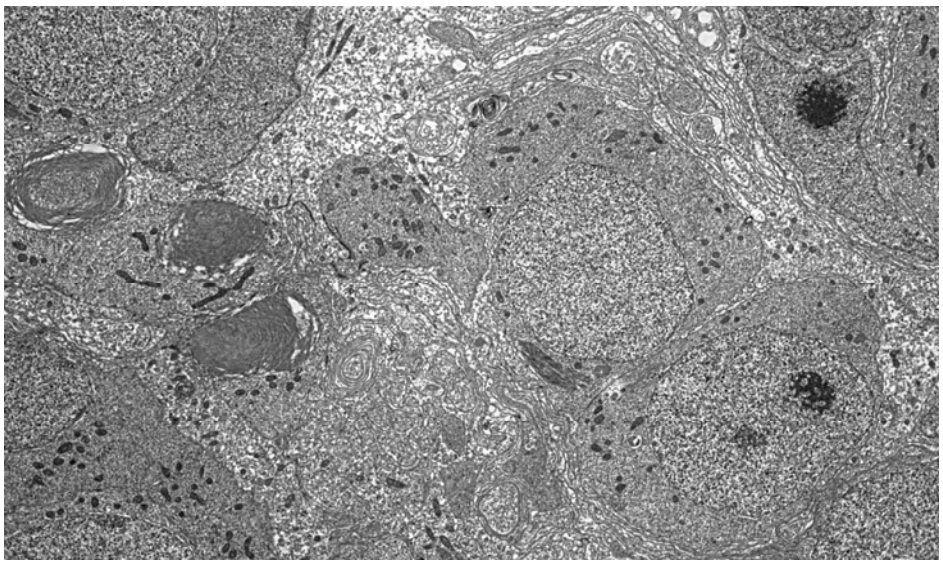
The pineal gland of Arctic charr (*Salvelinus alpinus* L.); a survey of seasonal melatonin production and gland ultrastructure



by
Fredrikke Johansen Musæus

Master thesis in biology
(60 credits)

Department of Arctic and Marine Biology
Faculty of Biosciences, Fisheries and Economics

University of Tromsø
November 2009

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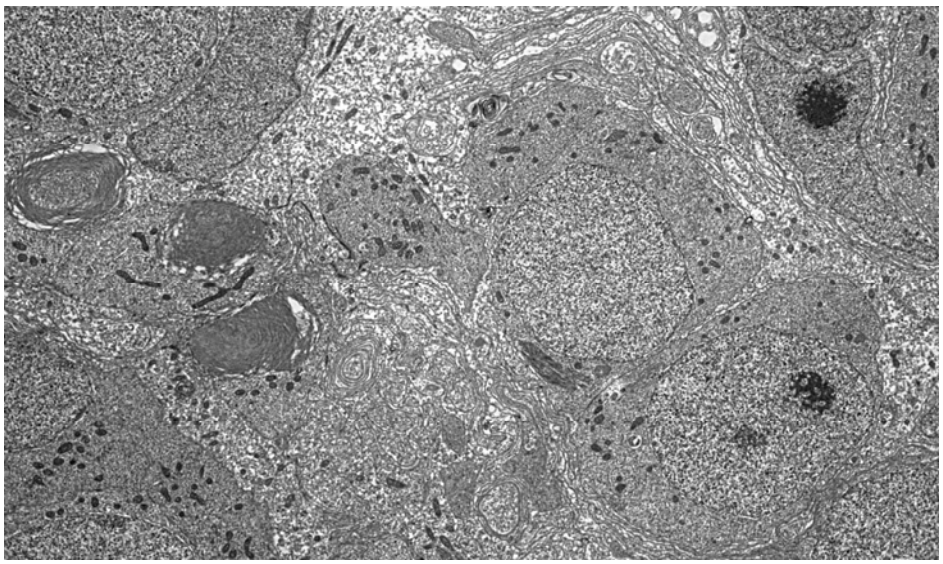

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Last, but not least, to my family and friends for believing in me, supporting me and just for being who you are.

Thank you,
Fredrikke

Tromsø,
November 2009

Summary

The pineal gland is part of the endocrine system involved with rhythmic activity in e.g. fish. The main product of the pineal gland is the indole hormone melatonin, synthesised from the amino acid tryptophan. Melatonin is mainly synthesized when it is dark, as light inhibit the production. For this reason melatonin is thought to be strongly involved in biological rhythms. Arctic charr (*Salvelinus alpinus*) is a circumpolar species and anadromous in parts of its distribution area. Arctic charr experience strong seasonal changes in environmental factors, such as light regime, temperature and nutrient availability. This may influence for example smoltification and spawning. The pineal gland and melatonin are considered important in the adaptation to the shifting environment. On this background the present study was conducted in order to reveal seasonal differences in diel plasma melatonin rhythms and putative associated changes in pineal morphology, pinealocytes, glial cells, blood vessels, mitochondria, endoplasmatic reticulum and lumen. In order to do so Arctic charr were held in freshwater under natural light conditions from August 2006 to June 2007. Blood samples taken at 7 time points through four 24 hour periods (August, December, February and June) during the year were analysed for plasma melatonin levels. Further, pineal glands were excised from Arctic charr at mid-day at the same dates for ultrastructure- and stereological analysis. Radioimmunoassay analysis of the plasma melatonin levels revealed higher peak values for the scotophase of December and February than was found during the photophase. August and June showed a consistent low level of plasma melatonin throughout the 24 hours period. No significant differences were found between the seasons with regard to ultrastructure and organelle volumes studied.

Key words: Arctic charr; seasonality; pineal gland; melatonin; ultrastructure; stereology

Table of contents

1. Introduction

Arctic charr (*Salvelinus alpinus* L.) is the worlds northernmost living freshwater fish. Its distribution is circumpolar, and further south it is found in alpine areas in central Europe (Johnson 1980). Anadromous (sea migratory) populations of Arctic charr is found in the northern parts of the distribution area, but in some lakes the Arctic charr remain in freshwater throughout their life even if it is possible to migrate to the sea (Johnson 1980). Populations and morphs of this species differ in many characteristics as a result of long term spatial separation. Examples of such traits are distribution with respect to depth, choice of prey, the size at maturity and the time and the place of spawning (Klemetsen *et al.* 2003). In many lakes sympatric populations (e.g. anadromous/resident) are found, which means that there is more than one form or morph found in one and the same lake (Johnson 1980; Rikardsen *et al.* 2000; Klemetsen *et al.* 2003). This reveals a substantial plasticity within this species regarding life-history strategies.

Light conditions in the Arctic show extreme variations throughout the year. During winter it is a long period of total darkness in the ice covered lakes, whereas in summer the sun is above the horizon for several months, resulting in high light intensity 24 hours a day. Food resources are abundant during summer, especially for the individuals that undertake migration to the sea. Fresh water lakes are very nutrient poor during winter (Gross *et al.* 1988). The extreme seasonal variations in light, food supply and water temperature in the high north represents substantial challenges for animals living in these areas, with regard to resource accumulation (feeding and growth) and reproduction. Arctic charr, as well as most other animals in these areas, need to accumulate as much energy as possible during the periods of abundant food supply for autumn reproduction and winter survival. This has resulted in a marked seasonal variation in food intake and growth in this species (Jørgensen *et al.* 1997). Immature fish feed intensively throughout summer and cease feeding in the autumn, apparently when a threshold condition factor and energy status have been attained (Tveiten *et al.* 1996). Maturing fish needs extra energy for gonad maturation, and in line with this it has been found that maturing fish start feeding earlier in spring than immature fish (Tveiten *et al.* 1996). Frantzen *et al.* (2004) studied the effects of photoperiod on plasma levels of sex steroid and gonad maturation, and found that a switch from long day to short day early in the

reproduction cycle resulted in a stronger synchronization in both ovulation and spermiation, and a temporal advanced maturation. Further it has been shown that photoperiod govern the timing of the process (smoltification) that prepare anadromous individuals for seaward migration (Johnsen *et al.* 2000). Hence, the Arctic charr show a strong seasonality, including rhythms in food intake, growth, adiposity, seawater tolerance, reproduction etc., which need to be synchronized with the changing seasons. These rhythms are expected to be controlled by phase-adjusted endogenous timing mechanisms in the Arctic charr (Sæther *et al.* 1996). For such phase adjustments (entrainment) animals in general use the change in daylength (photoperiod) as a reliable environmental cue for calendar information (Falcón *et al.* 1992; Reiter 1993). One of the organs that is involved in light perception and entrainment is the pineal gland, which produce and secrete the hormone melatonin. The pineal gland is both a sensory and a secretory organ (Rüdeberg 1970). The pineal gland is considered to be part of the system regulating biological rhythmicity, mainly due to its main secretory product, the indole hormone melatonin (Kulczykowska 2001). In birds and mammals melatonin is strongly involved in the synchronization of diurnal and annual rhythms (Reiter 1993) whereas the role of melatonin in fish is less clear (Falcón 1999).

The pineal organ in Arctic charr is located in the skull roof (Holmgren 1959; Rüdeberg 1968), and shown in figures 1 and 2.

Figure 1: Transparent window in the skull of Arctic charr, and the position of the pineal gland (arrow) under it. Photo by the author.

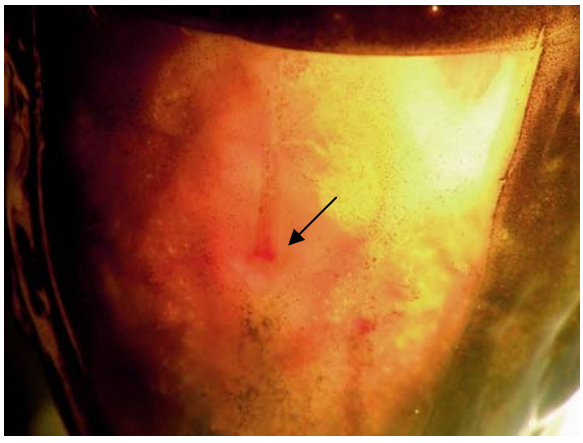


Figure 2: Close up picture of the pineal gland in Arctic charr (arrow). Photo: Jo Jorem Aarseth.

Above the gland is an area (the pineal window) where the cartilage is thinner and more transparent, and in this area there is a crevice for the pineal gland. It is close to the top of the head, in the most ideal position for receiving as much light information as possible (see figures 1 and 2). The pineal organ is developed from the same area as the thalamus and pituitary glands (Ekström and Meissl 1997).

The pineal gland in salmonids is directly light sensitive (Migaud *et al.* 2007). Light inhibit melatonin production, whereas darkness removes this inhibition. The responds time to light is rapid. Figure 3 shows the change in melatonin levels in an *in vitro* experiment as the light were changed. It is clear that light inhibit the melatonin production with immediate results (Strand *et al.* 2008).

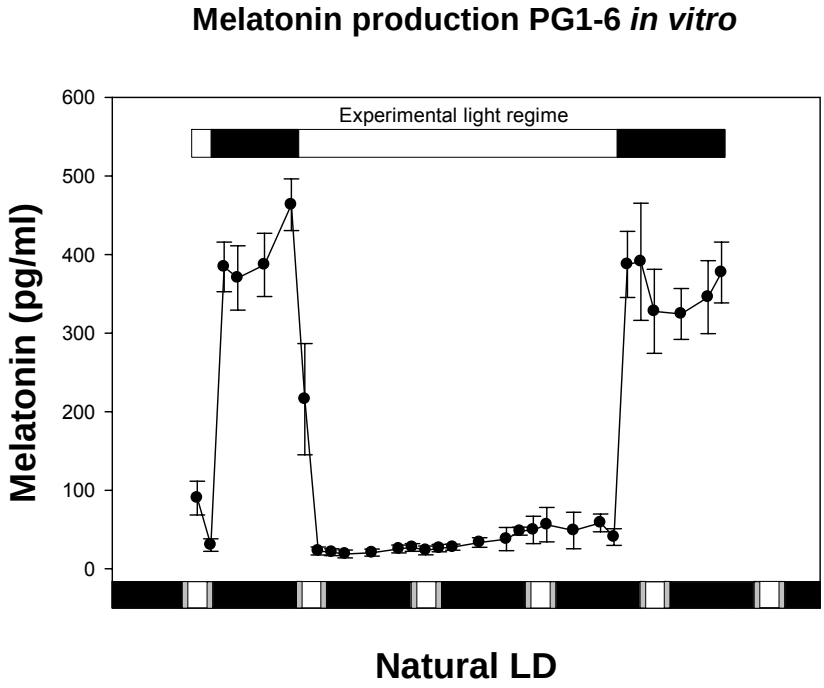


Figure 3: *In vitro* production of melatonin in pineal glands from Arctic charr exposed to an experimental light regime (top panel). Lower panel shows the natural LD at 69°N during the experiment (22.-27. January). Each value is the mean of 6 glands (±SEM). White, gray and black bars represent light, civil twilight and darkness, respectively. Light intensities; light 50 μW/cm^2 (752 lux), darkness 0.001 μW/cm^2 (0.015 lux). (From Strand *et al.* 2008, with permission).

The sack-like shape of the pineal gland is remarkable similar throughout the animal kingdom (Falcón 1999). A slender stalk connects the pineal gland to the diencephalon, and the lumen opens to the third ventricle, which is filled with cerebrospinal fluid (Falcón 1999). Generally the pineal gland in fish looks the same, but there are some species-specific variations with regard to morphology (Holmgren

1959) and folds in the central lumen (Ekström and Meissl 1997). The pineal organ consists of the pineal gland and the parapineal organ. The parapineal organ is present in embryologic stages of development but is lacking in adult fish (Holmgren 1959; McNulty 1984; Vighteichmann *et al.* 1991). The function of the parapineal organ in fishes is not well understood, and it will not be discussed in this thesis. Figure 4 show the whole pineal gland of the Arctic charr embedded in resin.

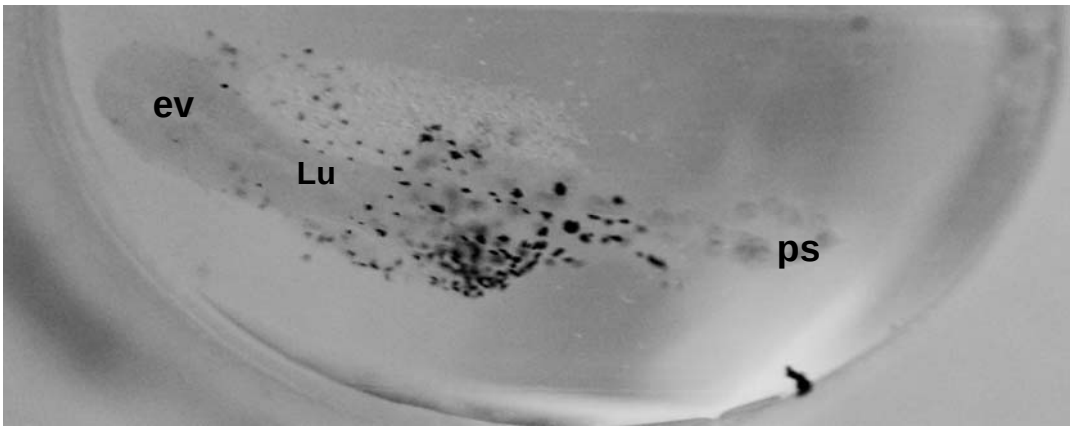


Figure 4: Pineal gland of the Arctic charr embedded in resin showing the entire gland with the end vesicle (ev), lumen (Lu, the lighter line in the centre), pineal stalk (ps) and the black dots (probably pigments. Diameter of the block is 8 mm.

Three types of cells are considered the main content of the pineal gland, i.e. pinealocytes (photoreceptor cells), glial (supporting) cells and second order neurons (ganglion cells; Ekström and Meissl 1997; Falcón 1999). There are blood vessels supplying blood to all parts of the pineal gland, but they do not penetrate into the parenchyma of the gland (Ekström and Meissl 1997; Gupta and Premabati 2002).

The pinealocytes are both photosensitive, containing photopigments, and secretory, producing chemical substances. Pinealocytes have been shown to undergo morphological changes in response to changes in photoperiod (Hafeez *et al.* 1978; McNulty 1982a; Ekström and Meissl 1997). The photoreceptors of the pinealocytes have similarities with the cone photoreceptors of the retina (Ekström and Meissl 1997). They differ, however, as the time it takes for a resting membrane potential (the dark potential) in the individual pineal photoreceptors to reach peak-potential (hyperpolarizrtion) and the recovery time (the return to the resting potential after a stimulus) is much longer in the pineal gland than in the retina (Meissl and Ekström 1988). This supports the theory that the pineal gland record gradual light

intensity changes rather than the rapid changes that the retina can perceive (Ekström and Meissl 1997). Further, the pineal pigments absorbs light at longer wavelengths than the retina (Bowmaker and Wagner 2004). Photoreceptors of lower vertebrates presumably release neurotransmitters constantly during darkness (Korf and Wicht 1992), and the size and numbers of synaptic ribbons (part of the photoreceptor axon terminal) change with time of day and ambient light (Ekström and Meissl 1997).

Glial cells (also called interstitial cells or supportive cells) create diffusion barriers between the extracellular fluid and the cerebrospinal fluid in the lumen, and show traits that generally are associated with synthetic activity (Rüdeberg 1968; McNulty 1978; Ekström and Meissl 1997). It has traditionally been difficult to pinpoint exactly what their function might be, as they differ between fish species (Ekström and Meissl 1997).

The second order neurons (ganglion cells) are intrapineal and most of them possess axonal projections to the brain. They are post synaptic to the pinealocytes, but some times they also form conventional synapses with each other and with pinealocytes, probably in order to provide feedback information in connection with light adaptive processes (Ekström and Meissl 1997). Bundles of neurons are found in the pineal stalk, the only neuronal information way to the brain (Ekström and Meissl 1997). Two different types of responses have been recorded in the neurons. Light inhibit the maintained ganglion cell discharges, and short wavelengths inhibit and longer wavelengths stimulate the response (Korf and Wicht 1992). The exact location of the terminal sites are not certain, but it seems to partly overlap with the axonal projections from the optic tract (Ekström and Meissl 1997). Neurons have not been studied in the present thesis.

Melatonin (N-acetyl-5-methoxytryptamine) is an indole hormone produced from the amino acid tryptophan. The biosynthesis of melatonin starts with conversion of tryptophan into 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPOH). Hydroxytryptophan is then decarboxylated by the aromatic amino acid decarboxylase to serotonin. Arylalkylamine N-acetyltransferase (AANAT) converts serotonin into N-acetylserotonin, and hydroxyindole-0-methyltransferase (HIOMT) methylates N-acetylserotonin to melatonin (Falcón 1999). The pineal gland is the main organ for melatonin production in fish, but there is melatonin production in the retina of the eye as well (Falcón 1999). In mammals and birds melatonin is also produced in the

gastrointestinal tract, and under specific nutrition-dependent circumstances this production may contribute to the levels of circulating melatonin, albeit not in a rhythmical fashion (Huether 1993; Ekström and Meissl 1997). No consistent information have been found for melatonin production outside the retina and pineal gland in fish (Kulczykowska *et al.* 2006), but pinealectomized trout have shown increased mid-scotophase plasma melatonin level (Gern *et al.* 1978). Kezuka *et al.* (1992) found that pinealectomy in goldfish did not completely abolish plasma melatonin levels. The production from the retina is considered to be mainly for local (paracrine) use and is not in phase with the plasma melatonin level (Falcón 1999). In fish the melatonin diffuses into the blood stream directly after the synthesis.

Melatonin is believed to be involved in behavioural, physiological and biochemical rhythmic activity. An indication of this is the diurnal variations in indole compounds (serotonin. 5-hydroxyindolacetic acid, 5-hydroxytryptophol and melatonin) in the pineal and that the melatonin levels are consistently higher during night time (Ekström and Meissl 1997; Falcón 1999). Light inhibit the production of melatonin, while darkness removes this inhibition. The limiting factor is AANAT, which show cyclic activity with higher activity in darkness (in pike, *Exos lucius*, Falcon *et al.* 1987). Abundance of AANAT messenger RNA transcripts varies in a manner parallel to the enzyme activity (Falcón 1999). Both the duration of the elevated night-time level and the amplitude of plasma melatonin rhythm change in a fashion consistent with the seasonal change in photoperiod (Falcón 1999), providing calendar information to the animal. It has been shown that Arctic charr maintains a diel- and seasonal melatonin rhythm perfectly reflecting the daylength even in lakes covered by ice and snow (Strand *et al.* 2008). A very high night-time plasma melatonin level was recorded in these fish in September, when the lake temperature was high (~10°C), whereas in June when there is constant light at high latitudes the 24 hour plasma melatonin levels in Arctic charr were constantly under the detection limit of the assay (Strand *et al.* 2008). Diel fluctuations of pineal melatonin production have also been described in species closely related to the Arctic charr such as rainbow trout (*Oncorhynchus mykiss*; Masuda *et al.* 2003) and Atlantic salmon (*Salmo salar*; Porter *et al.* 2001) as well as in many other fish species (Ekström and Meissl 1997; Falcón 1999).

The pineal gland is fairly well described on a general level, however several details regarding its specific mechanisms remain to be unravelled (McNulty 1984;

Ekström and Meissl 1997). As the production of melatonin in the Arctic charr pineal gland varies tremendously between seasons, the question arises weather there are any differences in the ultrastructure of the pineal gland throughout the year, and if putative ultrastructure changes is related to the changes in absolute production of melatonin between seasons. Energy saving is a crucial factor for animals living at the edge in the north and the Arctic charr would, at least theoretically, benefit from a reduction in the size of the gland and its melatonin production capacity during the season when melatonin production is minimal. For example has it been shown that there are changes in morphology of the structures of the pineal gland, with e.g. larger nuclear volumes, after continuous darkness (DD) in trout (Hafeez *et al.* 1978). They found larger nuclei and nucleoli in steelhead trout (*Salmo gairdneri*) exposed to DD in both pinealocytes and support cells. Correspondingly the size of support cells were significantly smaller in trout subjected to constant light (LL) as in those subjected not only to DD, but also to LD treatments.

Objectives of this study

On this background, the present study was set out to compare the diel melatonin production at different seasons with the ultrastructure (pinealocytes, glial cells, blood vessels, mitochondria, endoplasmatic reticulum and lumen) of the pineal gland of the Arctic charr. This was done in order to see weather there are any connection between the absolute melatonin production and the ultrastuctural components that is important for this production in the pineal gland.

2. Materials and methods

2.1 Fish and experimental design

The fish used in the present study hatched in 2004, and were offspring of wild, anadromous Arctic charr (*Salvelinus alpinus*) from a strain caught at Svalbard (79°N) in 1990. They had been reared at the Aquaculture Research Station in Kårvik, Tromsø (69°52'N, 18°55'E), under natural conditions. The experiment was carried out between August 2006 and June 2007 at the same research station.

100 fish of similar size was randomly selected from a stock tank and transferred to a 500L tank with fresh water. The amount of fresh water was continuously adjusted in order to maintain an oxygen saturation of 90 % in the tank and the water inlet was placed so that a circumferential current was created in the tank. By this arrangement the fish were forced to swim continuously (~1 bodylength/second) and this was done in order to prevent formation of social hierarchies among the fish (Christiansen *et al.* 1992). They were kept under natural light (light transparent roof) and ambient water temperature throughout the whole experiment. Feed (commercial dry feed; Skretting, Stavanger, Norway) was provided in excess by automatic disc feeders in accordance with routine praxis at the station.

Blood sampling

Four times throughout the year (August 9^{th} -10^{th}, December 18^{th} -19^{th}, February 14^{th}-15^{th} and June 13^{th}-14^{th}) blood samples were taken from subsamples of fish (n=10) at 4 hour intervals throughout 24 hours for plasma melatonin analysis (i.e. seven time points per day for each season). Before taking the blood samples the fish were removed carefully from the tank and transported to the laboratory area in a small bucket. A few (3-5) fish were then anesthetised at the time in Benzocain (60 ppm) for approximately 5 minutes. The fish were considered properly anesthetized when they showed no reaction to stimulus. Length and weight were registered for all the fish. Blood samples were taken from the caudal vein, using 2 ml vacutainers with lithium heparin (LH 34 I.U., BD Dianostics-Preanalytical Systems, Belliver Industrial Estate,Plymouth,UK). After the blood samples had been taken, the fish were transported back to another tank, where they were held until the last blood sampling

had been conducted. After the entire procedure for the day was finished, the fish were moved back to the original tank.

The blood samples were kept on ice before they were centrifuged at 3800 rpm for 10 minutes at 4°C, to separate the plasma from the blood cells. The plasma was then frozen at -80°C until later melatonin analysis. During the sampling times when it was dark, a head light with a red beam was used.

Collection of pineal glands

The pineal glands were dissected out for histological analysis from those fish that were sampled for blood at midday. The fish was killed by a blow to the neck before the neck was cut, and the pineal glands carefully excised from the fish heads. All together nine pineal glands were harvested at each season. Three of the pineal glands were preserved in 2.5 % Glutaraldehyde in 0.2 M cacodylic buffer. Three pineal glands that were to be used for light microscopy were fixed in 4 % formalin, and three were put in 8 % formaldehyde in 200 mM Hepes buffer. The pineal glands were stored in a refrigerator until embedding. The pineal glands for the ultrastructure analysis from August could not be used, so three new ones were collected at September 14th.

2.2 Plasma melatonin analysis

To analyse the blood samples for the daily and seasonal variations in plasma melatonin levels in the Arctic charr, a RadioImmunoAssay (RIA) method was used. This method is used for several different types of hormones, and it is validated for plasma melatonin analysis in Arctic charr (Strand *et al.* 2008).

Before samples could be analysed fat was extracted from plasma since it can interfere with the assay (primarily by obstructing binding of the hormone to the antibody). The extraction procedure was modified from Van't Hof and Gwinner (1996). Using this protocol approximately 20 % of the melatonin is lost (pilot studies) and all plasma melatonin levels presented are corrected for this. For details of the extraction process, see appendix 1.

The plasma melatonin levels can be measured indirectly by finding the ratio between the binding of the unknown amount of melatonin in the plasma ("cold

melatonin") and a known amount of added radioactive (^{3}H) melatonin (tracer) to a melatonin antibody. After washing out all unbound melatonin (both "cold melatonin" and tracer) with charcoal the remaining (bound) amount of ligands can be counted, and from that the amount of bound plasma melatonin to the antibody can be estimated. If there is little melatonin in the plasma there will be much tracer bound to the antibody, and vice versa. The plasma melatonin is estimated from the standard curve and the percentage binding of the known substances (tracer). The standard curve is made from an increasing dilution of standard melatonin, and this standard curve is also used to see if there are any irregularities with any of the solutions and/or with the binding capacity of the antibody.

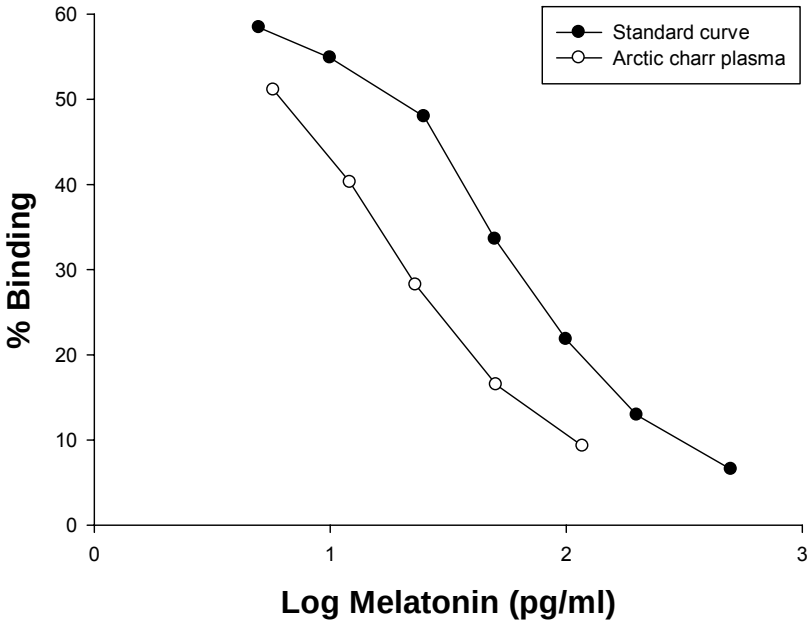


Figure 5: Parallelism between curves representing serial diluted Arctic charr plasma and the standard curve (from Aarseth et al. 2009, with permission).

The standard curve was made from total count (TC), none specific binding (NSB), total binding (TB) and the standard melatonin dilution range S1- S7. This is the basis for the calculations of the melatonin level in the plasma. The curve is sigmoid and ideally the values from the plasma samples should be in the steepest part of the curve. The binding should be above 40% for the results to be valid. In order to check if the antibody is able to bind melatonin specifically in a species, diluted species

plasma spiked with melatonin should display parallel displacement with the standard curve. In Arctic charr, this has been validated as shown in figure 5.

Several solutions are needed in an assay. The buffer solution is made special for the antibody so that its binding properties are optimal. The melatonin standard was pre-made, and the working solution was 10 ng/ml. 125 µl melatonin standard to 2,5 ml buffer made a solution of 0,5 ng/ml to be used for the standard curve and this was freshly made every day. The antiserum used was raised in sheep (Stockgrand Ltd., Guilford, Surrey, UK). The working solution was 50 µl (1:10 stored at -20°C) diluted with 20 ml buffer for 100 reagents. This gave a 1:4000 dilution of the original antiserum. ^{3}H-melatonin (^{3}H-Mel or tracer) was diluted from the stock, so 100 µl ^{3}H-Mel gave a disintegration activity of ~4000 cpm. Tracer was also made fresh every day.

Dextran coated charcoal was used to wash out the unbound components and other particles, so it would only be melatonin (^{3}H-Mel and cold) bound to the antibody left in the sample that is counted. Scintillation fluid in excess is needed to give accurate counting.

All samples were analysed in duplicate. In this way any extreme variations can be seen in the analysis. If two of the plasma samples are traded for two more S7 (one sample less in the assay) it is possible to measure intra-assay variation, to see if the counts are stable throughout the counting process, and if a theoretical known amount of melatonin is measured correctly. This is a security measure and insurance for the validity of the results. The S7 can also be used for inter-assay variation. If several assays are run, this is done to check that they are comparable. The extra S7 samples should in theory contain a melatonin level of 500 pg/ml, as that is the amount added to it. The amount of plasma in each sample should ideally be 500 µl per vial. This is difficult to achieve because of the rather large amounts of blood this would require (~1000 µl plasma from each fish). When there was too little plasma, the remaining amount was replaced with buffer. The plasma:buffer ratio was noted for final corrections of melatonin levels. Preferably the duplicates held similar amount of plasma, whenever possible. After correlation for the dilutions of the plasma samples the plasma melatonin level is given as pg/ml. For more details regarding the RIA procedure, see appendix 1.

2.3 Histology

Preparation for transmission electron microscopy (TEM)

Embedding

The pineal glands were blotted once on soft tissue paper and weighed at ±0,1 mg accuracy and the volume (ml) of the glands were estimated by fluid displacement in a 1 ml shot, clogged with cement. They were then immersed in Karnovsky fixative until embedding. As the glands were too large to be embedded as a whole, they were cut into smaller pieces (2-5 per gland) which were embedded separately. The method is standard for TEM, and contains several steps, including primary fixation, washing, second fixation, dehydration by graded series of alcohol, infiltration with transitional solvent and then infiltration of resin and embedding. The preparation helps to avoid changes in the cell and cell components size, shape and spatial distribution. The embedding also gives good cutting properties to the material. Chemicals are used as stabilizing factors. The initial fix (Karnovsky fix, right after dissection) stops biological activity, stabilizes protein and insures a good preservation of the cell structures. Osmium stabilizes fat (membranes), and uranyl acetate were added during the embedding process to get better contrasts in the preparation. The pieces of the pineal gland were embedded in Epon/Araldite. For more details regarding the embedding, see appendix 2.

Sectioning

The embedded samples were cut in a systematic random (the first point being random, the following at set intervals) fashion into ultra thin sections (~70 nm) on a ultramicrotome (Reichert-Jung Ultracut E; Vienna, Austria), using a diamond knife (Diatome, Switzerland). The sections were picked up with carbon coated formvar films on 200 square mesh copper grids (Agar Scientific, Essex, UK). A fixed distance was maintained between the sections, ~200 semi thin sections (0.99 µm). The semi thin sections were cut with a glass knife. Not all sections were used in the stereological count.

Contrasting

Uranyl acetate (Ur.Ac.) and Reynold's lead citrate were used for contrasting the sections on the grids because of their high atom numbers. They are ideal contrasts for TEM because they are unspecific, but different cell components are stained with different densities. This makes it easier to see the different structures in the section because the electron beam will be scattered by the high densities. Electrons that fail to hit the fluorescent screen create darker areas in the section. Electrons that pass through the section will be seen as fluorescent spots on the screen. Ur.Ac. binds mainly to phosphate groups and nucleic acid, lead citrate binds lipoproteins, proteins, glycogen and RNA. The copper grids with the tissue sections were left on the droplets of Ur.Ac. for 7 minutes, washed in distilled water, dried and placed on lead citrate for 5 minutes, washed and dried again. For details in the contrasting process, see appendix 2 for laboratory manual.

Microscopy and Micrographs

Transmission Electron Microscopy (Jeol JEM 1010, Tokyo, Japan), at 80kV, with a Morada camera system (Olympus Soft Imaging systems, Münster, Germany) was used to examine the sections so the structures could be accounted for. The sections were searched until an area containing biological material was found. The best magnification for this study was found to be 4000x for the pineal gland in Arctic charr. At this magnification a reasonable area could be covered, and the important structures could be separated. Micrographs were taken in a systematically random fashion (at 4000x magnification), starting at one end of the specimen, and taking pictures in the upper left corner of every second square, using the mesh of the copper grids as reference. Only the areas of the section where there was tissue were photographed, leaving out the areas only containing resin. In this way the entire section was covered, and no area was photographed twice. In some cases the upper left corner were not good for photographing due to artefacts or no tissue, and in these cases one of the other corners in the mesh were used.

Preparation for light microscopy

One pineal gland from each season was taken from the glands fixated in 4 % Formalin, and dehydrated in alcohol (100 %) and embedded (Technovit 8100, for details of the embedding process, see appendix 2).

After trimming the block with glass knife to get to the pineal gland at the right angel, semi thin sections were cut with a diamond knife, on a Leica EMUC6 Ultramicrotom. The sections were placed on a slide and stained with toluidine blue. A light microscope (Leitz aristoplan) with a Leica DFC320 camera was used to study the slides, and get the pictures.

2.4 Stereology and pineal ultrastructure

Stereology is a method for analyzing three dimensional (3-D) structures based on their two dimensional (2-D) histological sections (Weibel 1979; Inuwa 2005). The micrographs used for point counting were selected in a systematically random fashion (in a way so all the micrographs had the same chance of being chosen). Point count analysis was used to estimate the relative volume of selected pineal structures. The structures were chosen firstly for their assumed functional importance in the pineal gland, and secondly for how easily recognisable and detectable they are. The structures counted were pinealocytes, supporting cells (glial cells), mitochondria, endoplasmatic reticulum (ER), lumen and cilia and blood vessels (all vessel types included). The micrographs were analysed using the computer software imaging system iTEM (Olympus, Soft Imaging System, Münster, Germany). A mesh grid with squares 5000 nm both horizontally and vertically was added to the micrographs. This gave 66 hits per micrographs. The area in the lower left corner of the grids was used as hit as it is difficult to know what is directly under the cross of the lines, see figure 6 for an example of a micrograph with the overlaying grid.

The selection of the areas were systematic random at every step of the process from cutting the sections. When the entire pineal gland was cut (all the pieces), ten areas were chosen by dividing the total number of sections with ten to get the number of sections between each chosen area. One of the numbers was drawn and used as the starting point. In this way all the sections had an equal chance of being picked. After the ten sections were chosen, five sections were selected by taking every second section after drawing whether to start with the first or

the second. The final five sections were contrasted and photographed. From each section ~five micrographs were chosen by using the same selection system as were used to choose the initial ten sections. The number of micrographs varied somewhat between the pineal glands (see table 1).

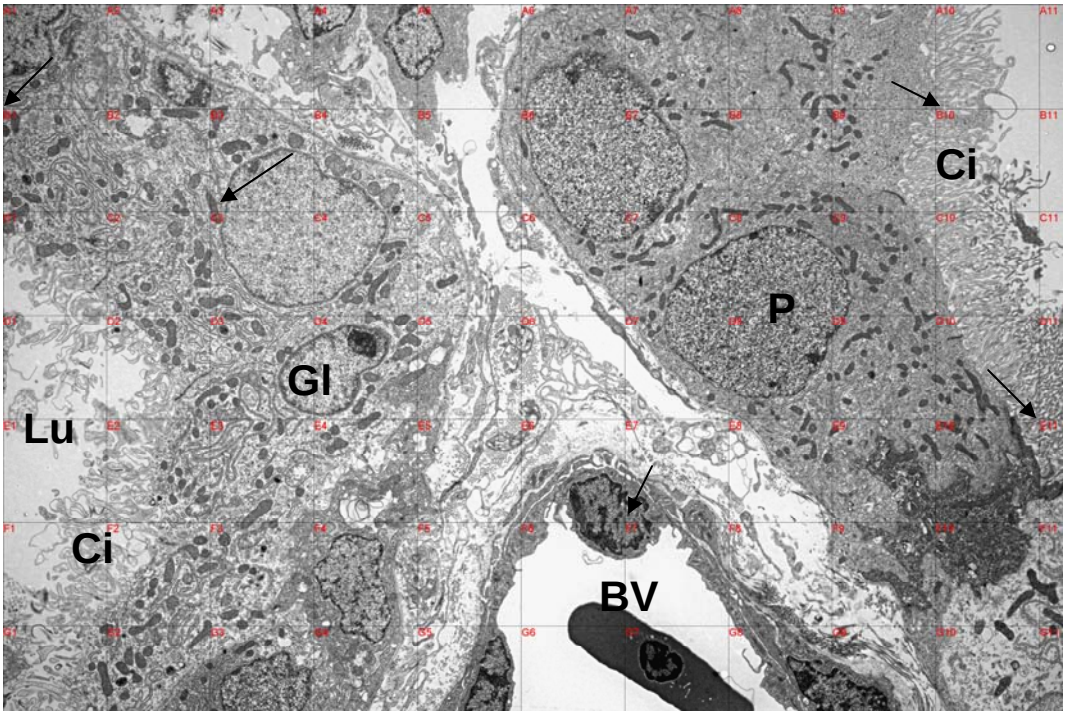


Figure 6: Micrograph with the point grid. Arrows point towards some of the areas that was counted, the lower left corner of the squares. Several structures can be seen, e.g. blood vessel (BV), pinealocyte nucleus (P), lumen (Lu), cilia (Ci) and glial cell nucleus (Gl).

Table 1: Number of micrographs used for counting per pineal gland and average per season

	PG	# of micrographs	average
September	09-133	26	
	09-134	19	
	09-135	23	22,7
December	07-208	16	
	07-209	22	
	07-210	22	20,0
February	07-1490	27	
	07-1491	20	
	07-1492	24	23,7
June	07-1493	26	
	07-1494	21	
	07-1495	24	23,7

Every pineal gland should have a total of approximately 200 hits of the relevant structures. This number is found to be ideal, as less gives an uncertainty in the result, whereas more does not give more accuracy, only more work (Gundersen and Jensen 1987). In this examination it is more hits, as it was difficult to estimate the number of micrographs needed before the counting started.

Volume calculation

The volume of each structure ($V_{(structure)}$) was calculated in relation to the total volume of the pineal gland, using the formula

$$V_{(structure)} = P_{(structure)}/P_{total} * V_{PG} * 1000$$

where

$P_{(structure)}$ is the number of hits of a structure, P_{total} is the number of hits in total and V_{PG} is the volume of the PG as measured before the embedding. Calculations were based upon standard procedures for stereology volume estimates (Weibel 1979; Gundersen 1986). The additional *1000 was to get µl instead of ml, as was the original denomination.

2.5 Statistical analysis

All data are presented as means and standard error of a mean (SEM). Statistical computations were made in Statistica 6.1 (Statsoft Inc., Tulsa, OK, USA) and Sigma plot 10.0 (Systat Software Inc.). A parametric, one-way analysis of variance (ANOVA) was used for revealing possible differences in the plasma melatonin levels between the sampling times for each separate date and for the fish growth, followed by a Turkey-HSD post hoc. test. Significant changes in PG volume vs. PG mass and BM vs. PG mass were performed by Multiple Regression Analysis. Due to the low number of pineal glands (n = 3) analyzed at each time point, possible differences of the structures between the seasons were analyzed by a non-parametric Kruskal-Wallis test for multiple comparison of mean ranks for all groups. A probability level of $p \leq 0.05$ was considered significant.

3. Results

3.1 Experimental fish

Water temperature in the fish tank during the experimental period is shown in figure 7.

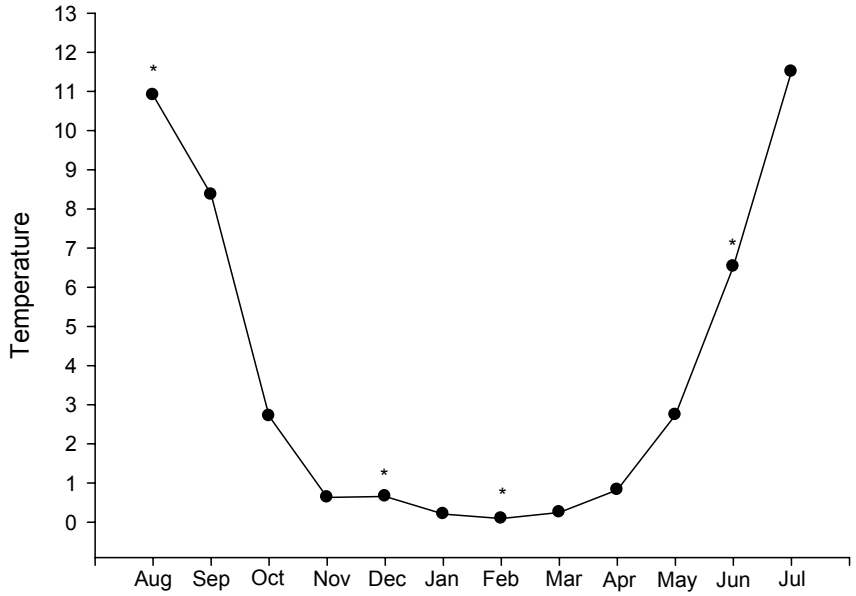


Figure 7: Water temperatures (°C) from August 2006 to July 2007. The sampling dates are marked with an asterisks.

Growth of the experimental fish throughout the experiment period is shown in table 2.

Table 2: Average body length (BL) and body mass (BM) ± SEM, throughout the study. N= the number of fish measured.

	BL (cm)	SEM	BM (g)	SEM	n
August	32,3 ±	0,95	425,5 ±	15,62	70
December	36,3 ±	0,29	616,2 ±	18,06	70
February	37,1 ±	0,43	679,7 ±	25,25	66
June	39,3 ±	0,50	875,9 ±	38,55	63

The fish showed a significant increase in body mass (BM) between the first (August) and the last (June) sampling dates ($p \leq 0.05$). The growth is smallest during the winter months (December and February). The body length (BL) showed significant

difference between August and June ($p \leq 0.05$), but none of the other months were significant from each other ($p \geq 0.05$).

The pineal gland (PG) mass and the body mass of the respective fish show a significant positive correlation ($p \leq 0.05$, $r^2=0.142$). The pineal gland size seems to follow the fish size, but the size of the fish can not be used to predict the mass of the gland (see figure 8).

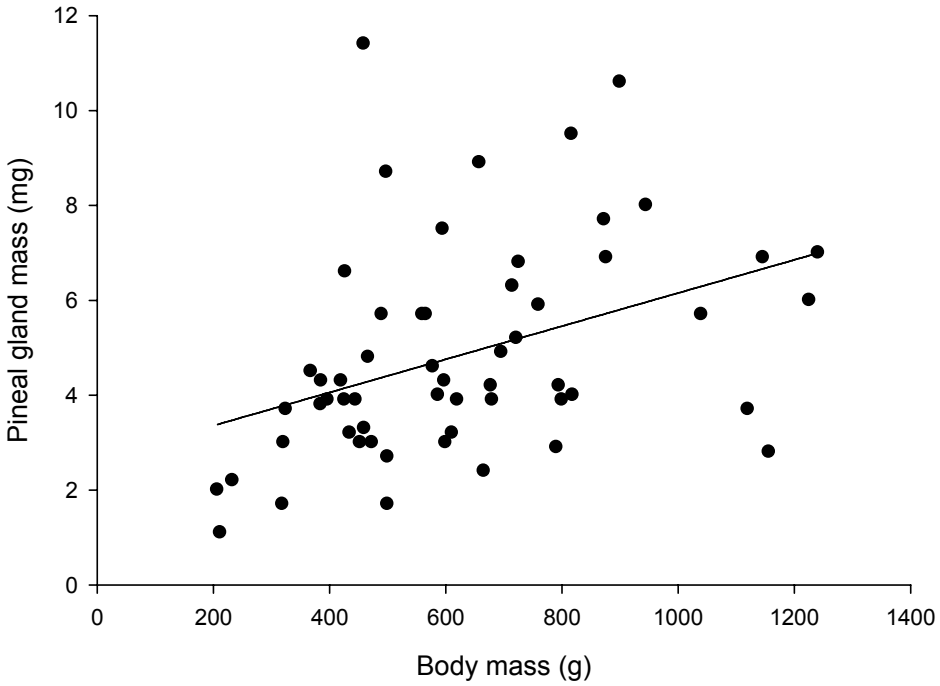


Figure 8: Pineal gland mass (in mg) in respective fish (BM in g; n=57). Regression line: y = 2.6603 + 0.0035x.

There is no correlation between the PG mass and the PG volume ($p \geq 0.05$), as shown in figure 9.

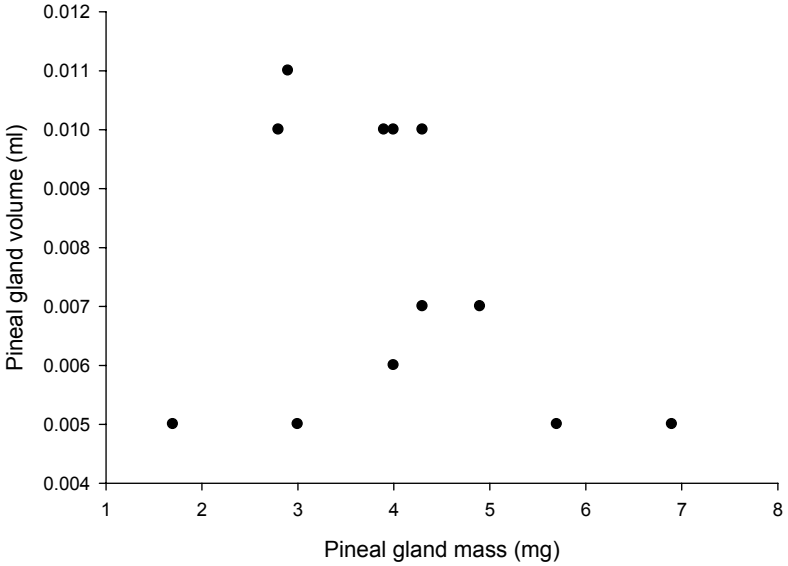


Figure 9: Correlation between mass (mg) and volume (ml) of the pineal gland in Arctic charr (n=12).

3.2 Plasma melatonin

Plasma melatonin levels during 24 hours in all experimental months are shown in figure 10. In August and June there is no significant difference between the melatonin levels at any given time point. The December and February curves on the other hand vary more throughout the day, with a peak in the dark period of the day. During daylight hours the melatonin levels are low. In February melatonin levels are at their highest, and the increase is stable during the mid-scotophase.

Inter- and intra- assay variations were 8,2 % and 7,2 %, respectively. The lower detection limit of the assay was 10 pg/ml, and all the values below the detection limit are set to half of this value, that is 5 pg/ml.

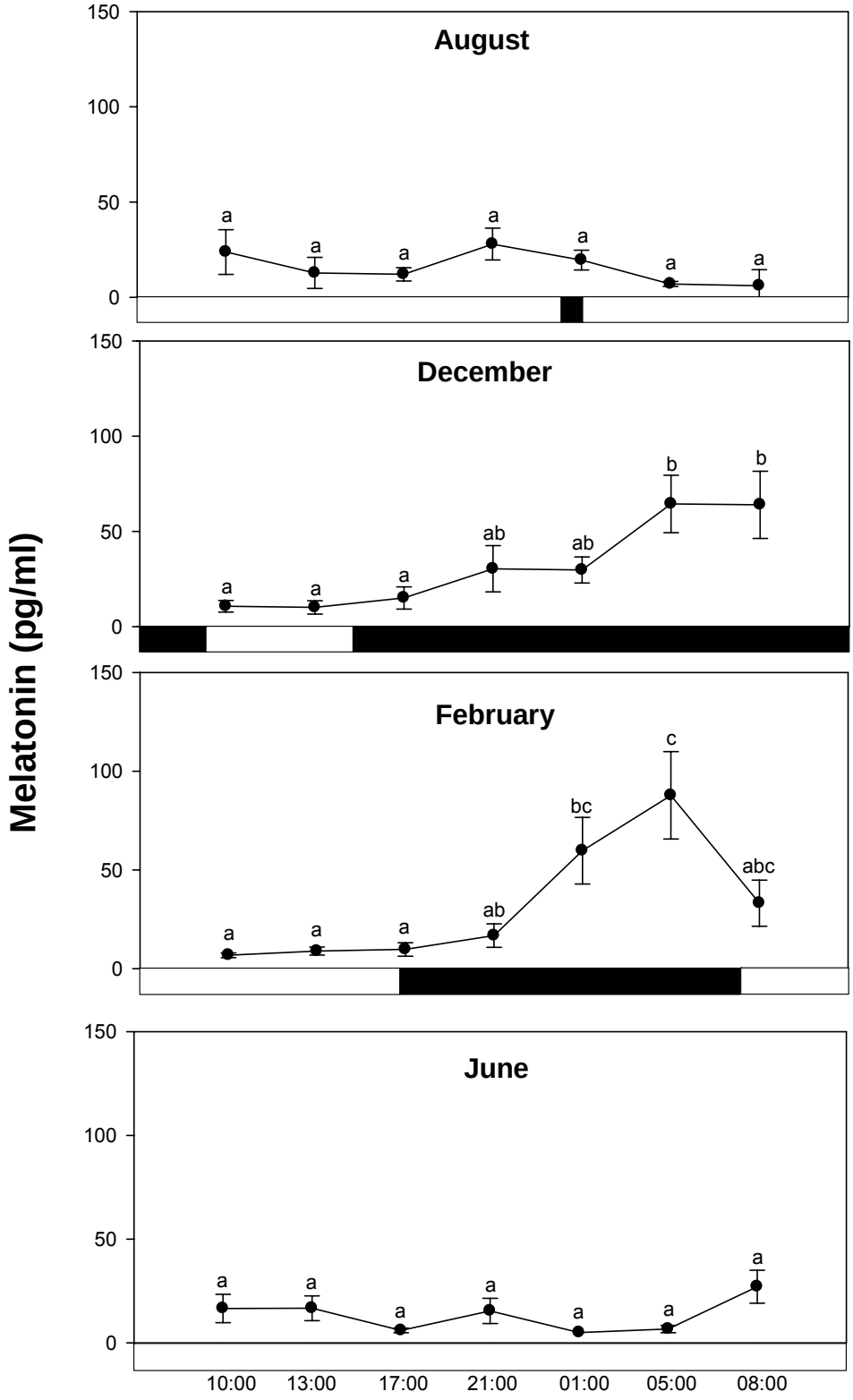


Figure 10: Plasma melatonin levels at seven different time points in four different months. The first point is at 10.00, the second 13.00, followed by 17.00, 21.00, 01.00, 05.00 and 08.00 o'clock. The black and white bars below each graph indicate duration of natural darkness and daylight, respectively. The letters indicate significant difference between different times ($p \leq 0.05$), that is, the times with different letters are significantly different from each other.

3.3 Pineal ultrastructure

TEM

Micrographs (figures 11 and 12) show some of the structures in the pineal gland, pinealocyte nucleus (P), glial cells nucleus (Gl), endoplasmatic reticulum (ER), mitochondria (Mit), photosegment (outer segment; Ph), cilia (Ci) and lumen (Lu). Blood vessels and the point count grid can be seen in the figure 6, in the materials and methods chapter.

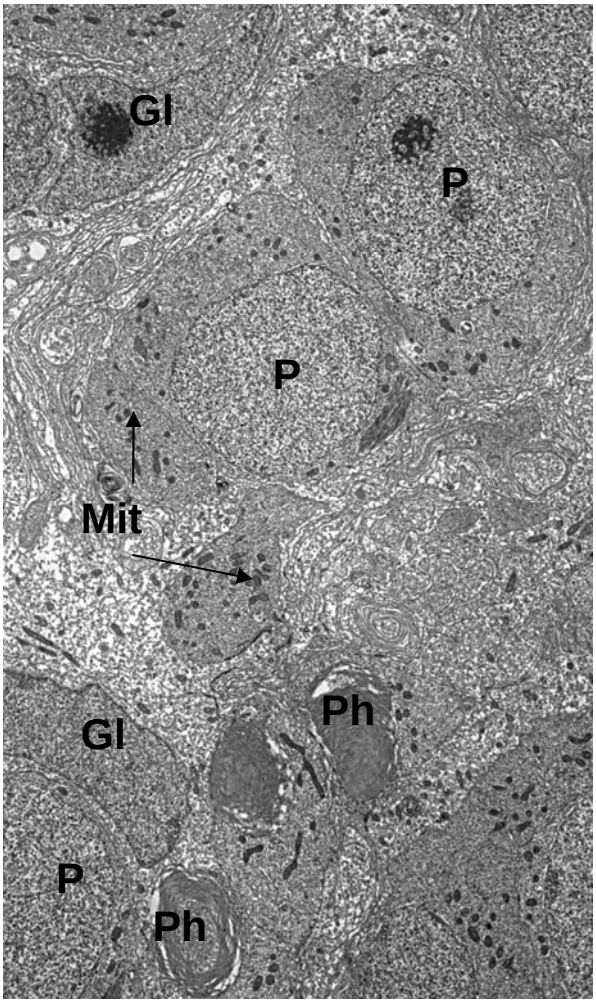


Figure 11: Micrograph from the pineal gland of Arctic charr showing pinealocyte nucleus (P), glial cell nucleus (Gl), mitochondria (Mit) and photopigments (Ph). Magnification: 4000x.

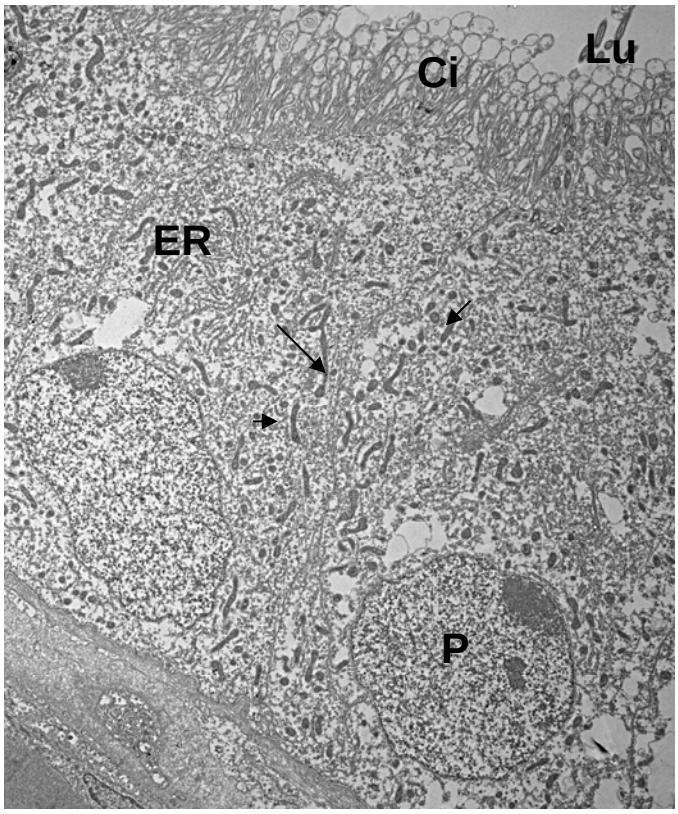


Figure 12: Micrograph from the pineal gland of Arctic charr showing pinealocyte nucleus (P), lumen (Lu), cilia (Ci), endoplasmatic reticulum (ER) and arrows points towards mitochondria (Mit). Magnification: 4000x.

Volumes of cells and organelles

The volumes of the structures in different seasons are shown in figure 13. There were no significant differences between the volumes of the structures in the pineal

gland throughout the year (p≥0.05). ER show lower volume in September than in the other months. The other five structures, pinealocytes, glial cells, mitochondria, blood vessels and lumen show some trends through the seasons, although not significant. September have higher volumes of pinealocytes and blood vessels, but comparatively lower ER, mitochondria and lumen volumes. February have the highest volumes of ER, mitochondria and lumen. In September ER and mitochondria volumes are lowest. For mitochondria and ER February are closely followed by June and December. The only structure with highest volume in June is the glial cells. The other seasons show lower, and almost equal volumes to each other for glial cells. February and June have similar volumes of pinealocytes, blood vessels and lumen. Their lumen volumes are much higher than September and December. The volume of blood vessels in February and June, on the other hand, are much lower than in September, and lower than in December.

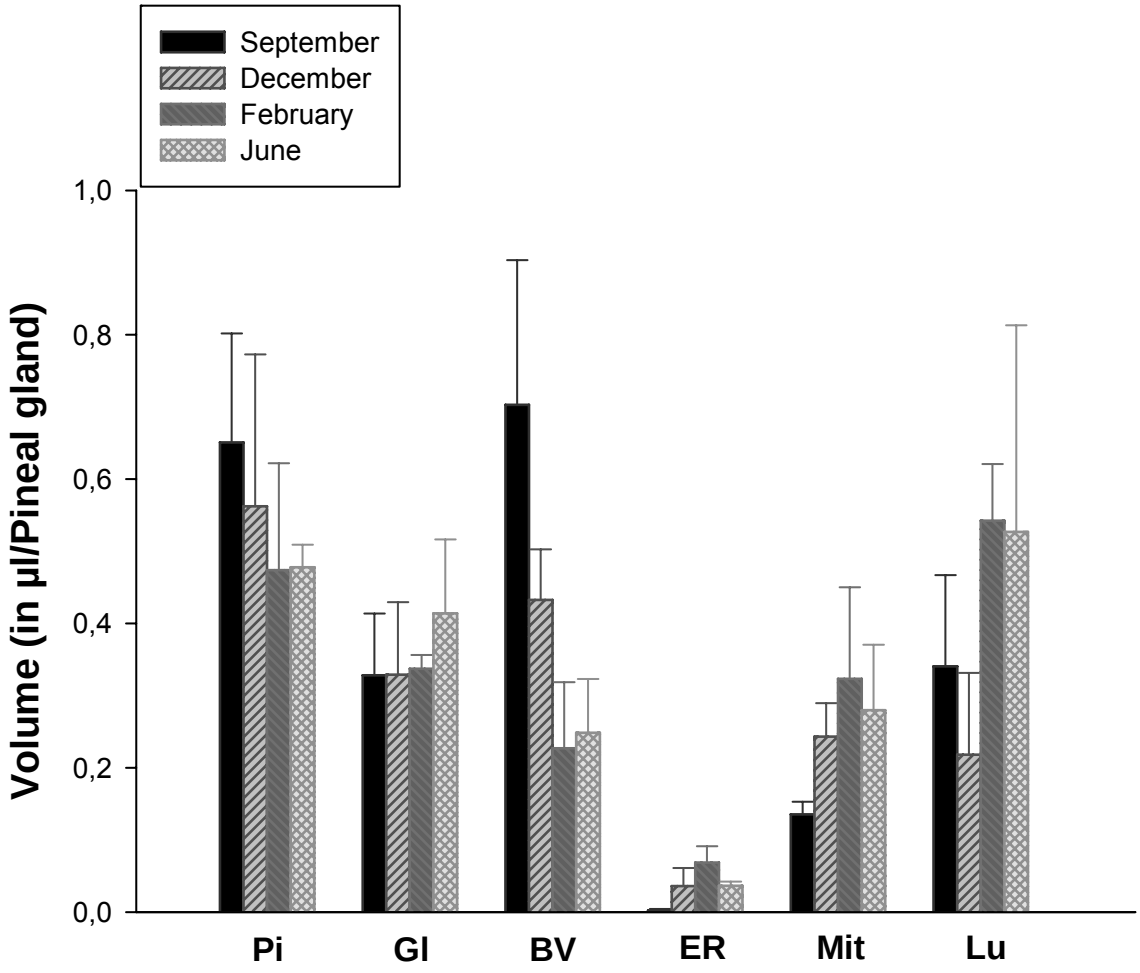
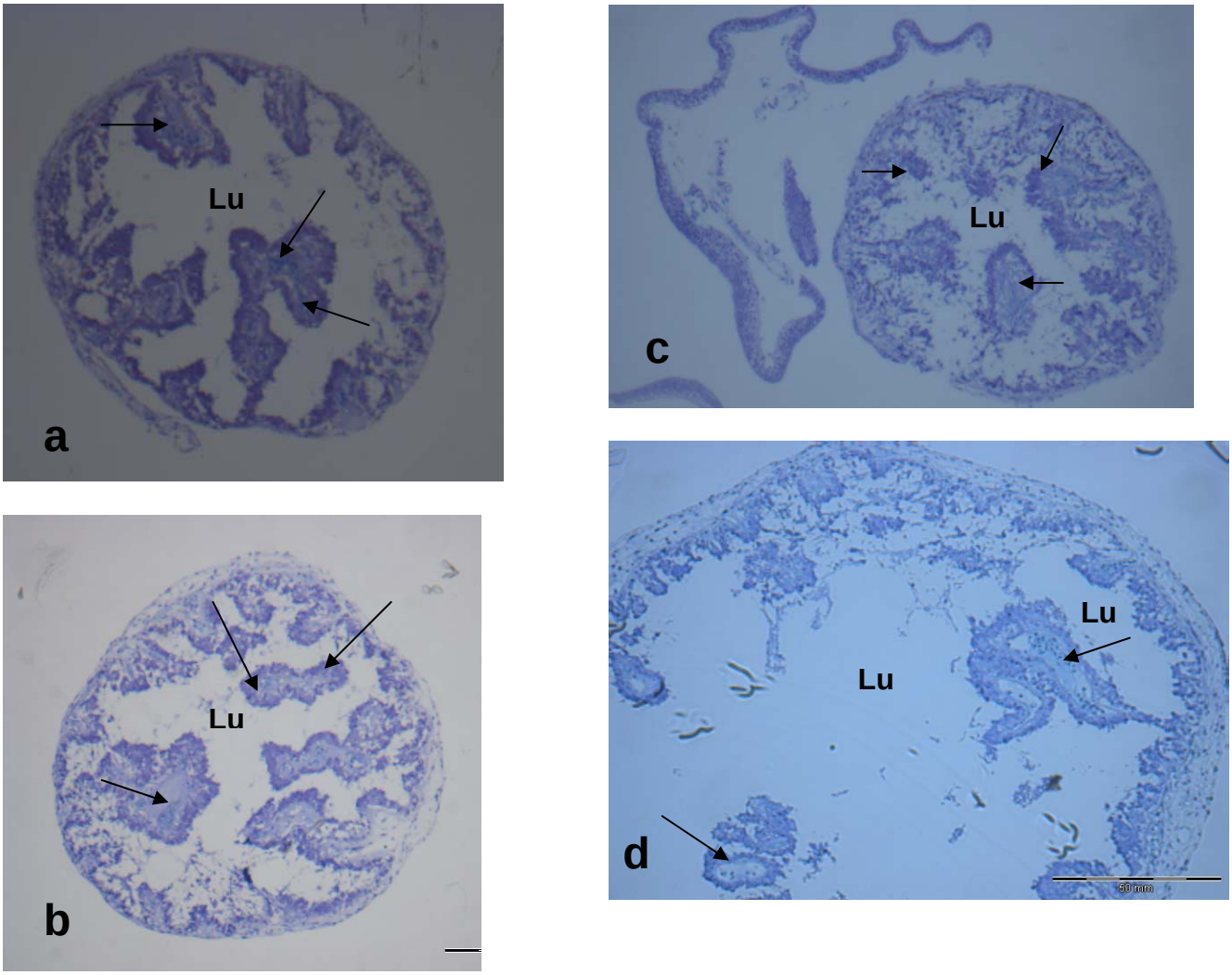


Figure 13: Relative volume of cells and organelles in the pineal gland at all sampling points ± SEM . Abbreviations: Pi, pinealocyte; Gl, glial cell; BV, blood vessel; ER, endoplasmatic reticulum; Mit, mitochondrium; Lu, lumen.

Light microscopy

Figures 14 a-d show light microscopy micrographs of the pineal gland at four different times, August, December, February and June. The lumen is convoluted, and the PG is surrounded by a membrane. The pinealocytes (round, light stained) and glial cells (darkest stained, both difficult to see in figures 14 a-d) are located towards the lumen, with a blood vessel in the middle. No statistical analysis has been conducted on the differences between the seasons.



Figures 14 a-d: Micrographs showing transverse sections of the end vesicle in the pineal gland of Arctic charr. Lumen (Lu) and arrows pointing towards blood vessels can be seen. Magnification: 10x
a) form August; b) from December; c) from February; d) from June. The section from June is ripped open, not giving the impression of a round gland.

4. Discussion

The fish grew throughout the experimental period (table 2). There was a slight decrease in growth rate during mid-winter (data not shown), in accordance with data obtained previously in both wild (Jørgensen *et al.* 1997) and captive Arctic charr (Sæther *et al.* 1996). The most intense feeding and growth period of the year (summer), was not included in the present study and hence the overall growth rate was moderate (data not shown).

Measurements of the pineal gland mass may be inaccurate due to the presence of several folds and membranes in the gland, and hence, different amounts of fluid could be trapped in the folds upon weighing. Nevertheless, there was a significant, positive relationship between the weight of the pineal gland and the fish body mass (figure 8), indicating that the size of the pineal gland increase with the fish size (or age). The relationship between body mass and pineal gland mass in fish has not, to the best of our knowledge, been reported before. This does not however, seem to be a general rend. In seals, for example, an inverse relationship between age (and therefore body mass) and pineal gland mass exists (Aarseth and Stokkan 2003). Further pubertal and early fertile sheep had larger pineal glands than infantile and adult individuals (Redondo *et al.* 2003). These findings suggest that the size of the pineal gland may depend on other factors than body size, and for example be more related to maturation stages. The pineal gland in fish is very small, making it difficult to get completely accurate measures. The measurements done in the present study were strictly standardized, and the number of measurements was quite high over a relatively large range of fish weights (207-1241 g). Hence, the results are considered valid. Since the pineal glands are from the same group of fish, the weight cannot be compared between the seasons, as the fish grew.

No trends could be found regarding the relationship between mass and volume of the pineal gland. This finding was highly unexpected, particularly since the pineal glands sampled differed so much in mass. It is not very likely that the density of the pineal gland tissue should differ very much, and although an accurate determination of the mass was difficult, obtaining an accurate volume of the gland was even more so with the 1 ml syringe used. The finding of a positive relationship between fish and pineal gland mass, and no relationship between the pineal gland mass and volume question strongly the accuracy of the volume measurements. This

would in so case influence the data on the volume of organelles in the gland, since these were related to the total volume of the gland. The fish body mass compared to pineal gland mass had more data (n=57) than the pineal glands which were available for the comparison between the pineal gland mass and volume (n=12) and hence it was more likely to reveal a relationship in the body- and pineal mass data.

Melatonin levels

Melatonin is released directly into the bloodstream after production in the pineal gland, and the plasma levels are therefore a reliable indication of production of melatonin in the pineal gland (Reiter 1991). The diel plasma melatonin rhythms seen at different seasons in the present study were somewhat inconsistent with those seen in previous experiments with Arctic charr (Strand *et al.* 2008). Strand *et al*. (2008) found very low plasma melatonin levels throughout the day in June in Arctic charr, consistent with the finding in the present study. There was an increase in plasma melatonin levels during night in the present study, consistent with findings in a large number of other fish species (Gern *et al.* 1978; Falcon *et al.* 1987; Kulczykowska 1999). However, the night-time peaks in December and February were seen late in the scotophase rather than early, as seen in the study by Strand *et al.* (2008). There is currently no reliable explanation for the mechanisms underlying these differences, but it cannot be excluded that the use of artificial light at the research station, both inside and outside, may have affected melatonin production in this experiment. In the room where the fish tank was kept there are lights which are switched on during working hours, and probably at other occasions if somebody needed to do something there during the natural scotophase. The light regime might therefore not be in complete accordance with the outside illumination, and hence influence the measured melatonin levels. This would be possible since the irradiance threshold for suppression of melatonin production seem to be very low in salmonid fish (Migaud *et al.* 2006), as well as in Arctic charr (between $1x10^{-2}$ and $1x10^{-3}$ W/m^2; Strand *et al.* 2008), and that there where no cover over the tank used in the study. On the other hand there may still be unknown factors involved in the regulation of plasma melatonin levels, since night time profile of plasma melatonin levels in intact fish both in the present study and in Arctic charr held in a natural system (Strand *et al.* 2008) deviate strongly from the mammalian C-like pattern (consistently high melatonin

levels during the whole scotophase; Reiter 1993) seen in Arctic charr pineal glands producing melatonin *in vitro* (Strand *et al.* 2008).

In August there was no significant difference in the plasma melatonin level throughout the 24 hour period. The measurement was, however, taken in early August, when nights are short at 70 °N and actually not completely dark. The lack of any peak in melatonin data for August may therefore either be due to irradiance during the night that was above the threshold for suppression of melatonin production, or the fact that blood samples were not taken during the short night. The amplitude of the diel plasma melatonin variation was similar in December and February, but expected to be higher earlier in autumn, due to higher water temperatures and a Q_{10} effect on melatonin synthesizing enzymes (Ekström and Meissl 1997; Porter *et al.* 2001; Strand *et al.* 2008). The results in the present study clearly show that there are strong seasonal differences in the pineal melatonin production, with a much lower production throughout the continuous light summer than during winter.

Pineal ultrastructure

The findings of the different cell types in the present study of the Arctic charr pineal gland are consistent with findings in other species. The cell nuclei seen are most likely identical to those described by Confente *et al.* (2008) for sole (*Solea senegalensis*), the round and light coloured nuclei being pinealocytes and the darker oval ones being glial cells (Rüdeberg 1968). Blood vessels are seen in the centre of the parenchyma, and the lumen is convoluted. In the outer areas there is mostly blood vessels and membranes, in the parenchyma the pinealocytes are found, and the stalk is compact with neurons (Gupta and Premabati 2002). The pineal gland of Arctic charr seems to fit this general description. Because of the differences in the localisation of different cell types, and the small size, biopsies were considered unspecific, as the chances of getting unrepresentative data were prominent. This is important for this study as volumes of different structures independent of cell types were analyzed, not the differences of organelles within different cells, which would allow random sampling of the cells as long as they were all represented.

In some of the pineal glands it was occasionally seen what could be melanocytes (pigment cells). Melanocytes are recognisable by their comparatively

huge size, even shape and many densely stained round contents. Rüdeberg (1969) found electron dense granules in dogfish (*Scyliorhinus canicula*) inside irregular shaped cells. Electron dense granules of what appeared to be pigments were described by McNulty (1978) in *Chologaster agassizi*. Pineal glands show some black spots around them when seen whole (see figure 4). They are found around and below the middle of the gland, seeing the upper area as the end vesicle, and towards the stalk as the lower part. Underlying the pineal gland there is a pigment layer (Rudeberg 1968). The "black spots" seen might be part of this layer and are not a part of the pineal gland itself. It is possible that these "spots" may get into the pineal membranes during the initial cutting and preparation for embedding. These spots could be what was found in this study, as they did not resemble the granules described by Rüdeberg (1968) and McNulty (1978).

The number of micrographs used for counting varied between the pineal glands (table 1). This should however not influence the results as there were more than enough hits for all the pineal glands for stereology, assuming ~200 hits is enough per sample (Gundersen and Jensen 1987).

The blood samples and the pineal gland were taken at the same time, except in early autumn when the melatonin data were obtained from blood samples taken in August (9^{th}), and pineal glands in September (14^{th}), some weeks later. It was assumed that August was too early for any morphological changes to have taken place and that September represents the months where long nights and "high" temperatures co-occur and hence is the month with the highest rate of (night time) melatonin production (Strand *et al.* 2008). Difference between night and day in melatonin production was not associated with differences in night and day pineal weight. This result does not correspond to those of Redondo *et al.* (2003) who found that the pineal gland of sheep were heavier, and had higher volume, when the glands were excised during the night than during the day (02.00 h vs 14.00 h). The difference between species may be related to ectothermy vs homeothermy; i.e. that the rate of changes may be much faster in the sheep with a body temperature of ~37°C than in a fish with a body temperature below 10°C.

McNulty (1982a) found a significant increase in the volume of photoreceptor cells and mitochondria and area of rough ER after exposing goldfish (*Carassius auratus*) to constant darkness for six months, compared to natural light:dark cycles, but no significant changes after six days. No significant changes were found after

exposing the fish to constant light. This shows that the changes in light conditions (and/or melatonin production) may be associated with changes in the pineal gland over time. Such long-term changes in organelle size involved in synthesis and secretion were not found in the Arctic charr, despite the strong seasonal change in light intensity and melatonin secretion between mid-summer and –winter. The relative glial cell and pinealocyte volumes varied little throughout the sampling period. There were differences between sampling dates in the other organelles, but these differences were not significant. It cannot be excluded, however, that the lack of statistical differences could be due to the low number of pineal glands analysed and huge inter-individual differences (cf. high SEM), and corresponding type 1 statistical errors.

There was a tendency toward higher blood vessel volumes in September and December than in February and June ($p=0.08$). Further there were a tendency to changes in the endoplasmatic reticulum ($p=0.055$), but this difference was in so case opposite of that found for blood vessels; higher in February than in September. Taken together, these results suggest that the present study did not reveal any quantitative, structural changes in the pineal gland of Arctic charr consistent with the changes in irradiation and melatonin production. This result is surprising, particularly since the season with high melatonin production (winter) also is the season where the temperature is low. Hence, a temperature compensation, brought about by alterations in the concentration and/or specific activity of melatonin synthesizing enzymes should be expected (Prosser 1990). Mean pinealocyte volume was somewhat higher in September as compared to June, but the difference was far from significant ($p=0.67$), and it may be suggested that the increased autumn/winter production of melatonin are associated with specific activity of synthesizing enzymes. John and George (1989) found significant changes in the area of pinealocytes, pinealocyte nucleus and mitochondria, between (some of the) different times of year (spring migration, breeding, moult and fall migration) in migratory Canada goose (*Branta canadensis interior*). They noted that the abundance of mitochondria (amongst other) appeared to be greatest during the spring post migratory phase, the phase with the lowest ambient temperature. In the present study, mean volumes of endoplasmatic reticulum and mitochondria was highest in the coldest sampling date. As mentioned before, type 1 errors cannot be excluded, and in September when

melatonin production is expected to be highest (Strand *et al.* 2008) both blood vessel and pinealocyte volumes were higher than in other dates.

Previously, the size of nuclei and nucleoli have been investigated (Hafeez *et al.* 1978). Both the nuclei and nucleoli of the support cells were significantly smaller in continuous light (LL) than in constant darkness (DD) and light:dark cycles (LD) in steelhead trout. If this is the case for Arctic charr, there is a possibility for increased activity even without increase in the volume of the cell itself. Another structure investigated, synaptic ribbons (part of the photoreceptor axon terminal; Ekström and Meissl 1997), seems to respond quickly to light changes. McNulty (1982a) found the synaptic ribbons in goldfish to change on the first day subjected to LL, and on every sampling they were markedly longer than the controls. There was also registered significant daily volume differences in endoplasmatic reticulum and golgi complex, but not mitochondria, in goldfish (McNulty 1982b).

As noted before, it is very unlikely that the method for measuring pineal gland volume was able to provide reliable volume data. The quality of the quantitative data on gland ultrastructure is therefore uncertain. However, when ultrastructure volumes were related to pineal weight (which is considered more correct than volumes) no clear-cut picture of seasonal differences appeared. It is therefore concluded that there is no differences between season in ultrastructure of the pineal gland in Arctic charr.

By comparing the micrographs from the four seasons by light microscopy, there were no clear differences to be seen, although no tests have been conducted to verify this. Taken together, these data indicate that the pineal gland of the Arctic charr does not undergo marked seasonal changes in ultrastructure and size. On the one hand this is surprising, taken into consideration that the organ size often is correlated with their metabolic activity (Jensen 1980) in order reduce the stationary cost of organ maintenance (Piersma 2002). On the other hand the low melatonin production in the Arctic charr occurs during a period with abundant food supply, making energy saving less important. Further, the pineal gland is a very small gland which probably represents an insignificant contribution to the total energy need in fish.

Conclusion

From this study it seems that the production machinery for melatonin in the pineal gland does not change its ultrastructure or components markedly with season, despite fluctuations in melatonin production throughout the year. This indicate that the pineal gland in it self is capable of increasing its production of melatonin without an increase in cell components/cells regarded as important for hormone synthesis in the gland.

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Appendix 1

Extraction for plasma

Day 1
Use conical glass extraction tubes. Acid washed or new.

1. Add 100 µl or more of plasma to the labelled extraction tubes and record amounts in standard assay forms (adjust amount of plasma depending upon the time of day you obtained the sample and/or the experimental treatment)
2. Bring all samples to an equal volume of 300 µl with 2x distilled water
3. Add 150 µl 1.0 M NaOH
4. Add 3 ml of reagent or HPLC grade chloroform

Vortex each vial individually and then set on vortex machine for at least 10 minutes continuous vortexing. Make sure the tubes are covered.
Set in 4°C overnight (or at least 4 hours)

Day 2
First extraction:

1. Vortex each vial individually, spin for 15 min at 1100 rpm (if they are persistent micelles then spin longer)
2. Carefully aspirate off chloroform layer into respective labelled glass tubes (12x75 mm) using a clean Pasteur pipet for each sample

2nd extraction: (3 and 4 only necessary if there is a lot of fat, e.g. seal)

3. Add 2 ml more of chloroform to extraction tubes. Vortex singly and set aside at 4°C for 1-2 hour
4. Spin again for 15 min at 1088 rpm. Repeat aspiration procedure and add to first respective extracted samples
5. Evaporate chloroform in 40°C water bath under nitrogen
6. Add 270 µl (or 160 µl when you do the assay in single tubes) of 0.1 M Tricine buffer
7. Vortex vigorously, pulse spin, and let equilibrate overnight at 4°C

To clean the extraction vials: Aspirate off water/plasma layer and discard in radioactive waste. Place dirty tubes into counts-off bucket, after at least 24 hrs into the acid wash.

Day 3:
Get dry ice, MEL-antibody and MEL label from the -70°C freezer (institute)
3rd extraction:

1. Wash with 250 µl or more of Petroleum ether (=petroleum benzine)

Vortex vigorously every single vial by hand until all of the fat comes off the glass and out of the buffer phase. Left over fat will interfere with the assay

2. Spin at 3000 rpm for 10 min
3. Freeze on dry ice setting the tubes at an acute angle but only long enough to freeze the buffer phase not the Pet ether phase
4. Using sink aspirator aspirate off upper Pet ether phase

Set in water bath for 5 minutes to let Pet ether evaporate. Pay attention to the time!

Check every tube carefully. If it is still milky or there are coloured crystals of fat, repeat the defattening procedure. There should be no ring of fat on the bottom of the tube. If there is you need to repeat the procedure and vortex longer. For some birds e.g. Redpolls, Ptarmigan and even some winter sparrows it is almost always necessary to wash twice.
Remove and set at 4°C for 30 minutes
Now you can continue with the Radioimmunoassay

RIA procedure
MELATONIN RIA

Buffer:
1 g gelatin dissolves in 200 ml distilled water, 50 minutes at 50°C.
17.9 g tricine and 9.0 g NaCl dissolves in 700 ml distilled water.
The two solvents are mixed and the volume adjusted to 1L with distilled water.

Storage and durability: Refrigerator 1 week.

Antiserum:
Anti-melatonin antiserum from sheep (product code: AB/S/01). Sufficient for 4000 assay vials.

Intermediate stock (1:10):	Contents resuspenderes in 2 ml distilled water. Distribute in eppindorf tubes á 50 µl and store at -20°C.
Working stock:	50 µl (1:10) to 20 ml with buffer. Sufficient for 100 vials, 1:4000 dilution

Dextran coated charcoal:
4 g activated charcoal dilutes in 200 ml buffer. Shake for 5 minutes on ice.
Distribute in 50 ml centrifuge vials and centrifuge at 1000 rpm, 4°C, 5 minutes.
Discard supernatanten and puss around the edges.
Resuspender the charcoal in 200 ml buffer and add 0,04 g dextran T70.
Stir for at least 1 hour at 4°C.

Storage and durability: Refrigerator 1 week.

^{3}H-melatonin:

Intermediate stock:	20 µl from the bomb in 2 ml abs. EtOH. Stored at -20°C.
Working stock:	Dilution in buffer so that 100 µl contains 4000 cpm. Make fresh every day.

Standard:

Melatonin stock (1 mg/ml):	Dissolve 10 mg melatonin in 0.5 ml abs.EtOH. Adjust the volume to 10 ml with distilled water
	Durability at least 1 year in a refrigerator.

Working stock: 100 µl (1 mg/ml) to 100 ml with distilled water = 1 µg/ml.
500 µl (1 µg/ml) to 50 ml with distilled water = 10 ng/ml.
125 µl (10 ng/ml) to 2.5 ml with buffer = 0.5 ng/ml.

MT standard 0.5 ng/ml (µl)	MT fritt plasma (µl)	MT concentration Pg/0.5 ml
0	500	0
5	495	2,5
10	490	5,0
25	475	12,5
50	450	25,0
100	400	50,0
200	300	100,0
500	0	250,0

Scintillation fluid:
5 g PPO (2,5-diphenyloxazole)
0.3 g dimethyl-POPOP
To 1L toluene (with low sulphur content)

PROCEDURE
At the start of new round:

Make all the working solutions.
Run one standard curve before you start with your samples.

Day 1
Make tracer.
Make antibody solution.
Make std stock (0.5 ng/ml) in buffer.
Defrost the samples to be analysed.
Pipette out standard curve according to table.
Vortex all vials and incubate for 30 minutes in room temperature.
Add tracer according to table.
Vortex all vials and incubate over night in refrigerator, 4°C.

Day 2
Place the charcoal solvent for stiring on ice for 30 minutes.
Add 500 µl buffer to the TC vials.
Add 500 µl charcoal to all vials except TC.
Vortex all vials and incubate for 15 minutes at 4°C.
Centrifuge for 15 min at 500 g (~2700 rpm), 4°C.
Transfer 700 µl from each vial to counting vials.
Add 4 ml scintilation fluid in each vial and put on lid.
Shake for 1 hour on a shaker.
Count each sample in the β-counter (scintillation counter) for 5 minutes.
Melatonin concentration in each sample is calculated from the std. curve.

	MT clean plasma (buffer)	MT std 0.5 ng/ml	Sample	AS	Buffer	^{3}H-MT	Dextr.c.Charcoal	Buffer	Dilution factor	Comments
TC buff					700	100		500		
NSB buff					700	100	500			
TC	500				200	100		500		
NSB	500				200	100	500			
4x TB	500			200		100	500			
S1	495	5		200		100	500			
S2	490	10		200		100	500			
S3	475	25		200		100	500			
S4	450	50		200		100	500			
S5	400	100		200		100	500			
S6	300	200		200		100	500			
S7		500		200		100	500			
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Appendix 2

Preparation for TEM
Protocol for embedding in Epon/araldite
Safety:
Separate procedure for OsO_4
Always use gloves
The resin is carcinogen and allergen and shall only be used in disposable equipment
Waste:
Fix, buffer and alcohol discharge in the sink
OsO_4 , uranyl acetate and propylene oxide in special bottles
Epoxy resin evaporate under a hood and polymerize at 60°C

1. Fixation, minimum 4 hours
2. 2x 15 min wash with buffer, corresponding to the initial fix used
3. 1 % OsO_4 in distilled water for 1½ hours
4. 2x 15 min wash with buffer (the same as above)
5. 2x wash in distilled water, quick changes
6. 2 % uranyl acetate in water 1½ hours
7. Dehydration
   30 % ethanol 5 min
   60 % ethanol 10 min
   (70 % ethanol if a stop in the protocol is needed, otherwise no)
   90 % ethanol 10 min
   96 % ethanol 10 min
   2x absolute ethanol 10 min
   3x propylene oxide 5 min
8. epoxy:propylene oxide 1:2 30 min
9. epoxy:propylene oxide 1:1 1 hour without lid
10. pure epoxy 1 hour
11. pure epoxy over night (in mats or vials)
12. Polymerization Epon/araldite over night at 60°C

EPON (Glycidether)/ARALDIT

Glycidether	6ml	18ml	24ml
DDSA	11ml	33ml	44ml
Araldite 502	3ml	9ml	12ml
DMP 30	0.4ml	1.2ml	1.6l

Shake well and stir ½ hour on a magnetic stirrer
Store at -20°C
Leftovers polymerizes at 60°C over night

Conrasting
Contrasting is done on a piece of parafilm with pits made in it (to stop the droplets from moving into each other), in a petri dish with lutperler to absorb any moist. A droplet of Ur.Ac. is placed in each of the pits, and the grids placed on the droplets, with the tissue down, for 7 minutes. The grids are picked up with forceps and washed by dipping 10-15 times in five different cups of distilled water. Small pieces of blotting paper are placed in the forceps to absorb water droplets. The drying period is at least 15 minutes, before the procedure is repeated with a new parafilm for the lead citrate droplets. The drying time between the contrast solutions are important to avoid crystallisation of the solutions. The grids are placed one on each droplet, with the tissue side down, for 5 minutes. Wash as with Ur.Ac. and let the grids dry for at least 30 minutes before use in the microscope or storage in a grid box.

Embedding using Technovit 8100
Dehydration
The tissue is dehydrated in 100 p.c. acetone for 60 min at 4°C. During the first 5 min acetone should be renewed a few times until the solution remains clear.
Infiltration
Using Technovit 8100 the infiltration time lasts between 6-10 hours at 4°C. The infiltration solution consists of
100 ml base-liquid Technovit 8100
0.6 g hardener I (1 bag)
The infiltration solution is stored at a maximum of 4 weeks at 4°C.
To avoid a change in temperature, the Histoform should be kept in cold store (ice).
Embedding
Embedding solution: 15 ml infiltration solution (base-liquid and hardener) and 0.5 ml hardener II are mixed thoroughly at 4°C. After that the tissue to be embedded is immersed and the solution is agitated for 5 min. The well mixed embedding solution is poured into the embedding mould and the tissue specimen is properly placed. Right afterwards, the mould is sealed hermetically with the cover foil and placed on crushed ice (refrigerator) at 4°C.
When using the above mentioned ratio, the curing time at a temperature of max. 11°C is at least 3 hours.