsin was isolated from *X. laevis* dermal melanophores cDNA, and termed melanopsin *opn4* (Provencio et al., 1998). The gene was detected to be expressed in the retinal horizontal cells, the retinal pigment epithelium (RPE) and iris. In the brain, it has been localized within magnocellular preoptic nucleus and the suprachiasmatic nucleus, moreover, all these areas have been suggested as photoreceptive (Provencio et al., 1998). In contrast to VA opsin, melanopsin orthologues were isolated from mammals and it was strictly expressed in human and mice photosensitive retinal ganglion cells (pRGCs) (Provencio et al., 2000; Provencio, Rollag, & Castrucci, 2002). Furthermore, many other orthologues have been identified later in a wide range of species as reviewed by (Davies et al., 2010) including Atlantic salmon (Sandbakken et al., 2012). Currently, there are two established forms of melanopsin in nonmammalian vertebrates, *Xenopus*-like form (*Opn4x*) which was identified in the *X. laevis* (Provencio et al., 1998) and mammalian-like form (*Opn4m*) human retina (Provencio et al., 2000). The former is expressed in nonmammalian vertebrates while the latter is widely detected in all vertebrates (Bellingham et al., 2006).

1.2.2 Vertebrate ancient (VA) opsin

Vertebrate ancient opsin (VA opsin) was identified originally from ocular cDNA from Atlantic salmon (Soni and Foster, 1997). Phylogenetically, it was assumed to have diverged from a common ancestor early in vertebrate evolution, hence the name ancient. Though, a closer insight on the phylogenetic tree of vertebrate opsins indicates that many opsins pre-date VA opsin (Davies et al., 2010; Max et al., 1995; Okano et al., 1994) . After being discovered in salmon, other VA family members have been identified in other teleost species. A very long

carboxyl-terminus VA in common carp (Moutsaki et al., 2000) regarding the short one in salmon. However, both short and long isoforms were identified later in zebrafish (Kojima et al., 2000), and, a medium isoform was identified in smelt fish (Minamoto and Shimizu, 2002). Variable carboxyl-tail considered as a feature of other non-cone non-rod opsins even though the clear cut functionality is not revealed yet (Davies et al., 2010). Currently, many VA opsin orthologues have been identified in the majority of vertebrate classes. The exception from the role is the mammals as none of the mammalian lineages in the genome database revealed any VA opsin orthologues. This might be attributed to that, VA opsin gene was lost early in the evolution of the modern mammals (Davies et al., 2010).

1.3 Non-image forming photoreception in teleost

Teleost and other studied nonmammalian vertebrates have revealed multiple photoreceptors structures which include the retina, pineal organ and deep brain areas (Ekström and Meissl, 1997; Falcón et al., 2009), though, the privilege of having multiple photoreceptors is not clear so far. Several studies have attempted to elucidate the role of these regions. Fernandes et al. (2012) narrowed the photosensitive region to neurons in the preoptic area, and suggested that it is the *opn4a* positive cells in this area that mediates dark photokinesis. Kokel et al. 2013 found that "the hindbrain is both necessary and sufficient to drive a photomotor response, a robust and reproducible series of motor behaviors in zebrafish that is elicited by visual wavelengths of light but does not require the eyes, pineal gland, or other canonical deep-brain photoreceptive organs". More recent work reported *opn4*, neuropsin *(opn5)* and VA opsin as possible deep-brain photoreceptors that might be responsible for the onset a development of reproduction (Kang and Kuenzel, 2015). Nevertheless, the answer to the questions like 'what are the functional roles of vertebrate photoreceptors, or which photoreceptors are responsible for important biological processes' remained unknown, therefore (Davies et al., 2015) suggested a further work to determine the full functional role of vertebrate photoreceptors.

1.3.1 Photoreception in the retina

The retina of vertebrate's eye is considered as a conserved structure through the evolution and it is composed of different cell types which organized in a highly ordered layers (Pujic and Malicki, 2004), in which rod and cone photoreceptors (image forming structure) occupy the outer retina, while the retinal ganglion cells occupy the inner retina and relay visual information to the brain through the optic nerve (Butler and Hodos, 2005). A small subset of retinal ganglion cells express melanopsin as had been shown in many studies (Berson, Dunn, & Takao, 2002; Hattar, Liao, Takao, Berson, & Yau, 2002; Sekaran, Foster, Lucas, & Hankins, 2003). A recent

study identified six melanopsin genes from Atlantic salmon, these genes have found to be belong to two different groups, mammalian-like (*Opn4m*) and Xenopus-like (*Opn4x*). This study showed a differential co-expression of *Opn4m* and *Opn4x* in retinal ganglion, amacrine and horizontal cells (Sandbakken et al., 2012). On the other hand, *opn4* was not the only photoreceptor to be detected in the retina hence VA opsin was isolated also from salmon and later from other teleost and other nonmammalian vertebrates (Pierce et al., 2008).

1.3.2 Photoreception by the pineal organ

Pineal organ is responsible of production and releasing of melatonin (Ekström and Meissl, 1997), in Atlantic salmon, it is regulated by the intensity of ambient light with a high level at night and low levels at day (Ekström and Meissl, 1997). The normal fluctuation of melatonin levels in the blood reflects the daily rhythm of light and thus has a potential to conduct light information to hypothalamus-pituitary axis (Porter et al., 1998), Furthermore, multiple photoreceptor receptors detected to be expressed in Atlantic salmon and Atlantic halibut pineal organ including VA opsin (Philp et al., 2000) and melanopsin (Eilertsen et al., 2014) respectively.

1.3.3 Photoreception by deep brain photoreceptors

As reviewed by (Foster et al., 1994), the results of Karl Von Frisch work in 1911 was the first to suggest the presence of deep brain photoreceptors in vertebrate from which he summarized that, the response involved a photoreceptor which is localized in the basal hypothalamus. Furthermore, other functional studies implicated the role of this photoreceptor in behavioural light responses and photoperiodic regulation of reproduction in teleost experimentally (Day and Taylor, 1983). Later, more specific studies identified multiple photoreceptor families in the brain of teleost like VA in the hypothalamic region in Atlantic salmon (Philp et al., 2000; Soni and Foster, 1997). Melanopsin was also identified in Atlantic cod from different areas including supraoptic/chiasmatic nucleus (SOC) and habenula of the brain (Drivenes et al., 2003). Moreover, recent study detected the presence of melanopsin in the habenula, suprachiasmatic nucleus, dorsal thalamus, and lateral tubular nucleus of first feeding larvae of Atlantic halibut (Eilertsen et al., 2014). Whilst in Atlantic salmon, Opn4m was found to be expressed in the dorsal thalamus, the nucleus lateralis tuberis of the hypothalamus, and Opn4x is expressed in the dopaminergic, hypophysiotrophic cell population of the supraoptic/chiasmatic nucleus and in the serotonergic cell population of the left habenula (Sandbakken et al., 2012).

1.4 Influence of light on hatching

In halibut, hatching was found to be affected negatively by light, as light was arresting the hatching process (Helvik and Walther, 1992). Later, hatching was found to be regulated by nonvisual opsin in hindbrain of Atlantic halibut, where a transient bilateral cells cluster has shown an expression of VA opsin and melanopsin at embryonic stages (Eilertsen et al., 2014). Furthermore, studying zebrafish development under different visible light wavelength revealed a high hatching rate under blue and violet light (Villamizar et al., 2014). Not much have been elucidated about the hatching of Atlantic salmon regarding the influence of light. However, studying light periodicity revealed that, in a light regime of 16L:8D (16 hours light:8 hours dark) results in a rhythmic hatching pattern, in which most eggs will be hatching during the light hours. Furthermore, in the same study, in DD regime, the eggs hatched continuously during the 24-hour periods, with no significant difference in hatching between the previous natural day and night periods was found. Again, the emergence of alevins was well synchronized to the external LD cycle, where they started to leave the gravel just before the lights were turned off and the highest number of fry emerged during the first hour of darkness (Brännäs, 1987). Additionally, Villamizar et al., (2013) conducted a study on three fish species with different daily rhythms (nocturnal, diurnal, and neutral/blind). Their results pointed to the existence of daily rhythm of hatching vary among the different species.

Light emitting diodes (LEDs) which are used to produce light in this study is considered as a new lighting technology system established for the fish farming industry. It can be adjusted to fulfil environment and species requirements through narrow bandwidth outputs (Migaud et al., 2007). This speciality would provide a major advantage of manipulating the light to produce specific wavelength to induce the different photoreceptors in variable areas in the brain.

Here in this study, the periodicity, the intensity, and the wavelength will be enrolled to detect the possible influence of light on the hatching of Atlantic salmon.

1.5 Atlantic salmon

Atlantic salmon farming industry in Norway considered the largest of its kind in the world, moreover, great efforts are oriented toward fulfilling the maximum health and economy benefits. Therefore, continuous improvement of the quality and quantity of the production is needed (Liu et al., 2011). In this context, integrated farming methods are required from the early points of production process. The control of photoperiod to achieve the optimal growth has been intensively studied during salmon's post hatching stage (Good and Davidson, 2016).

Many studies have shown a significant light influence on early life stages of different fish species (Villamizar et al., 2011).

Photoperiod regulates many developmental event during the life cycle of Atlantic salmon including growth, migration and maturation (McCormick et al., 1998). For instance, time of hatching and emergence of alevins reported to be affected by photoperiod as discussed in the study conducted by (Brännäs, 1987). Economic-wise, controlling the pre-harvesting maturation is critical to flourishing of the salmon farming industry, which has been proved to be controlled by photoperiod (Leclercq et al., 2011). However, the specific photoreception mechanism by which these important processes is not clear yet, though a suggestion that enrol the involvement of pineal organ which has been shown to be the first differentiated light receptor of Atlantic salmon (Östholm et al., 1987). However, new promising results from RT-PCR and *in situ* hybridization showed early expression of melanopsin and VA opsin during development in Atlantic salmon, and may be the first functional light receptors in the organism as they have been found prior to the development of visual photoreceptors in retina (Sandbakken, 2011), which has argued by (Östholm et al., 1987) not differentiated before hatching.

(Sandbakken, 2011) detected melanopsin in RGCs and horizontal layer as well as hypothalamus and suggested that, the expression of these nonvisual receptors in different cell populations reinforces the possibility of functional diversification of the melanopsins groups, moreover, the different timing of the onset of expression of the melanopsins likely reflects the differential rate of development of these cell types.

1.6 Project objectives

The nonvisual opsins melanopsin and VA opsin are known to be expressed in the eye and brain of larval and adult stages of Atlantic salmon, but little is known about the expression early in development even though the nonvisual photoreceptor system seems to be the first light detectors. The project has two aims. The first is to characterize the expression pattern of melanopsin and VA opsin at the early developmental stages around hatching. The second is to use stimulation by narrow bended LED light to potentially relate the function of these genes to light-regulated processes such as hatching.

1.6.1 Study questions

- Do the light parameters, periodicity, intensity, and spectrum, influence the timing of hatching?

- Does the timing of hatching depend on the early developed nonvisual photoreceptors in the brain?

2 Material and Method

2.1 Place and period of the study

The experiments were conducted at the laboratory facility of Marin developmental biology (Helvik-team), Høyteknologisenteret (HiB), University of Bergen, Norway, according to the local animal care guidelines. The study was a part of a project entitled: Photobiology and muscle development. The experiments and data analysis was carried out between May 2016 and May 2017.

2.2 Animal housing

Eggs were collected from one females and sperms were collected from three males in January 21st, 2016 from Marine Harvest Tveitevågen, Askøy. Fertilization was conducted at the light lab facility, HiB, Department of Biology, University of Bergen, Norway.

After the fertilization, the eggs were placed on hatching pads (Fish Tec AS) inside the incubation chambers at the light lab facility. The water was supplied from Svartediket with a flow rate of approximately 1 litre/minutes to ensure good oxygen conditions and that hatching enzymes are continuously washed away from the experimental unit to avoid affecting unhatched eggs.

The lighting conditions were applied though light-emitting diodes (LEDs) from January 30th, 2016 when the eggs were at early cleavage. Hatching was expected to be between May 6th, 2016 (450-day degree-dd) and May 16th, 2016 and (500 dd). Experiments and the handling of embryonic animals do not require ethical clearance as stated by the Norwegian Veterinary Authorities guidelines.

2.3 Experimental design

2.3.1 Experimental conditions

The experiment was conducted in the light lab facility. Eleven light chambers (Figure 2.1) were distributed in U-shape as shown by Figure 2.2. Each chamber dimensions were as following: length = 45 cm; width = 45 cm; Hight (distance to water surface) = 15 cm. Water temperature were adjusted by a header tank by mixing hot and cold water. Temperature was recorded every 10 minutes using 5 probes inserted in 5 tanks (Figure 2.2).

Total number of 1980 eggs were distributed equally into three replicates per chamber (180 egg per chamber).

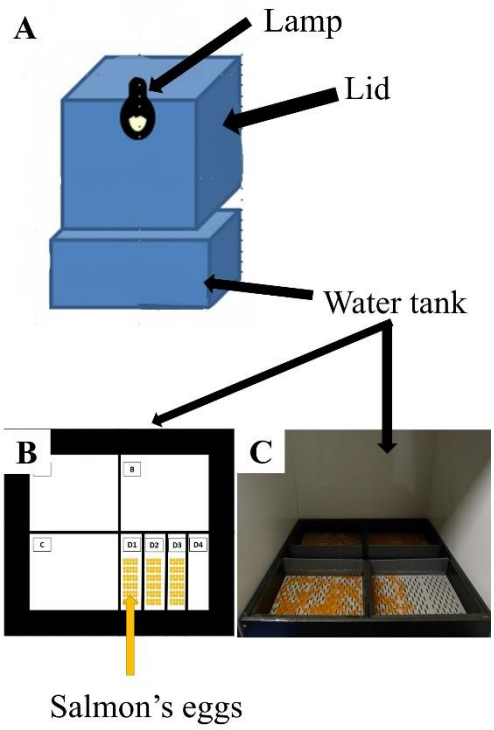

Figure 2.1 Illustrate the chamber design. A: the chamber composed of the water tank and a lid, both are light proof, lamp was installed in the chamber lid. B: schematic diagram of the water tank from inside shows the three partitions (replicates). C: the photo of the chamber, showing the 4-cells per chamber. In the experiment, only one cell was partitioned to be used.

2.3.2 Light conditions

Chambers were equipped with LEDs to provide light of different intensities and wavelengths. The experiments were done by three different intensities of white light (W), high (W_{High}) 1 W/m^2, medium (W_{Med}) 0.1 W/m^2, low (W_{Low}) 0.01 W/m^2 and six different light spectra of a medium intensities (0.1 W/m^2), deep red (DR_{Med}), amber (A_{Med}), green (G_{Med}), blue (B_{Med}), royal blue (RB_{Med}), and ultra violet (UV_{Med}) (Figure 2.2). Photon flux was adjusted in µE/m2/s using optical sensor (RAMSES ACC-VIS) (Table 2.1). Lastly one chamber, the control, equipped with no LED light (DD).

The lighting periods applied were 14-hours of light: 10-hours of darkness (LD), 24-hours constant light (LL), and 24-hours of constant darkness (DD). Dawn and dusk were imitated by dimming up and down the light 30 minutes before it goes on or off. Light intensity was adjusted using uEinstein measurements to ensure similar photon flux in the different light spectrum.

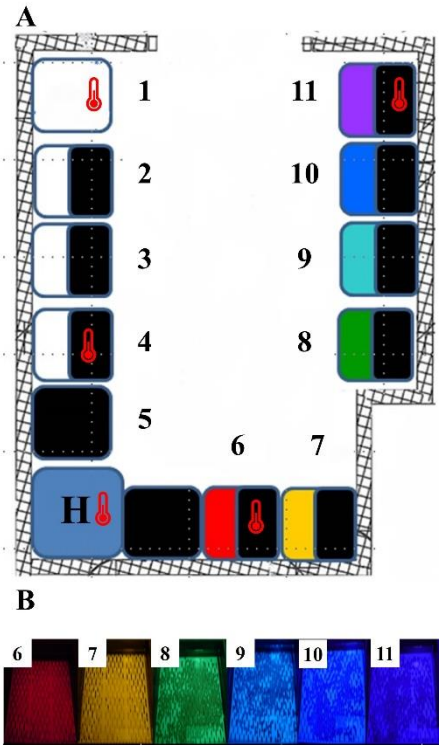

Figure 2.2 Showing the different wavelength used during the hatching experiment. A: schematic diagram of the light lab, chambers distributed in U-shaped design. 11 chambers were used. H: the header tank. 1: continuous light (LL). 2: Light/dark cycles of white light (high intensity). 3: Light/dark cycles of white light (medium intensity). 4: Light/dark cycles of white light (low intensity). 5: continuous darkness. 6: Light/dark cycles of red light (medium intensity). 7: Light/dark cycles of amber light (medium intensity). 8: Light/dark cycles of green light (medium intensity). 9: Light/dark cycles of blue light (medium intensity). 10: Light/dark cycles of royal blue light (medium intensity). 11: Light/dark cycles of ultra violet light (medium intensity). Thermometer mark in chambers (1, 4, 6, 11, and H) indicate where the temperature probes were installed. B: the photo of the chambers under different lights. Number corresponds to A.

Table 2.1 The photon flux for each light intensity.

Colour (light cycle)	Photon flux µE/m^2/s
W(LL_{Med})	0.4925
W(LD_{High})	4.9035
W(LD_{Med})	0.4932
W(LD_{Low})	0.0507
DR(LD_{Med})	0.4505
A(LD_{Med})	0.4536
G(LD_{Med})	0.4509
B(LD_{Med})	0.4503
RB(LD_{Med})	0.4504
UV(LD_{Med})	0.4501
DD	-

2.4 Hatching experiment

Hatching experiments were carried out to test the effect of the light periodicity, light intensity, and light spectrum on the hatching process. They were conducted during the period from the 2nd of May 2016 (428 dd) to 20th of May 2016 (521 dd).

The eggs were monitored daily, whilst monitoring, a special red-light lamp was used to prevent undesirable light exposure which might interfere with the experiment settings. Hatching was recorded manually in a printed paper with the same experiment design (appendix).

2.4.1 Hatching percentage experiment

Eggs were reared in the following light regimes: LLW_{Med}, LDW_{High} LDW_{Med}, LDW_{Low}, LDR_{Med}, LDA_{Med}, LDG_{Med}, LDB_{Med}, $LDRB_{Med}$, $LDUV_{Med}$, and DD. The hatching rate was calculated as the total number of hatched eggs divided by the total number of fertilized eggs. Result was analysed to reveal if there are differences between treatment (light regimes) and control groups (LL_wMed and DD).

2.4.2 Hatching period experiment

Eggs were reared in the following light regimes: LLW_{Med}, LDW_{High} LDW_{Med}, LDW_{Low}, LDR_{Med}, LDA_{Med}, LDG_{Med}, LDB_{Med}, $LDRB_{Med}$, $LDUV_{Med}$, and DD. The hatching span (the number of days took all the eggs to hatch during the experiment period) for each light regime was recorded. Result was analysed to reveal if there are differences between treatment (light regimes) and control groups (LL_wMed and DD).

2.4.3 50% of hatching

Data for all replicates was plotted using Microsoft excel. Day degrees were plotted against percentage of accumulative hatching for all the groups. A line was generated at 50% to estimate the corresponding value for each replicate. These values were then tested for significant difference.

2.4.4 Hatching rhythmicity experiment

Eggs were reared in the following light regimes: LLW_{Med}, LDW_{High} LDW_{Med}, LDW_{Low}, LDR_{Med}, LDA_{Med}, LDG_{Med}, LDB_{Med}, $LDRB_{Med}$, $LDUV_{Med}$, and DD. The hatching during light and during dark was monitored at two times, 30 minutes before light goes off (to check hatching during light period) and on (to check hatching during dark period). Total number of eggs from both periods was then analysed to reveal if there are differences between treatment (light regimes) and control groups (LL_wMed and DD).

2.5 Melanopsin and Vertebrate Ancient opsin characterization

In situ hybridization was used to characterise the two nonvisual opsins. The purpose of the study was not to express differentially the paralogues and isoforms., Therefore, a mixture of melanopsin probes (opn4m1a1, opn4xa1, opn4x1b1/2) was used and the probe for VA opsin was a general probe, detecting different paralogues and isoforms of the VA opsin gene (Table 2.2). These probes were used throughout all the expression experiment.

Initially, the two photoreceptors were localised in the mid-hatch stage to determine their expression within the brain, then potential co-localisation within the same brain region was identified. Additionally, the expression was investigated in different developmental stages to characterise the temporal expression. Eventually, analysis of the immediate early gene *c-fos* was done to show the potential neural activation upon light stimulation to correlate the expression pattern to the nonvisual opsins. For all the experiments (localization, co-localization, temporal expression, and *c-fos* activation), eggs from DD were used for *in situ* hybridization (ISH) on sections to characterize the expression pattern of melanopsin and VA opsin in the brain.

2.5.1 Localization and co-localization and temporal expression of melanopsin and VA opsin

At every collection time, 30 eggs were collected in 50 ml pre-labelled tubes, anesthetized by buffered MS-222 (Vnr. 140729, Finquel vet. 100%. Tricainmesiat 100%, Metacain, for Atlantic salmon, rainbow trout and Atlantic cod) for 5 to 10 min, the yolk was flushed with 4% paraformaldehyde-buffered (4% PF) in PBS (pH 7.4), and the embryos were fixed in 4% PF for 48 hours at 4°C. Then they were briefly washed with 1XPBS, dechorinated, and treated with 25 % sucrose: 25% Tissue-tek in 1xPBSfor 24 hours at 4°C. Then they were moved into new sucrose solution and stored at – 80°C until mounting and sectioning.

Embryos were mounted in a mould of 20% sucrose: 80 % Tissue-Tek and rapidly frozen on an iron block which was precooled in liquid nitrogen. Parallel sectioning (10 µM) was done with a Leica CM 3050S cryostat. Two parallels were produced from each developmental stage. Sections were collected on SuperFrost Ultra Plus glasses. Before storage at - 20°C, the tissue was air dried for 45 minutes at room temperature and for 45 minutes at 65° C.

For the localization experiment, two parallels from mid-hatch stage sections were used. One for ISH the other for Nissl's staining 0.5 % Cresyl Violet. For co-localization experiment two

parallels from mid-hatch stage were used for ISH, one for melanopsin probe, the other for VA opsin probe.

2.5.2 *C-fos* activation experiment

At every collection point, 18 eggs from DD regime were transferred equally into two 500 mL beakers which filled with water from DD chamber (the water level in the beaker matches the one in the chamber water volume). One beaker containing 9 eggs was re-placed into DD (control group), while the other 9 eggs were placed into the LDW_{Med} regime (treatment group). They were both left for 120 minutes then the eggs were separately collected into 50 ml pre-labelled tubes and processed the same way as the previously described in 2.4.1. section.

For *c-fos* activation experiment, four parallels from mid-hatch stage were used (two for each the control and the treatment). One of the parallels used for *c-fos* probe (sense/antisense) and the other for VA opsin probe.

2.6 Probe preparation

Digoxigenin (DIG)-labelled riboprobes (Table 2.2) for three melanopsins, two VA opsin, and *c-fos* were made following the manufacturer's instructions (Roche Diagnostics, Germany). In the synthesis of the riboprobes, PCR product was used as template for the reaction as described in (Thisse and Thisse, 2008) and the synthesised probes were precipitated by LiCl and EtOH together with tRNA (Roche Diagnostics, Germany).

Table 2.2 The primers used in PCR to generate sense/antisense probes to detect the expression.

Probe (length)	**Primer**	**Antisense and sense (AS, S)**
opn4m1a1 (704 bp)	F:5' gctccatcctcttctgcgattg 3' R: 5' tgcatggaggtggtgaagaag 3'	AS:5' taatacgactcactatagggtgcatggaggtggtgaagaag 3' S:5' cattaaccctcactaaagggaagctccatcctcttctgcgattg 3
opn4xa1 (1032 bp)	F:5'tggcaatactaatggtgtggctt3' R:5'ggctgatgatgatttgtgggatac 3'	AS:5' taatacgactcactataggggctgatgatgatttgtgggatac 3' S:5' cattaaccctcactaaagggaatggcaatactaatggtgtggctt 3'
opn4x1b1/2 (887 bp)	F:5' aggcatggacaacatggacc 3' R: 5' ggttatagatggctgaggctttgg 3'	AS:5'taatacgactcactataggggttatagatggctgaggctttgg 3' S:5' cattaaccctcactaaagggaaaggcatggacaacatggacc 3'
VA opsin (1040 bp)	F: 5' cgaggagkagagtcyaaattaag 3' R: 5' tagatkactgggttgtagactgc 3'	AS: 5' taatacgactcactatagggtagatkactgggttgtagactgc 3' S:5'cattaaccctcactaaagggaacgaggagkagagtcyaaattaag 3'
***C fos* (1095-1098)**	F1:5'acgatgatgtactcsgctttc 3' R1:5'gctgagggartcagaggaytg 3'	AS:5'taatacgactcactataggggctgagggartcagaggaytg 3' S:5'cattaaccctcactaaagggaaacgatgatgtactcsgctttc 3'

2.7 *In situ* hybridization

In situ hybridization protocol was provided from Helvik lab facility (Sandbakken et al., 2012). No modifications were made on the protocol unless otherwise stated.

2.7.1 Hybridization (day one)

Prior to *in situ* hybridization, the sections were air-dried at room temperature for 45 minutes then baked at 65°C for 45 minutes. They rehydrated in descending gradient of ethanol (90, 70, and 50%) for 1 min, and were washed for 1 minute in 2X saline-sodium citrate (SSC), then permeabilized with proteinase K (10 µg/ml in 0.1 M Tris-HCl pH 7.5) for 3.5 minutes, and post-fixed in 4% paraformaldehyde (PF) for 5 minutes, followed by rinsing 2X2 minutes in 1X phosphate-buffered saline (PBS). To reduce background staining, tissue was treated with 0.1 M triethanolamine (TEA) pH 8.0 for 3 minutes then with 0.25% acetic anhydride in 0.1 M TEA for 10 minutes, followed by rinsing for 1 minutes in 2X SSC. Finally, tissue was dehydrated in ascending gradient of ethanol (50, 70, 90, and 2X2 100%) and air-dried for 1 hour during which a hydrophobic frame was marked around the tissue using a PAP pen.

For hybridization, approximately 200 ng DIG-labelled probe in 100 µL hybridization solution was applied to each slide. The composition of the hybridization solution was: 10 mM Tris-HCl, 300 mM NaCl, 20 mM EDTA, 0.2% tween-20, 1% blocking solution (15 g Blocking Reagent in 30 ml 5x Maleate pH 7.5), 0.1% dextransulphate, and formamide. Incubation was carried out at 65°C overnight (16 hours) using preheated humidity chambers in which 2X SSC-wet paper was applied. Hybri-slips were used to cover each slide to prevent evaporation.

All hybridization solutions were made/diluted in Diethylpyrocarbonate (DEPC) water, and for all probes, sense probe was applied as a control for nonspecific staining.

2.7.2 Post-hybridization treatment (day two)

After hybridization, tissue was washed 2 X 30 minutes in 2X SSC, 30 minutes in 50% deionized formamide in 2X SSC at 65°C, and 2X10 minutes in 2X SSC at 37°C. Then the tissue was treated 20 minutes with RNase A (0.02 mg/ml) at 37°C, and washed 20 minutes with RNase buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 M NaCl) at 65°C. The sections were incubated 2 hours with 2% blocking solution in 2X SSC with 0.05% Triton X-100 and then overnight (16 hours) with alkaline phosphatase-conjugated sheep anti-DIG goat antibody (1:2000) in a plastic box with water-wet paper to keep the humidity.

2.7.3 Visualization (day three)

To remove redundant antibody, tissue was washed 2X10 minutes in 1X maleate buffer (20 mM maleic acid, 30 mM NaCl, pH 7.5) and then 10 minutes in visualization buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The staining reaction with chromogen substrate (3.4 µL Nitro-blue Tetrazolium, 3.5 µL 5-bromo-4-chloro-3-indoylphosphate) was carried out for 24 hours for (VA probe) and 48 hours for (*Opn4* probes) in darkness at 4°C. The reaction was terminated with stop solution (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0) and tissue was mounted in 70% glycerol (in 1X PBS). Photographs were taken using a digital camera CoolSNAP-PRO, attached to Leica M420 microscope. Images were taken using the image acquisition and processing software Image-Pro Plus, version 7.0. Image were gathered and enhanced using Microsoft paint and Microsoft photo editor. Scale bars were added to the photos using imageJ software.

2.8 Nissel's staining

Sections were air-dried for 1 hour and they were baked in 65°C for 10 minutes. Then they were rehydrated in an ethanol-series (96%, 70%, 50% in dH2O) for 1 minute in each solution, afterward, they were rinsed for 1 minute in dH2O. The stain was developed by dipping the object glass with cryo-sections for 1-2 seconds in 0,5% Cresyl violet in dH2O (Cresyl Fast Violet). To remove excess colour, the object glasses were dipped in 70 % EtOH in dH2O. Differentiation of the colour was done in 96 % EtOH in dH2O for 3-5 seconds. When achieving the desired colour (nerve cells strongly violet against a colourless background), the sections were dehydrated in 100 % EtOH in 2x 2 minutes. Then they were transferred to xylol for 5 minutes followed by 2 minutes for clearing. Lastly, DPX was used to glue the cover glass.

2.9 Data analysis

Data analysis and graphs was done by graphpad prism software (version 7) unless otherwise stated.

2.9.1 Light period

Light period was tested to detect the potential influence on hatching period, percentage, and the time to 50% of hatching. One way ANOVA was used to compare the mean between the groups. Variables analysed were light regime (predictor) and number of days for each group for all hatch events, percentage of hatched eggs, and time to 50% of hatching (responses).

2.9.2 Light intensity

Light intensity was tested to detect the potential influence on hatching period, percentage, and the time to 50% of hatching. One way ANOVA was used to compare the mean between the groups. Variables analysed were light regime (predictor) and number of days for each group for all hatch events, percentage of hatched eggs, and time to 50% of hatching (responses).

2.9.3 Light spectrum

Light spectrum was tested to detect the potential influence on hatching period, percentage, and the time to 50% of hatching. One way ANOVA was used to compare the mean between the groups. Variables analysed were light regime (predictor) and number of days for each group for all hatch events, percentage of hatched eggs, and time to 50% of hatching (responses).

2.9.4 Hatching rhythmicity

Hatching rhythmicity experiment were analysed using multi-proportion analysis. Chi square test (χ^2) was performed to compare between the number of hatched eggs during the light or dark periods in all groups. Variables analysed were period of treatment, light/dark (predictor) and number of hatched egg (response).

3 Results

3.1 Hatching experiment

The hatching experiment was done to investigate the effect of light period (Table 3.1), intensity (Table 3.2), and spectrum (Table 3.3) on hatching. The number of hatched eggs, the percentage, the number of days, and the day degrees are expressed as Mean±S.E.M.

3.1.1 Light period

One way ANOVA was calculated on the light period to test the effect on hatching percentage, hatching period, and time to 50% of hatching. The result in (Figure 3.1) has revealed that, light period does not seem to affect the hatching percentage or the time to 50% of hatching, however, it influences significantly the hatching period.

For the hatching period, LLW Med was significantly different from the control P = 0.001. In the test for hatching percentage, no significant difference detected between the treatments LDW Med, LLW Med, and the control DD, P = 0.200. Regarding the 50% of hatching, no significant difference was also detected, P = 0.302.

Table 3.1 Light periodicity. Effect of light periods on the hatching period (in days), percentage (% of hatched eggs) and the time to 50% of hatching (day degree). Value are expressed as Mean±S.E.M. groups with different superscripts in the same row are significantly different (P < 0.05)

	MEAN+SEM		
	DD	LLW MED	LDW MED
HATCHING PERIOD	8±0.8[a]	14±0.3[a]	11.17±0.6
HATCHING PERCENTAGE	96.7±2.0	91±4.6	99.3±0. 7
50% OF HATCHING	481±2.1	478.7±1.8	476±2.3

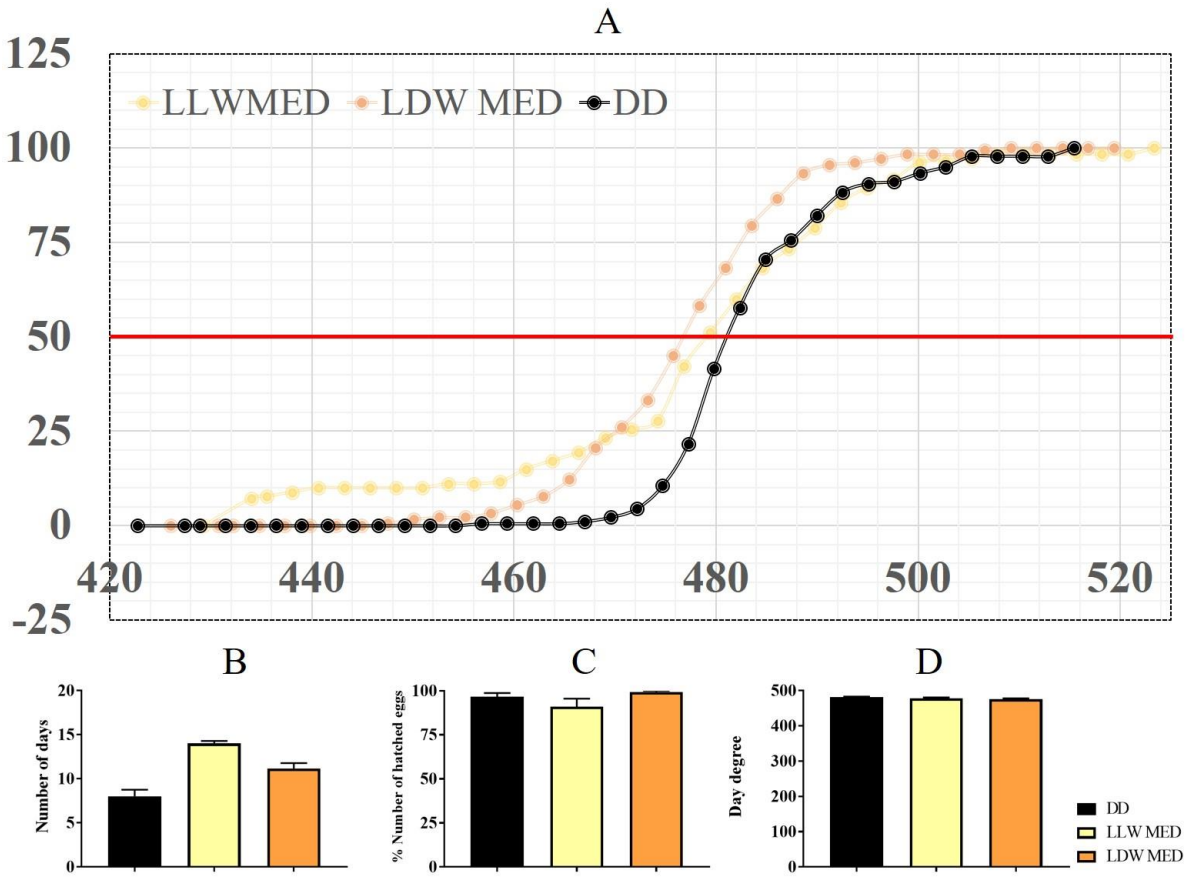

Figure 3.1 Shows the effect of light period treatments, the continuous dark DD (control), continuous light LLW MED, and the light dark cycles LDW MED. A: the % of cumulative hatching per treatment, the red line drawn to calculate the 50% of hatching. B: the number of days for all hatching period per treatment. C: the percentage of hatching in the three treatments. D: the time to 50% of hatching. Values in B, C, and D demonstrated as mean ± S.E.M.

3.1.2 Light intensity

One way ANOVA was calculated on the light intensity to test the effect on hatching percentage, hatching period, and time to 50% of hatching. The result in (Figure 3.2) has revealed that, the intensity of light does not seem to affect the hatching percentage, yet, it influences significantly the hatching period, and the time to 50% of hatching.

For the hatching period, LDW Med was significantly different from the control DD, and LDW Low ($P = 0.007$). However, no difference between the treatments LDW Med, LDW High and LDW Low and control DD was detected regarding the hatching percentage $P = 0.504$.

The test for time to 50% of hatching has shown significant difference between the treatment and the control $P = 0.002$.

Table 3.2 Light intensity. Effect of light intensity on the hatching period (in days), percentage (% of hatched eggs) and the time to 50% of hatching (day degree). Value are expressed as Mean±S.E.M. groups with different superscripts in the same row are significantly different ($P < 0.05$).

	MEAN+SEM			
	DD	LDW HIGH	LDW MED	LDW LOW
HATCHING PERIOD	8 ± 0.8^{a}	9.6 ± 0.8	$11.8\pm0.6^{a\,b}$	7.8 ± 0.7^{b}
HATCHING PERCENTAGE	96.7±2.0	97.7±1.5	99. 3±0. 7	96. 7±0.9
50% OF HATCHING	481 ± 2.1^{a}	481 ± 0.9^{b}	476 ± 2.3^{c}	490.7 ± 1.3^{abc}

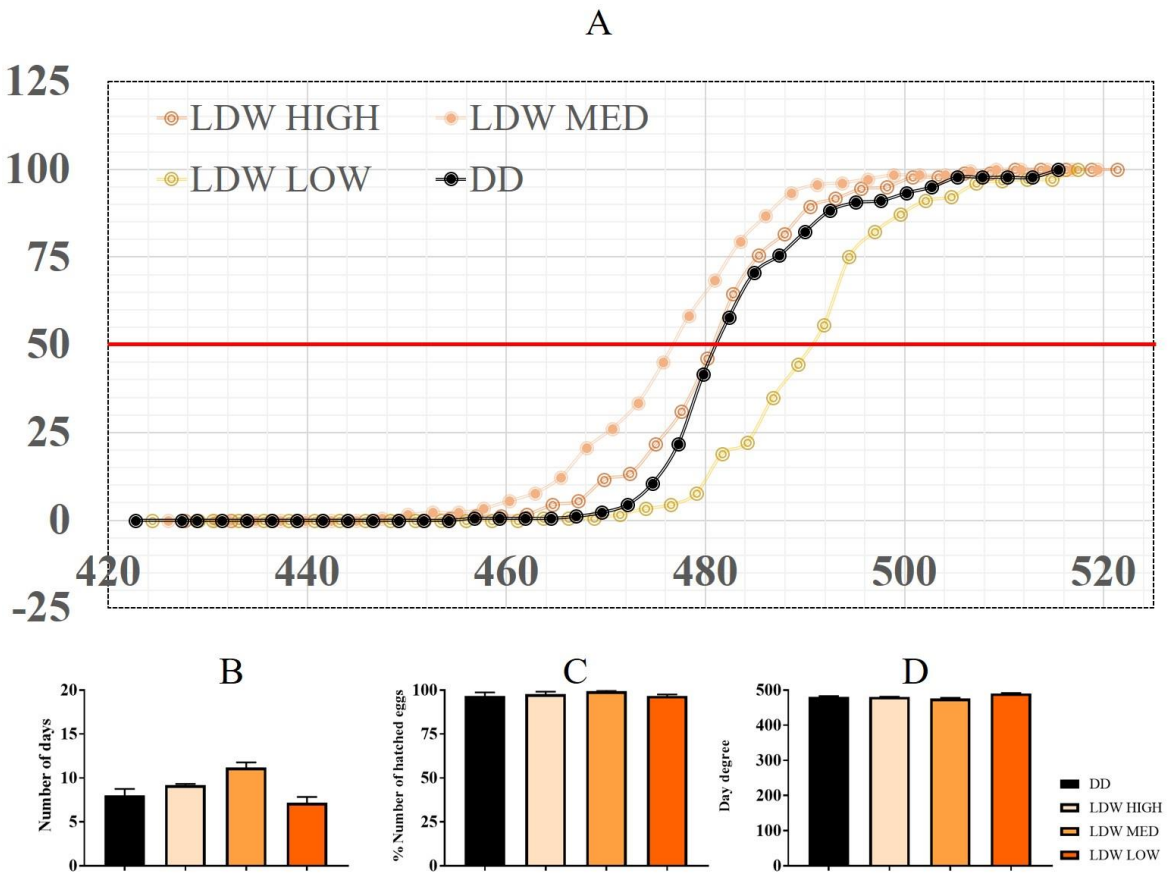

Figure 3.2 Shows the effect of light intensity treatments, the continuous dark DD (control), and the light dark cycles of high (LDW HIGH), medium (LDW MED), and low light intensity (LDW LOW). A: the % of cumulative hatching per treatment, the red line drawn to calculate the 50% of hatching. B: the number of days for all hatching period per treatment. C: the percentage of hatching in the three treatments. D: the time to 50% of hatching. Values in B, C, and D demonstrated as mean ± S.E.M.

3.1.3 Light spectrum

One way ANOVA was calculated on the light spectrum to test the effect on hatching percentage, hatching period, and time to 50% of hatching. The result in (Figure 3.3) has revealed that, neither the hatching period nor the percentage of hatching are affected by the light spectrum. However, it influences significantly the time to 50% of hatching.

Tests for hatching period and the percentage of hatching did not show significant difference, P = 0.073, 0.582 respectively. However, the test for time to 50% of hatching has revealed significant difference between the control and the treatments LDGR MED and LDRB MED. Additionally, significant difference was also detected between the treatments LDGR MED and LDUV MED was detected P = 0.005.

Table 3.3 Light spectrum. Effect of light spectrum on the hatching period (in days), percentage (% of hatched eggs) and the time to 50% of hatching (day degree). Value are expressed as Mean±S.E.M. groups with different superscripts in the same row are significantly different (P < 0.05).

	MEAN+SEM						
	DD	LDDR MED	LDAM MED	LDGR MED	LDBL MED	LDRB MED	LDUV MED
HATCHING PERIOD	8±0.8	7±0.9	8.7±1.9	12.7±1.8	8.5±0.3	10.8±0.4	7.2±1.9
HATCHING PERCENTAGE	96.7±2.0	98.3±0.9	97.7±0.3	99.3±0.7	98±0.0	99.3±0.7	97.7±0.1
50% OF HATCHING	481±2.1^{ab}	490.7±2.3	490.3±1.3	495.3±4.8^{ac}	490.7±0.9	493.3±2.3^{b}	481.7±0.7^{c}

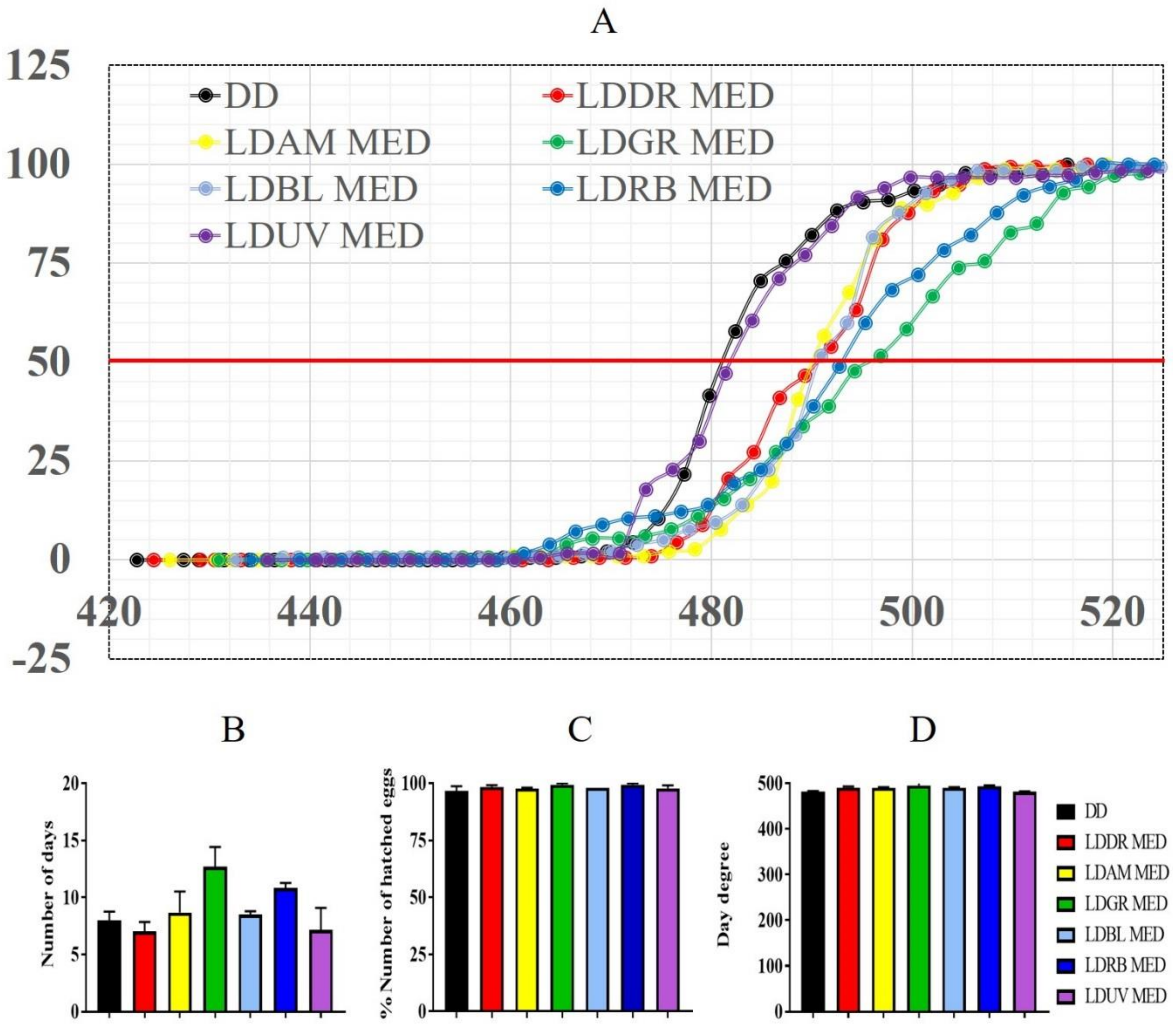

Figure 3.3 Shows the effect of light spectrum treatments, the continuous dark DD (control), and the light dark cycles of medium intensity of red light (LDDR MED), amber light (LDAM MED), green light (LDGR MED), blue light (LDBL MED), royal blue light (LDRB MED), and the ultra violet light (LDUV MED). A: the % of cumulative hatching per treatment, the red line drawn to calculate the 50% of hatching. B: the number of days for all hatching period per treatment. C: the percentage of hatching in the three treatments. D: the time to 50% of hatching. Values in B, C, and D demonstrated as mean ± S.E.M.

3.1.4 Hatching rhythmicity

Hatching rhythmicity tested for the effect of light period, light intensity, or light wavelength (Figure 3.4). T test for significant difference, chi square (χ^2) test implied on the proportion of hatched eggs during the light and the dark period.

The results of χ^2 revealed that, the light period did not affect the number of eggs hatched during the light or the dark periods. The proportion of eggs number that hatched during the light period in the treatment (LLwMed and LDwMed) and the control group (DD) was 0.46 whereas the proportion of eggs number that hatched during the dark period was 0.54 The difference in proportions was not significant, χ^2 (2, N = 517) = 0.1096, p = 0.9467.

The light intensity as well did not reveal any significant effect on the hatching period. As indicated by χ^2, the proportion of eggs number that hatched during the light period in the treatment (LDWHigh, LDwMed, and LDWLow) and the control group (DD) was 0.45, whereas the proportion of eggs number that hatched during the dark period was 0.55 The difference in proportions was not significant, χ^2 (3, N = 703) = 1.774, p = 0.6206.

Lastly, no effect of light spectrum was detected between the treatments (different light wavelengths) and the control. The proportion of eggs number that hatched during the light period in the treatment (LDwMed, LDRMed, and LDAMed, LDGMed, LDBMed, and LDRBMed, and LDUVMed) and the control group (DD) was 0.43, whereas the proportion of eggs number that hatched during the dark period was 0.57 The difference in proportions was not significant, χ^2 (7, N = 1417) = 10.46, p = 0.1642. Figure 3.1 show the proportion of hatched eggs in the light and dark period.

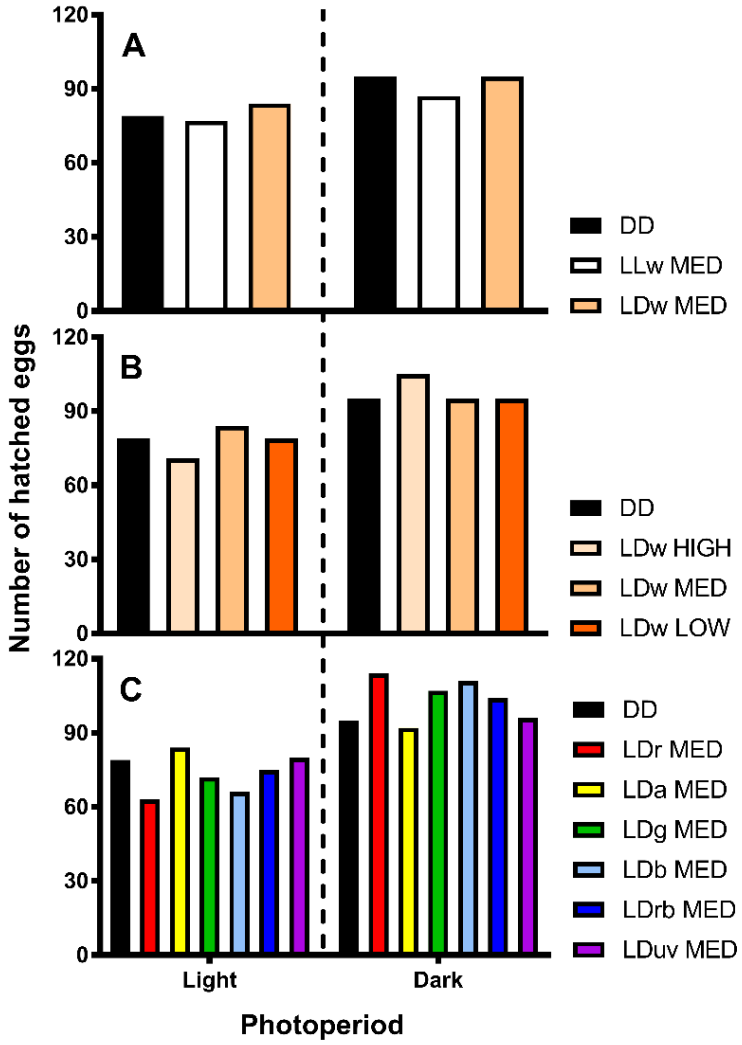

Figure 3.4 The effect of light periods (A), intensities (B) and wavelengths (C) on the rhythmicity of hatching. Each bar represents the total number of eggs hatched during the light or dark period.

3.2 Expression of Melanopsin and Vertebrate ancient opsin

In situ hybridization was performed to detect the expression of melanopsin and vertebrate ancient opsin in the brain of early developmental stage of Atlantic salmon. Additionally, the co-localization of both nonvisual photoreceptors was investigated and detection of the temporal expression in three early developmental stages was investigated. Lastly, the activation of immediate early gene *c-fos* was studied to explore the presence of general neural activity related to photoreceptors under the study.

3.2.1 Localization and temporal expression of melanopsin

The nondifferential localization of melanopsin by *in situ* hybridization revealed different clusters in the brain of post-hatch stage of Atlantic salmon (Figure 3.5). These clusters were identified from rostral to caudal direction as following, asymmetrical cluster was localized in the left habenula. Two successive symmetrical clusters extending caudally were identified in the thalamus region. They were centrally located. The former one in the caudal area of the dorsal thalamus and relatively longer than the later one which was narrower and close in distance. One symmetrical, short, and caudally oriented cluster was identified in the mesencephalon. This cluster was, extremely narrow, and centrally-located. One cluster was localised in the hindbrain however, the precise location could not have been identified (1^{st} or 2^{nd} rhombomeres). It was symmetrical and short in length, wide however centrally-located. The last cluster was moderately long and was showing a narrow spatial expression in the central area of the spinal cord.

The expression analysis for the temporal expression in the pre, mid, and post-hatching stages (Figure 3.6) revealed the same results in the all developmental stages as described above.

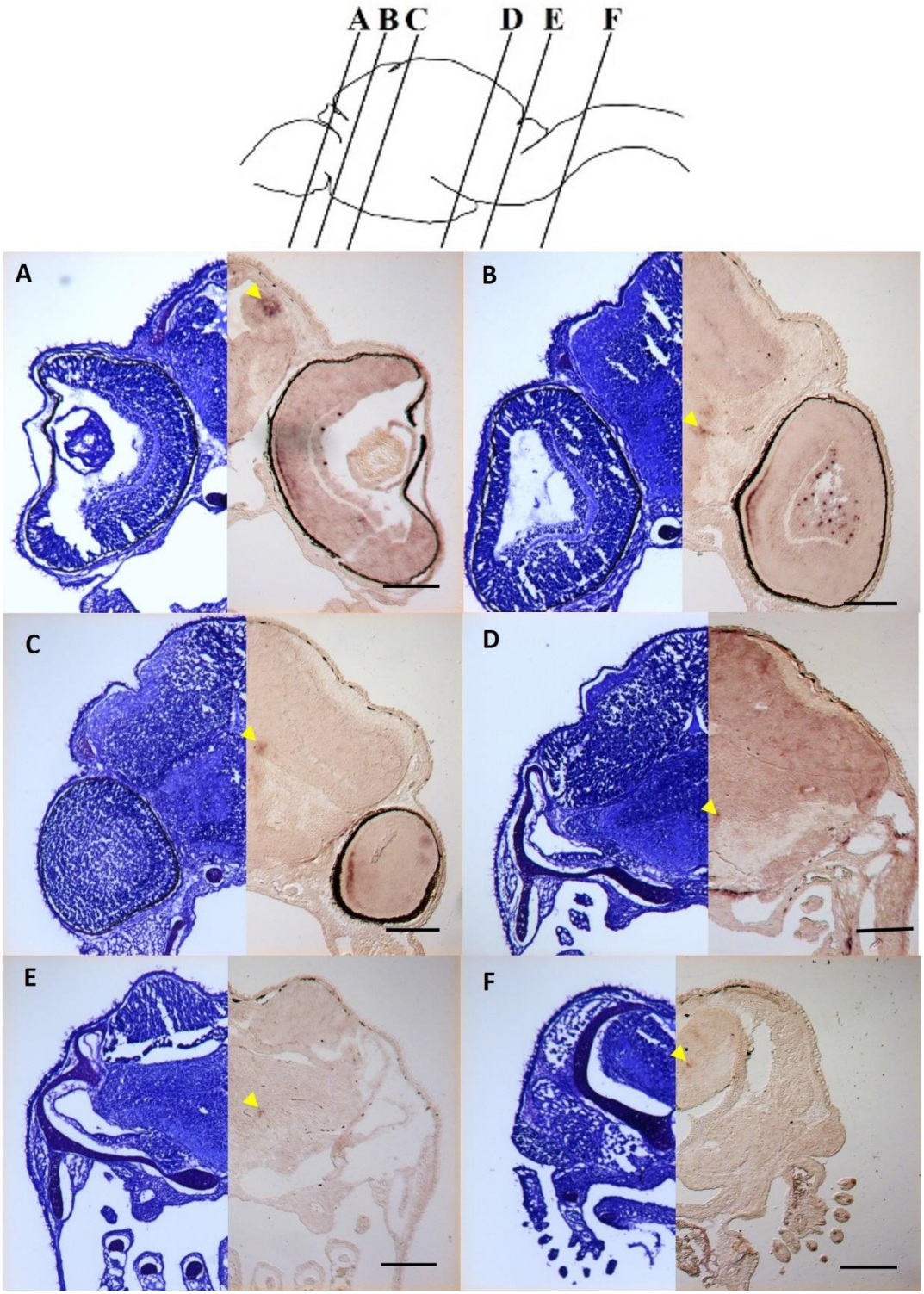

Figure 3.5 Spatial distribution melanopsin expression in the brain of Atlantic salmon (post-hatch stage). A-F: Nissl-stained sections (left) at the equivalent level of melanopsin expression (right). A: expression of melanopsin in the habenula (Hb). B, C: Expression of melanopsin in the Thalamus (Th). D: Expression of melanopsin in the mesencephalon (probably NLT region however the brain is not well-developed hence it is difficult to precisely confirm the location). E: Expression of melanopsin in the hindbrain. F: Expression of melanopsin in the spinal cord. Yellow arrowheads indicate clusters. Scale bars = 250µM

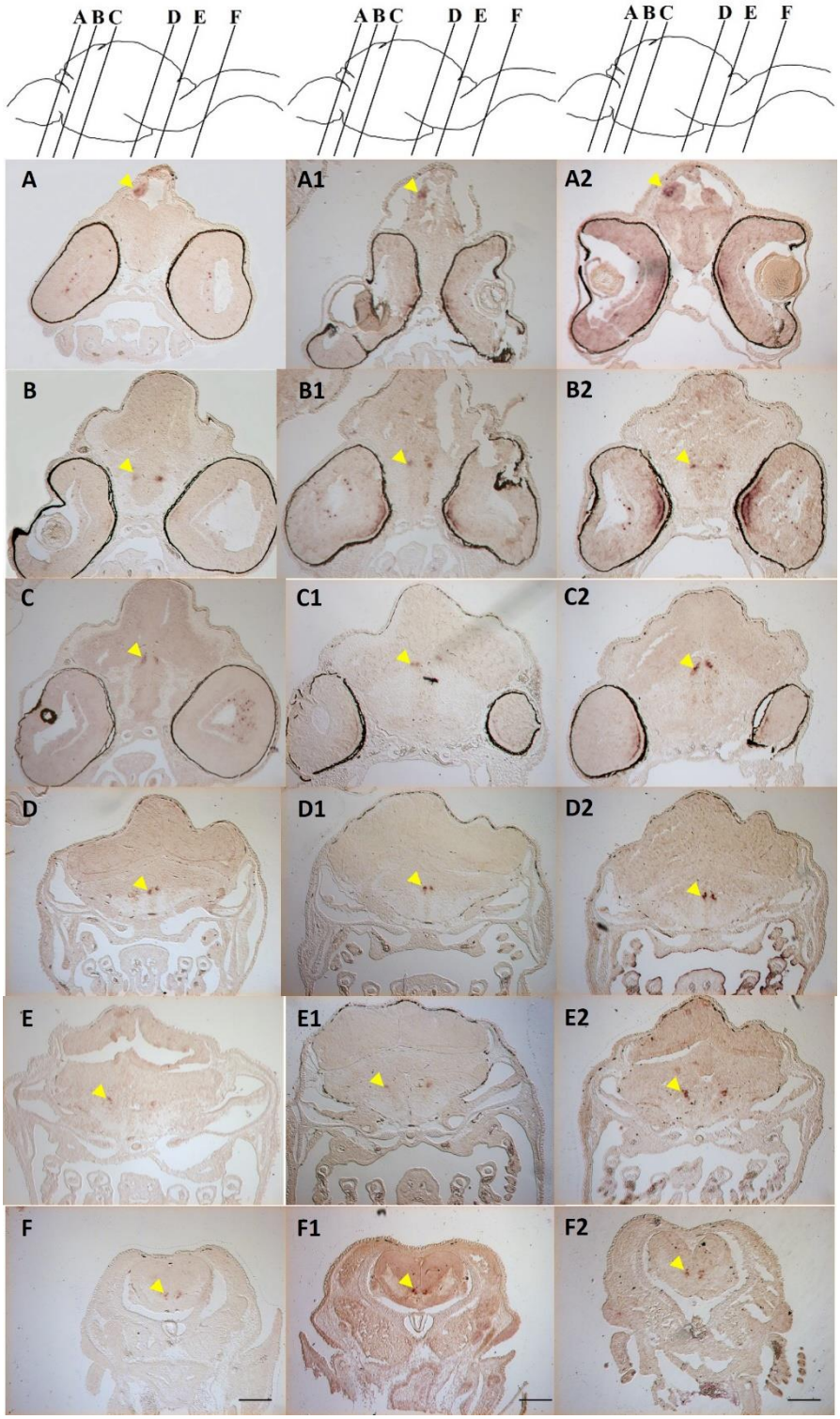

Figure 3.6 *In situ* hybridization on cryosection of 10 µm thickness showing melanopsin expression in Atlantic salmon brain at pre-hatch (A-F), mid-hatch (A1-F1), and post-hatch (A2-F2) stages. Rows A-F show 6 clusters of melanopsin at (A, A1, A2: the expression at the habenula. B, B1, B2: the expression at the thalamus. C, C1, C2: thalamus. D, D1, D2: the expression at mesencephalon. E, E1, E2: the expression at the hindbrain, (most likely 1st or 2nd rhombomere) F, F1, F2: the expression at the spinal cord). Yellow arrowheads indicate clusters. Scale bars = 250µM.

3.2.2 Localization and temporal expression of VA opsin

The results of *in situ* hybridization on the brain of the post-hatch stage of Atlantic salmon have shown seven different clusters of VA opsin (Figure 3.7). These clusters are distributed from the rostral area toward the caudal area in the following order: first, one cluster was identified only in the left habenula, followed by three symmetrical, caudally extended clusters in the thalamus region. The first one which is the longest was in the dorsal thalamus (THd), this was followed by another cluster which was at the same level behind however it was more centrally-located (narrower). The last one was in dorso-lateral to the previous one (probably near to the tegmentum), and both later clusters were short in length. Another symmetrical caudally extended cluster was short in length laterally-located and was identified in the mesencephalon region just beneath the boundary between the tegmentum and the diencephalon probably. One laterally-located, long, and symmetrical cluster was identified in the hindbrain. Again, the precise location was difficult to be identified. The last cluster was centrally-located (very narrow) and it was found in the spinal cord area. Moreover, it was very long (approximately the same length as the first cluster in the thalamus region.

The expression analysis for the temporal expression in the pre, mid, and post-hatching stages (Figure 3.8) revealed the same results in the all developmental stages as described above.

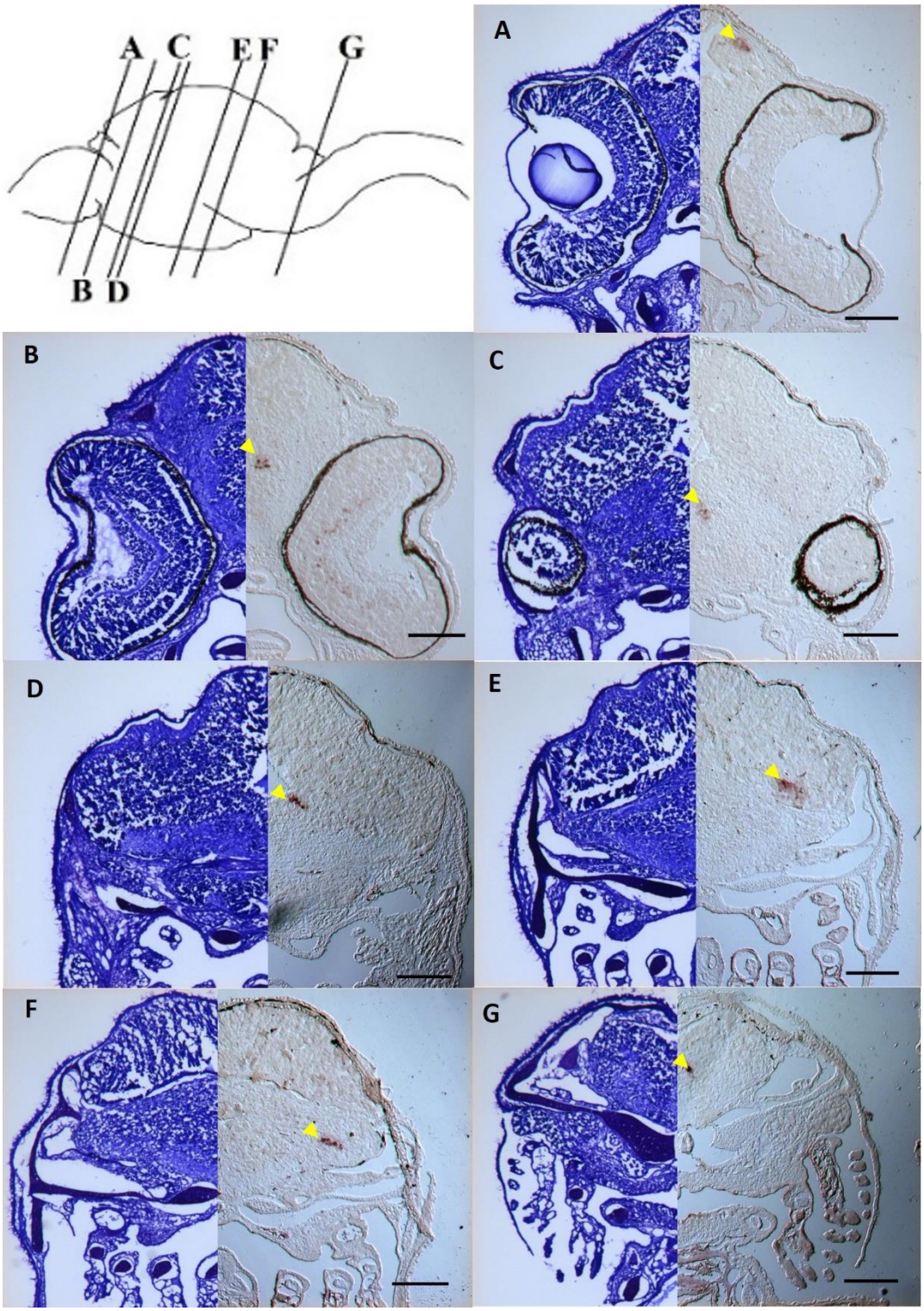

Figure 3.7 Spatial distribution of VA opsin expression in the brain of Atlantic salmon (post-hatch stage). A-G: Nissl-stained sections (left) at the equivalent level of VA expression (right). A: Expression of VA opsin in the habenula. B, C, and D: Expression in the thalamus. E: Expression in the mesencephalon. F: Expression in the hindbrain. G: Expression in the spinal cord (SC). Scale bars = 250µM.

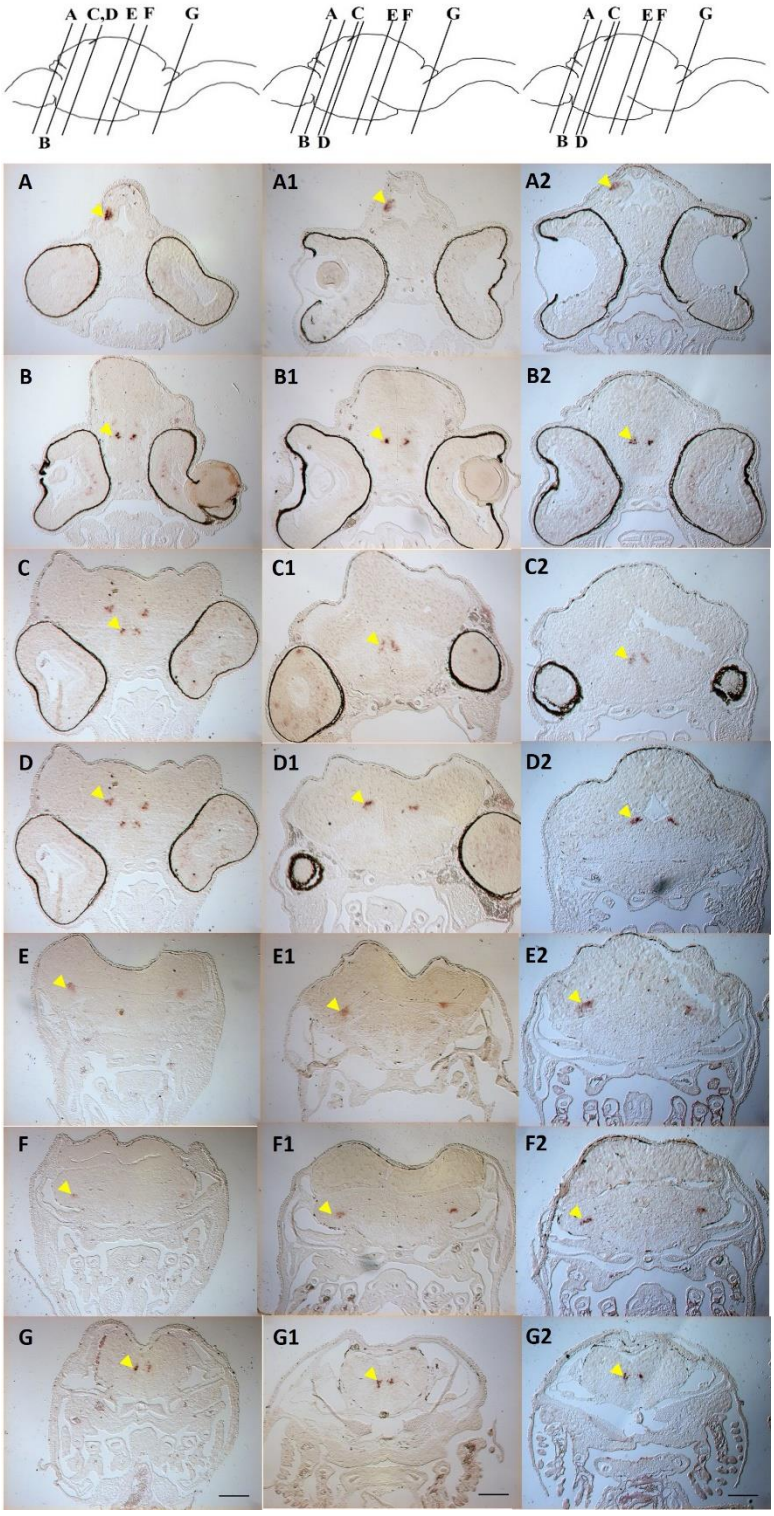

Figure 3.8 *In situ* hybridization on cryosection of 10 µm thickness showing vertebrate ancient opsin expression in Atlantic salmon brain at pre-hatch (A-G), mid-hatch (A1-G1), and post-hatch (A2-G2) stages. Rows A-G show 7 clusters of VA opsin at: A, A1, A2: expression at the habenula. B, B1, B2: expression at the thalamus. C, C1, C2: expression at the thalamus. D, D1, D2: expression at the thalamus. E, E1, E2: expression at the mesencephalon. F, F1, F2: expression at the hindbrain. G, G1, G2: expression at the spinal cord. Yellow arrowheads indicate clusters. Scale bars = 250µM.

3.2.3 Co-localization

The co-localization of the melanopsin and VA opsin (Figure 3.9) was investigated mid-hatch stage of Atlantic salmon using *in situ* hybridization technique. The results have demonstrated a mutual spatial distribution in some areas while other areas have shown co-localization at different level in the brain or did not show co-expression. The 1st co-localized expression was identified in the habenula, where both pigment were exclusively found on the left habenula. The 2nd region was the THd, here the tail of the 2nd VA opsin cluster was co-localized with the head of the 2nd melanopsin cluster, moreover both expression were approximately in the same region. Again, in the thalamus, the posterior part of the 3rd cluster of melanopsin was co-localized with the 3rd cluster of VA opsin at the same level in the brain however, the former, the melanopsin was more dorsally-located. The 4th cluster of VA opsin on the other hand did not show any spatial co-localization with melanopsin in the thalamus region. In the mesencephalon, the two photoreceptors were co-localized in the brain however, the 4th melanopsin was in the central compared to the 5th cluster of VA which was extremely laterally-located in mesencephalon, the melanopsin cluster did not show any co-localization with the VA opsin. In the hindbrain, there was co-localization of the and the 6th clusters of melanopsin and VA opsin respectively. However, the melanopsin cluster was more central than the VA which was laterally-located. Lastly, the tail of the 7th cluster of VA overlapped with the head of the 6th cluster of melanopsin in the spinal cord region, moreover, both have shown to be expressed at the same level.

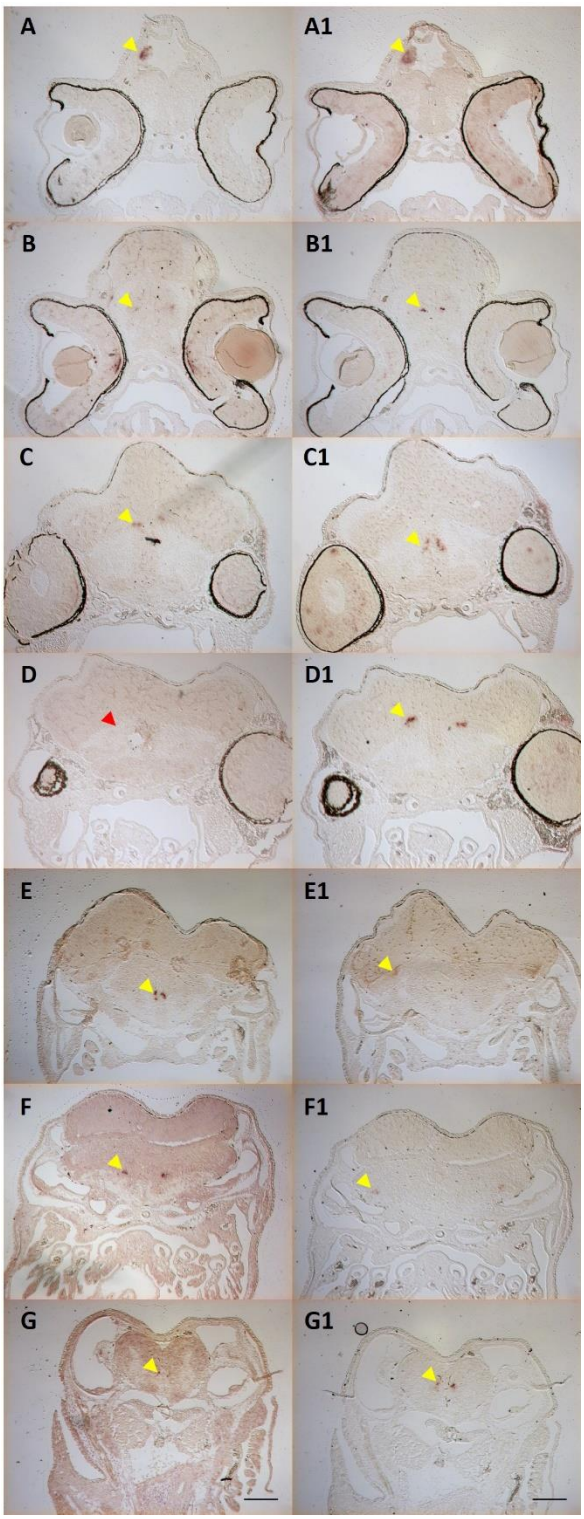

Figure 3.9 Co-localization of melanopsin and vertebrate ancient opsin expression in Atlantic salmon's brain at mid-hatch stage. A-H illustrate melanopsin expression; A1-H1 show VA opsin expression. A, A1: co-localization of the 1st clusters of melanopsin and VA in the habenula. B, B1:co-localization of the 2nd melanopsin and VA clusters in thalamus (the tail of VA cluster overlapped by the melanopsin cluster). C, C1: co-localization in thalamus (the tail of melanopsin 3rd cluster overlapped by the VA 3rd cluster). D1, D2: the 4th cluster of VA in the thalamus, no co-localization detected. E1, E2: the 4th cluster of melanopsin (narrow, central) is co-localized with the 5th cluster of the VA (lateral) in the mesencephalon. F1, F2: the 5th cluster of melanopsin (wide, central) is co-localized with the 6th cluster of the VA in the hindbrain (lateral). G1, G2: the 6th cluster of melanopsin is co-localized with the 7th cluster of VA (the tail of VA cluster overlaps with the head of the melanopsin cluster). Yellow arrowheads indicate clusters. Red arrowhead indicates the absence of clusters. Scale bars = 250µM.

3.2.4 *c-fos* activation

The early immediate gene *c-fos* was used to indicate the neural activity in the brain of mid-hatch stage of Atlantic salmon (Figure 3.10). The result of *c-fos* activation after 120 minutes has shown a neural activation pattern of VA opsin and/or melanopsin in some regions of brain compared to the control group which was kept in dark. The first region was detected in the left habenula, here a co-localized expression of the *c-fos* and VA opsin was found. In the next two regions, the thalamus and the mesencephalon, no neural activation was detected. However, there was slight regional activation in the hindbrain region where *c-fos* was found to be expressed in the treatment but not the control. Lastly, there was no activation in the spinal cord are as the result of *c-fos* expression were similar in the treatment and the control.

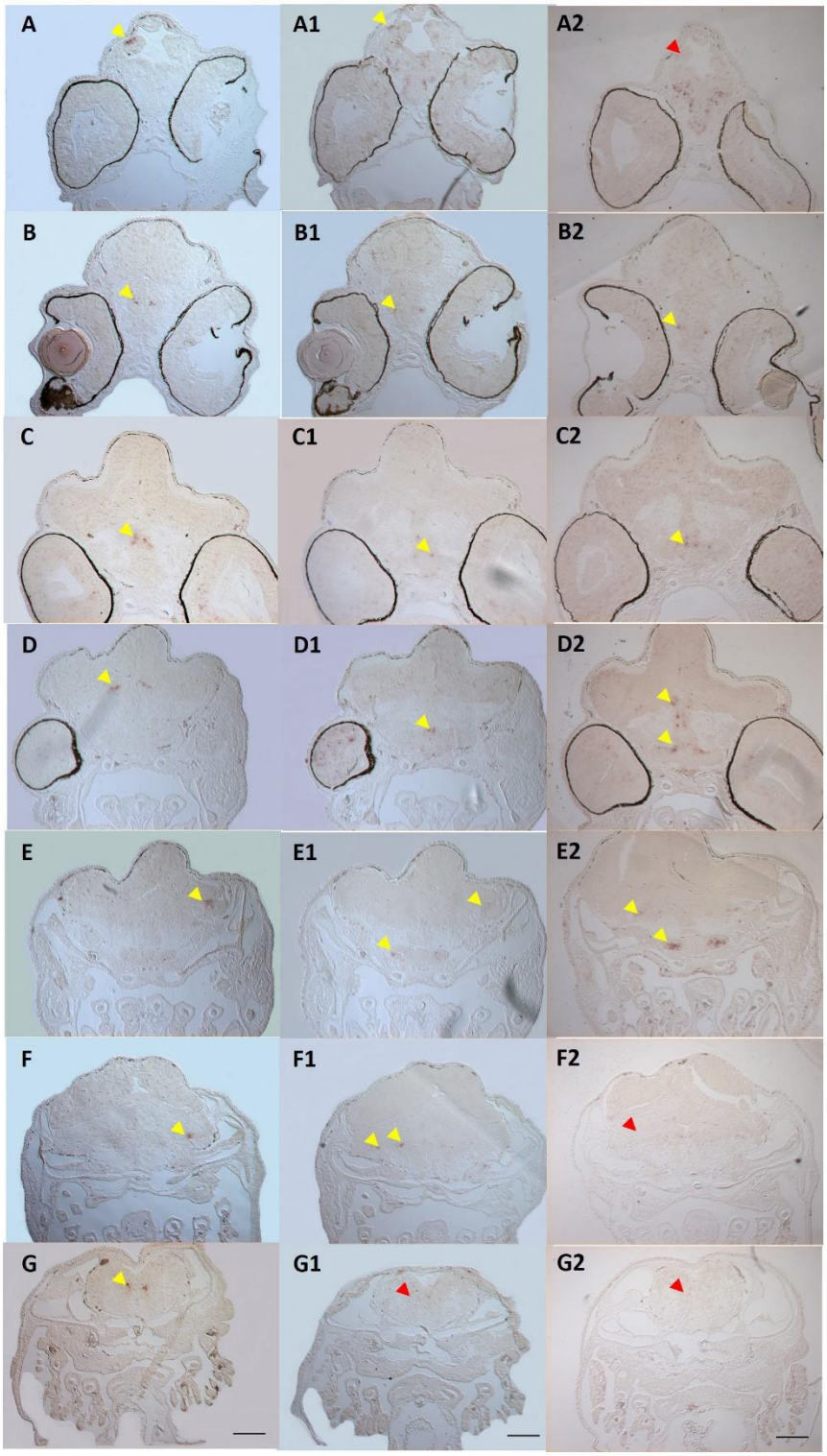

Figure 3.10 Co-localization of vertebrate ancient opsin and the immediate early gene *c-fo*s expression in Atlantic salmon's brain at mid-hatch stage. A-H illustrate VA expression. A1-H1 show *c-fos* expression (treatment) at the equivalent level. A2-H2 show *c-fos* expression (control) at the equivalent level. A, A1, A2: the *c fos* and the VA expression at the habenula, no expression in the control. B, B1, B2: expression at the thalamus region. C, C1, C2: expression at the thalamus region. D, D1, D2: expression at the thalamus. E, E1, E2: expression at the mesencephalon. F, F1, F2: expression of the VA and *c-fos* in the hindbrain, the absence of expression in the control. G, G1, G2: expression of the VA in the spinal cord, no *c-fos* expression in both the treatment and the control at the equivalent section. Yellow arrowheads indicate clusters. Red arrowhead indicates the absence of expression. Scale bars = 250µM.

4 Discussion

4.1 Methodological considerations

4.1.1 The first experiment (hatching)

This experiment was performed to investigate the influence of different artificial lighting condition on the process of hatching in Atlantic salmon. Here, different light periods, intensities, and spectra were tested (for all hatching data see Appendix 1). To evaluate the effect of light, hatching parameters were considered. First, whether light conditions would influence the hatching into one period (either light or dark). Second, if the hatching rate would be affected by different light quantities and qualities. Third, whether the total period of hatching would be decreased or increased because of different light treatments. Last, if the light conditions would influence negatively or positively the time to 50% of hatching in different treatments.

The fact that all eggs were from one female might have influence the true replica of the experiment, moreover, the water flow was shared by the three replicates in each group. Ideally, the eggs should be collected from more than one female and the water flow should be completely separated between the three replicates within the group. Additionally, there was temperature fluctuation during the experiment (adjusted day degree (age) according to the temperature fluctuation is available in Appendix 2), which could also be considered as essential factor the experiment.

The temperature has been monitored every 10 minutes using 5 probes distributed in 5 tanks, header tank, LL, LDWLow, DR, and UV. There was unexpected elevation in the temperatures detected during the hatching experiment, therefore, the temperature at each time point during hatching monitoring was estimated by calculating the mean of all temperatures recorded.

4.1.2 The second experiment (Melanopsin and VA expression)

In situ hybridization is a well-known technique to identify specific DNA or RNA in a cell, tissue, or a whole mount samples. The concept of the technique is to hybridize the complementary strand of the designed probe to a sequence. Consequently, the results could be visualised in different methods. Among these methods is by development of a histochemical chromogen for a labelled probe (Coulton and de Belleroche, 1992). The advantages of the techniques are that it provides a cellular resolution for the targeted sequence (Jensen, 2014) while the qPCR technique for example does not give.

Motivated by the previous work of Sandbakken, (2011) who have detected melanopsin expression during the early developmental stage of Atlantic salmon and based on the effect of light on the hatching and presence of transient cluster in the hindbrain of halibut during hatching (Eilertsen et al., 2014; Helvik and Walther, 1992), the desired results from the experiment is to verify the presence of nonvisual photoreceptor in the brain of early developmental stages of salmon and to link it to the hatching process.

The experiment was conducted to investigate the spatial and temporal expression pattern of two nonvisual photoreceptors in the brain of Atlantic salmon as well as the possible regional neural activation presence. To perform this experiment, three sampling point were selected by calculating the age of the eggs (day degree dd). Based on the egg's age, the three points were named pre-hatch, mid-hatch, and post-hatch. Eggs were sampled from one group DD, and random selection for the replica was done by lottery. The desired results from the experiment is to verify the non-differential presence of nonvisual photoreceptor in the brain of early developmental stages of salmon therefore, mixture of melanopsin probes was used and general VA probe was used also to detect all paralogs of melanopsin and VA opsin, respectively.

Neural activation of the proto-oncogene *c-fos* in the brain is well known method to identify the responses in the brain as a result of different stimulators (Hoffman et al., 1993), e.g. the effect of light on the deep brain photoreceptors in Atlantic halibut (Eilertsen, 2014) or the light avoidance behaviour in zebrafish (Moore and Whitmore, 2014). In halibut, the activation was recognised effectively after 120 minutes of light exposure, therefore, this time was used during the current study without modifications. From this study, it is apparent that expression of *c-fos* can be detected as a response to 120 minutes of light exposure in early stages of salmon.

The experiment was conducted to test the possible regional neuro-activity by comparing treatment and control group. Again, only one stage (mid-hatch) has been selected as it represents the time of hatching. Moreover, as most of the expression regions were co-localized, only one probe (VA opsin) was used.

The results from the experiment has shown a regional neural activation in some region of the brain, however, it is not possible from this study to relate the activation to specific photoreceptor.

4.2 Hatching experiment

The purpose of the hatching experiment was to investigate if light influence the hatching process in Atlantic salmon. We analyse effects of light periodicity, intensity, and spectrum. To evaluate the influence on the hatching process, hatching rhythm, hatching percentage, hatching period, and time to 50% of hatching were monitored.

4.2.1 Hatching period

The result from current study revealed that, light periodicity (Figure 3.1) and intensity (Figure 3.2) influence the span of hatching significantly. This was also been found previously in Baltic salmon where the light period reported to accelerate hatching contrary to continuous darkness, as 50% of eggs hatched within 2 days and 6 days respectively (Brännäs, 1987).

A study on haddock tested the effect of light photoperiod (24:0, 18:6, or 12:12 h light/dark), intensity (high and low), and spectrum (blue, green, and white) with a control groups reared in continues light or darkness on the hatching time. The result revealed that, embryos hatching occur earlier in continuous light followed by the LD cycles and DD. However, the size of both embryos reared under LL and DD were significantly smaller (Downing and Litvak, 2002).

4.2.2 Hatching percentage

The result from the current study revealed around 99% hatching in all light treatments and there was no significant influence of light period (Figure 3.1), light intensity (Figure 3.2), and light spectrum (Figure 3.3).

In marine fish like Sole, light period has shown different hatching rate in 12L:12D cycles of white light compare to continuous white light continuous darkness (48.5%) (Blanco-Vives et al., 2011). Moreover, these rates were higher than the control. A study on catfish reported that, exposure to different photoperiods might induce the hatching rate. The eggs reared in 12L:12D and 6L:18D revealed higher hatching rate than the ones that reared under natural light period and other different cycles (18L:6D, 24L:0D, and 0L:24D) (Mino et al., 2008).

Hatching rate was also found to be the highest under 12L:12D cycles compared to continuous light or darkness in three fishes of different daily rhythms. Zebrafish (diurnal), sole fish, nocturnal, and cavefish neutral (Villamizar et al., 2013).

The hatching rate was found to be influenced by light spectrum in zebrafish, with the highest rate reported in the LD cycles of blue light (Villamizar et al., 2014) as well as sole fish in which the LD cycles of red light also reported the lowest hatching rate (Blanco-Vives et al., 2011).

Although only one egg group was used in this experiment, our results indicate that the light seems not to have any negative effects on the development to such degree that it influences the ability to hatch.

4.2.3 Hatching rhythmicity

The results of this study did not reveal any difference in hatching between the dark and light periods (Figure 3.4). The effect of light on the hatching rhythmicity has been investigated earlier in salmon as well as other teleost species. The hatching has been found to be affected by light period. One study has found that, Baltic salmon eggs that reared under white lighting condition of 16L:8D has demonstrated hatching rhythmicity where eggs tend to hatch during the light period, in contrast, the ones that kept under continuous darkness have hatched continuously. Moreover, no difference has been found in in the number of eggs that hatched during the light or the dark periods (Brännäs, 1987).

Study on halibut demonstrated that, the light has negatively affected (inhibit) the hatching process, the effect involves the intensity and the wavelength of light. Nevertheless, the transfer of the light-arrested eggs to darkness results in recurring the of hatching process within 80 to 140 minutes (Helvik and Walther, 1992).

Villamizar and colleagues, 2013 have also investigated the presence of circadian rhythms regarding the effect of light and temperature in hatching of three teleost species with different daily patterns of activity; zebrafish, Senegalese sole, and the blind somalian cavefish, these fishes' express diurnal, nocturnal, and neutral (not entrained by light) activity respectively. In their study, the eggs were exposed to three light regimes, 12L:12D cycles of white light, continuous white light, and continuous darkness. Their results have shown a trend of daily rhythms of hatching which was synchronized to the LD cycles, additionally, there was a species-specific acrophases. Moreover, they noticed that, hatching rhythms are governed by a clock mechanism that restricting/orienting hatching to a particular time of day/night, consequently, if embryo has reached certain developmental state on time hatch, while the one that has not postponed until the next available window. Eggs reared in LL and DD conditions revealed hatching rhythms and this "gating phenomenon" persevered in cavefish, in contrast, zebrafish eggs split into ultradian bouts of hatching occurring at 12–18-h intervals, lastly, in sole egg's DD and LL produced a 24-h delay and advance. Another study on walleye pollock (*Theragra chalcogramma*) has shown that, the in eggs reared in continuous dark or diel light, hatching has started at the same stage (Olla and Davis, 1993).

In contrast to the results from this study which has shown no rhythmicity in hatching, longer light periods appear to influence the rhythmicity of hatching in Baltic salmon (Brännäs, 1987). While in another, the Atlantic halibut the light might turn off or delay the hatching process (Helvik and Walther, 1992). Bearing in mind that, some species live in different daily patterns (Olla and Davis, 1993), it seems to be a species-specific preference for hatching regarding different hatching periods, therefore to profile the rhythmicity, it would be more efficient to test different light period.

Apparently, mature hatching rely on other factors beside the light parameters (period, intensity, and spectrum) (Helvik and Walther, 1993). Naturally, salmon females lay the eggs inside the gravel (Brännäs, 1987) where the intensity of light is low, and the deeper the egg the harder to be reached by light. Therefore in salmon, the oxygen consumption (Oppen-Berntsen et al., 1990) as well as the temperature (Brännäs, 1987) might be more important and reliable environmental signal to regulate hatching of the eggs rather than the solely light (Helvik and Walther, 1992).

4.3 Spatial, temporal distribution, and neural activation of melanopsin and VA opsin in the brain

This study has verified the existence (Figure 3.5 and Figure 3.7) and co-localization (Figure 3.9) of the two nonvisual photoreceptors melanopsin and VA opsin in the brain of Atlantic salmon, moreover, it has revealed that, these photoreceptors are expressed in the brain of different early developmental stages (Figure 3.6, Figure 3.8). Additionally, it had also shown regional neural activation in the habenula and the hindbrain (Figure 3.10).

The study of temporal expression of these photoreceptor has not received much research attention however, two studies on zebrafish and halibut early life stages has been reported (Eilertsen et al., 2014; Matos-Cruz et al., 2011).

Previous study on the brain of Atlantic salmon parr has shown a differential expression of melanopsin (Sandbakken et al., 2012) and non-differential expression of VA opsin in the brain (Philp et al., 2000). Melanopsins have also been identified in the brain of halibut (Eilertsen et al., 2014), and cod (Drivenes et al., 2003).

4.3.1 Expression in the habenula

Melanopsin and VA opsin were found in the left habenula at the early developmental stage. This has been previously found in later stages, the parr stages of salmon (Sandbakken et al.,

2012), also in early developmental stages of Atlantic halibut (Eilertsen et al., 2014). The evolution of the habenular circuit is known to be highly conserved (Beretta et al., 2012). Hence, habenula have been detected to be innervated from the parapinal organ (Beretta et al., 2012; Ekström and Ebbesson, 1988; Ekström and Meissl, 1997; Servili et al., 2011), thus, the co-existence of two photoreceptive pigments in the habenula indicates internal photoreceptive function that may modulate and influence the function of the habenula. (Yanez and Anadon, 1996).

4.3.2 Expression in the thalamus

The study has shown that melanopsin and VA opsin appears early in thalamus, which is similar to the expression studies of older stages (parr) by Sandbakken et al., (2012) where they identified the melanopsin the dorsal thalamus. Other studies on different teleost species have identified its expression in the thalamus, as described in halibut and cod, zebrafish (Drivenes et al., 2003; Eilertsen et al., 2014; Fischer et al., 2013).

VA opsin has been identified in salmon (Philp et al., 2000; Sandbakken et al., 2012) as well as in halibut (Eilertsen et al., 2014), and zebrafish (Fischer et al., 2013; Hang et al., 2014; Kojima et al., 2000).

This region, the thalamus, has been suggested as essential brain region regarding the process of photoreception (Fernandes et al., 2013). Studies on zebrafish linked the light avoidance behaviour in zebrafish telencephalon through the thalamus (Mueller, 2012). For example, some melanopsin-expressing cells in the posterior tuberculum are part of dopaminergic cell clusters. These has been found forming long projections reaching the hindbrain and spinal cord, presumably, they may disturbingly contribute in modulating the locomotion (Tay et al., 2011). Functional divergence between the photoreceptors in thalamus and in other brain regions as it has been found that expression of VA opsin in the thalamus is light-regulated whereas in other region no effect was detected (Hang et al., 2016).

4.3.3 Expression in the hindbrain

The salmon hindbrain contains clusters of melanopsin and VA opsin expressing cells at the time of hatching. Studies by Sandbakken et al., (2012) on older stages did no describe clusters in the hindbrain. Expression of melanopsin in the hindbrain has previously been describe in medaka (Fischer et al., 2013), zebrafish (Matos-Cruz et al., 2011) and halibut (Eilertsen et al., 2014).

Studies on other teleost (halibut and zebrafish) have shown the VA opsin cluster expression in the hindbrain (Eilertsen, 2014; Hang et al., 2014; Kojima et al., 2008). In hatching stage of halibut, the presence of the expression in the hindbrain has been link to neural network projected into the yolk sac, moreover, neural activation was also detected in the hindbrain and the hatching gland (Eilertsen et al., 2014). Interestingly, the hindbrain cluster of halibut was shown to be transient around hatching, with a differential disappearance of melanopsin and VA opsin (Eilertsen. 2014). In this study, we do not see a similar transient expression, as the expression of both persists after hatching.

The hindbrain is conventionally known as a photomotor response region in the brain (Kokel et al., 2013). Moreover, this response was detected in zebrafish while the eggs still unhatched however, it has attributed to the development of the neural plasticity rather than hatching process directly (Fernandes et al., 2013). Presumably, the hindbrain expression demonstrates a photomotor response through unknown photosensitive neurons within the hindbrain (Kokel et al., 2013).

4.3.4 Expression in the spinal cord

This study also identified the presence of melanopsin and VA opsin in the spinal cord. Melanopsin has been found in halibut previously (Eilertsen et al., 2014). Accordingly, it has been suggested that, melanopsin-expressing cells might be primary sensory cells or interneurons representing the first example of dispersed high-order photoreceptor cell. No other studies have shown the expression of the VA opsin in the spinal cord of Atlantic salmon. However, other studies on zebrafish has shown positive results and it has been detected in bilateral location during specific stage which make its expression transient (Kojima et al., 2008).

Although partly overlapped in the spinal cord, the presence of both melanopsin and VA opsin suggest that the area of the expression are primary sensory neurons as previously indicated by Eilertsen et al., (2014). Furthermore, recent study in zebrafish has attributed the photo-sensitivity of spinal central pattern generator (CPG) circuit in the spinal cord to the long version VA opsin (Friedmann et al., 2015).

4.3.5 Co-expression of melanopsin and VA opsin

Melanopsin and VA opsin were found to be co-localised in different regions of the early developmental stage of Atlantic salmon (Figure 3.9). This study verifies that the habenula, the

thalamus, the hindbrain and the spinal cord contain both clusters of melanopsin and VA expressing cells.

Previous studies on salmon has also revealed a co-localization in the habenula and the thalamus (Sandbakken et al., 2012). Both regions in the brain of teleost has received reasonable attention. The habenula, for instance, is known to have an evolutionary conserved circuit, (Beretta et al., 2012), moreover, it is highly innervated from the parapinal organ (Beretta et al., 2012; Ekström and Ebbesson, 1988; Ekström and Meissl, 1997; Servili et al., 2011), therefore, a reasonable argument would assume a photoreceptive function might exist (Yañez and Anadon, 1996).

On the other hand, the thalamus is known as photoreceptive region in the brain of teleost (Fernandes et al., 2013). The projection from thalamus toward the hindbrain and spinal cord modulate locomotion (Tay et al., 2011) and light avoidance behaviour was also involves thalamus intervention (Mueller, 2012). The one would suggest that, thalamus has its own functional specialty as expression of the nonvisual opsins might be affected by light while the same expression in another region might not (Hang et al., 2016).

The current study has provided a profile for the expression during the early developmental stages which could be added to the previous work on parr stages (Sandbakken et al., 2012) (Figure 4.1). The figure illustrates that the expression of nonvisual opsins in district clusters already at early developmental stages and that the expression persists in the brain.

In the hindbrain, the co-localization has been detected in other teleost but not the salmon. In halibut, both photoreceptors were found to be co-localized in a hindbrain transient cluster (Eilertsen et al., 2014). Other studies on zebrafish have shown separately that, the two photoreceptors are found in the hindbrain (Hang et al., 2014; Matos-Cruz et al., 2011). The hindbrain is a photoreceptive region in the brain of teleost, and it has been detected that, photomotor response resulting from intense light exposure in zebrafish is mediated though it (Kokel et al., 2013).

No previous work on salmon has indicated the co-localization of melanopsin and VA opsin in the spinal cord. However, the melanopsin has been found as dispersed bilateral in the spinal cord of early stages of Atlantic halibut, hence, melanopsin-expressing cells was then suggested as the first dispersed high-order photoreceptor cell (Eilertsen et al., 2014) while the VA opsin was detected in the spinal cord of zebrafish where it has been involved in light detection during early developmental stages even before functional retina (Friedmann et al., 2015).

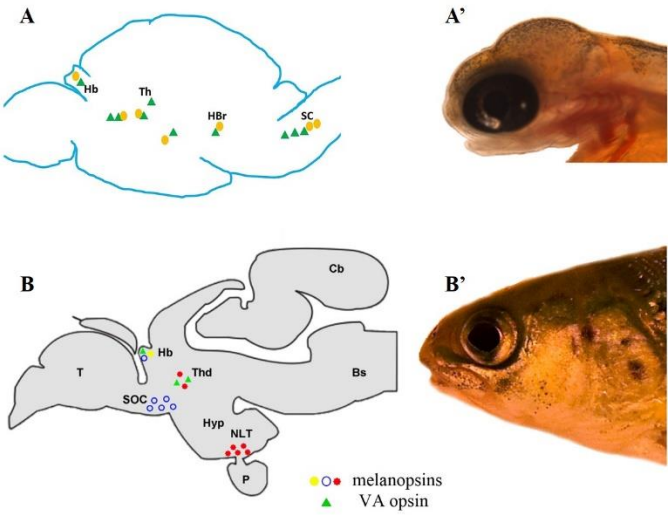

Figure 4.1 Schematic drawing summarizes the results of melanopsin and VA opsin expression in the brain of Atlantic salmon. A: represent the characterization in this current study (early developmental stages); The areas show the expression are represented by different shapes and colours. Melanopsin (yellow circles), VA opsin (green triangles). B: represent the characterization from (Sandbakken et al., 2012) in which they have characterized the expression in the later developmental stage (parr). SOC: Supraoptic /suprachiasmatic nucleus. Hb: the habenula. Th: thalamus. Thd: dorsal thalamus. Hyb: Hypothalamus. NLT: Nucleus lateralis tuberis. Bs: Brainstem. HBr: hindbrain. Cb: Cerebellum. SC: spinal cord.

4.3.6 Neural activation

The current study revealed regional neural activation in the brain during the period of hatching by using the immediate early gene *c-fos*. The regions where the neural activation was detected include the habenula and the hindbrain. Other studies have also found neural activation in different brain regions e.g. habenula, thalamus, ventral hindbrain and spinal cord in response to light in halibut and zebrafish (Dreosti et al., 2014; Eilertsen, 2014; Randlett et al., 2015)

In the habenula, neural activation was slightly detected in the embryos that was photo activated, but not in the control that were kept at darkness. The activated region of habenula co-localized with VA opsin expression which indicate a direct light activation of habenula photoreceptors. Recently, the light-response in zebrafish larvae was attributed to habenular neuron, specifically in the dorsal interpeduncular nuclei (dIPN) (Dreosti et al., 2014). Additionally, Randlett et al., (2015) found that light can increase extracellular signal-regulated kinases (pERK) expression within channel-rhodopsin expressing neuron (ChR2) in different cells including habenula. This finding enhance the theory of asymmetricity in the brain of teleost as reviewed previously (Aizawa, 2013).

These important responses during larval stage are requires probably during the early development of zebrafish's brain for generating the lateralization/asymmetry (Budaev and Andrew, 2009).

In Atlantic halibut Eilertsen et al., (2014) show neural activation of a hindbrain cluster with melanopsin and VA opsin expressing cells. This cluster seems to have projections to the hatching glands. In our analysis in Atlantic salmon there are presence of both VA and melanopsin expressing neurons in the hindbrain, but they seem not to be organised in a massive cluster as the one found in Atlantic halibut. Nevertheless, correlating the hatching directly to the neural activation of salmon's hindbrain is more complicated as the hatching cannot be arrested as in halibut (Helvik and Walther, 1992). Moreover, the activation was not found to have a cellular co-localization with the VA opsin and melanopsin expression, therefore, further work in the hindbrain cluster of salmon's brain is a substantial necessity.

4.4 Concluding remarks

A light-related hatching process has earlier been identified in halibut (Helvik and Walther, 1992). Generally, the hatching in fish is a complicated process involving a great coordination between the embryo and the environment (Oppen-Berntsen et al., 1990). Moreover, these environmental factors might vary, ranging from oxygen concentration, pH level, temperature, light or other factors (Brännäs, 1987; Helvik and Walther, 1992; Oppen-Berntsen et al., 1990). The current study has identified many areas in the brain expressing melanopsin and VA opsin. Moreover, the neural activation in the habenula and the hindbrain at the stage of hatching might indicate that the nonvisual system is functional and may contribute with environmental light cues that are important for modulation of neural signalling.

Light-influenced hatching through the nonvisual photoreception in the deep brain need more effort to be elucidated. However, this study could not indicate clear link as it has found previously in halibut.

This study was the first to indicate the presence of photoreceptors clusters in the early brain of Atlantic salmon. The study has provided evidence for the role of deep brain photoreceptors in light detection by reporting the presence of the expression of nonvisual photoreceptors, melanopsin and VA opsin, before the functional eye has developed. Furthermore, the study has shown regional neural activation in the habenula and the hindbrain during hatching.

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5 Appendices

Appendix 1. Number of hatched eggs in all light treatments. P= light period. DD= continuous dark. LL=continuous light. D1, D2, and D3 are the replicates within each light treatment.

		LLWMED			LDW HIGH			LDW MID			LDW LOW			DD			LDDR MED			LDAM MED			LDGR MED			LDBL MED			LDRB MED			LDUV MED		
Day degree	P	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
428.05031	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
432.74832	D	8	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
434.24314	L	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
436.80016	D	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
439.35881	L	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
441.91474	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
444.47148	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
447.04801	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
449.61872	L	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
452.18083	D	0	2	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
454.74546	L	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0	1	0
457.32129	D	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	3	0	0	2	0
459.89382	L	0	4	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
462.47878	D	1	1	2	0	1	0	3	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	2	4	1	0	0	0	1	2	0	0	0
465.06008	L	3	0	1	5	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	1	0	0	1	2	0	13	12	4
467.66610	D	2	1	4	1	1	0	2	6	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	1	0	1	6	2
470.26485	L	1	3	0	7	2	2	3	9	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	2	0	0	2	0	0	3	5	5
472.85487	D	2	2	0	2	1	0	1	8	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	2	3	1	1	0	1	2	6	6	19
475.44746	L	14	8	4	3	8	4	4	5	4	1	1	0	1	0	1	0	0	0	0	0	0	0	4	2	1	2	0	3	3	4	12	5	7
478.04534	D	7	5	4	2	10	5	11	4	6	1	2	0	2	2	0	0	0	1	0	1	1	2	1	5	5	1	2	1	2	3	6	2	11
480.64166	L	2	4	10	9	6	12	14	5	5	0	1	1	8	3	0	2	0	4	0	0	1	4	0	5	8	7	1	3	3	6	6	3	2
483.21756	D	4	3	8	13	9	11	2	5	11	4	2	0	13	6	1	4	0	4	1	8	0	0	3	9	6	5	5	6	7	4	5	7	1
485.79947	L	2	2	5	6	7	7	4	6	10	6	9	5	13	16	7	5	10	6	7	2	2	5	3	4	6	13	17	7	6	5	5	4	4
488.37450	D	3	3	4	4	4	3	5	2	6	1	2	3	6	10	13	2	4	6	5	6	0	3	2	4	6	5	4	3	8	9	2	2	0
490.95155	L	3	2	7	4	3	7	4	4	4	13	8	2	10	4	9	7	7	11	13	11	13	7	5	4	11	11	17	5	3	7	0	3	2
493.52859	D	1	4	2	1	1	2	1	0	3	10	2	5	1	4	4	2	2	6	14	7	8	2	5	0	1	7	3	4	2	1	0	0	0
496.1056	L	1	2	1	1	2	2	0	1	0	6	1	13	0	7	5	2	5	6	4	8	8	2	9	1	3	3	3	4	0	7	0	0	0
498.68884	D	0	5	3	0	0	1	0	0	2	9	9	17	0	1	10	13	4	0	6	2	16	4	6	5	0	3	3	3	3	1	0	0	0
501.27023	L	1	0	0	1	2	2	0	0	2	1	8	4	0	2	2	11	14	7	8	4	2	8	1	4	3	0	1	6	1	3	0	0	0
503.86170	D	1	0	0	0	0	0	0	0	0	3	4	2	0	1	0	4	5	3	0	1	1	1	2	0	0	0	0	4	2	2	0	1	0
506.45021	L	0	1	1	0	2	0	0	0	0	1	3	3	1	2	1	2	4	4	1	4	0	4	3	6	0	0	0	2	0	2	0	0	0
509.02335	D	0	0	0	0	0	0	1	1	0	0	2	0	1	1	1	1	1	1	0	1	6	1	3	0	0	0	0	3	0	0	0	1	0
511.60100	L	0	0	0	0	1	1	0	0	1	2	1	4	1	1	3	3	3	1	1	3	0	6	6	2	0	1	1	2	3	2	0	0	1
514.15013	D	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
516.70764	L	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0
519.28680	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
521.85960	L	1	2	0	0	0	0	0	0	0	2	2	1	1	0	3	1	0	0	0	1	1	2	2	0	1	0	0	0	0	0	1	0	2

Appendix 2. illustrates the record of the day degrees (age) calculated based on the temperature in the header tank and the four monitored light chambers during the study period.

Day	Header tank	Probe2 LLw Med	Probe3 LDw Low	Probe4 Deep Red	Probe5 UV dim	Avprobe	Diff Header/prob	Avg. probe
102.0	402.1	429.1	421.1	426.7	435.4	428.1	26.0	428
102.9	406.5	433.8	425.7	431.3	440.1	432.7	26.2	433
103.2	407.9	435.3	427.2	432.8	441.6	434.2	26.3	434
103.7	410.4	437.9	429.7	435.4	444.2	436.8	26.4	437
104.2	412.8	440.4	432.3	437.9	446.8	439.4	26.6	439
104.7	415.2	443.0	434.8	440.5	449.4	441.9	26.7	442
105.2	417.6	445.6	437.3	443.0	452.0	444.5	26.8	444
105.7	420.1	448.1	439.8	445.6	454.6	447.0	27.0	447
106.2	422.5	450.7	442.4	448.1	457.3	449.6	27.1	450
106.7	424.9	453.3	444.9	450.7	459.9	452.2	27.2	452
107.2	427.4	455.9	447.4	453.2	462.5	454.7	27.4	455
107.7	429.8	458.4	450.0	455.8	465.1	457.3	27.5	457
108.2	432.3	461.0	452.5	458.3	467.7	459.9	27.6	460
108.7	434.7	463.6	455.1	460.9	470.3	462.5	27.8	462
109.2	437.1	466.2	457.6	463.5	472.9	465.1	27.9	465
109.7	439.6	468.8	460.2	466.1	475.6	467.7	28.1	468
110.2	442.1	471.4	462.8	468.6	478.2	470.3	28.2	470
110.7	444.5	474.0	465.3	471.2	480.9	472.9	28.3	473
111.2	447.0	476.6	467.9	473.8	483.5	475.4	28.5	475
111.7	449.4	479.2	470.4	476.4	486.2	478.0	28.6	478
112.2	451.9	481.8	473.0	479.0	488.8	480.6	28.8	481
112.7	454.3	484.4	475.6	481.5	491.4	483.2	28.9	483
113.2	456.8	487.0	478.1	484.1	494.0	485.8	29.0	486
113.7	459.2	489.5	480.7	486.7	496.6	488.4	29.2	488
114.2	461.7	492.1	483.2	489.2	499.3	491.0	29.3	491
114.7	464.1	494.7	485.7	491.8	501.9	493.5	29.4	494
115.2	466.5	497.3	488.3	494.3	504.5	496.1	29.6	496
115.7	469.0	499.9	490.8	496.9	507.1	498.7	29.7	499
116.2	471.4	502.5	493.4	499.5	509.8	501.3	29.8	501
116.7	473.9	505.0	495.9	502.1	512.4	503.9	30.0	504
117.2	476.3	507.6	498.5	504.6	515.0	506.5	30.1	506
117.7	478.8	510.2	501.0	507.2	517.6	509.0	30.2	509
118.2	481.2	512.8	503.6	509.8	520.2	511.6	30.4	512
118.7	483.7	515.4	506.1	512.3	522.8	514.2	30.5	514
119.2	486.1	517.9	508.6	514.9	525.4	516.7	30.6	517
119.7	488.6	520.5	511.2	517.4	528.0	519.3	30.7	519
120.2	491.0	523.1	513.7	520.0	530.7	521.9	30.9	522