DIFFERENTIAL LIGHT INTENSITY AND SPECTRAL SENSITIVITIES OF ATLANTIC SALMON, EUROPEAN SEA BASS AND ATLANTIC COD PINEAL GLANDS *EX VIVO*.

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ABSTRACT

Photoperiod is perceived by pineal photoreceptors and transduced into rhythmic melatonin signals. These rhythms can be influenced by light intensity and spectral content. In this study we compared the light sensitivity of Atlantic salmon, European sea bass and Atlantic cod by testing *ex vivo* the effect of different intensities and narrow bandwidth lights on nocturnal melatonin suppression by isolated pineal glands in a flow-through culture system. Using combinations of neutral density and bandpass interference filters we tested a range of light intensities (ranging from 1.22×10^{13} – 3.85×10^{6} photons.s^{-1}.cm^{-2}) and three wavelengths of 80 nm width (472, 555 and 661 nm corresponding to blue, green and red, respectively). Results showed clear species specific light intensity and spectral sensitivities, with cod being from 100 to 1000 times more sensitive than sea bass and salmon. Regarding the influence of spectrum, red light was less efficient on suppressing melatonin than blue and green in salmon but results were not as clear in the two other species studied. Finally, the first evidence of relative photoreception in teleosts was obtained in cod suggesting that the definition of illuminance thresholds (day/night perception) would depend on the day intensity. Indeed, a single order of magnitude increase or decrease in day intensity was shown to elicit a significant shift in the intensity response curve of night-time melatonin suppression. Taken together, this study demonstrated species specific light intensity and spectral sensitivities within temperate teleosts.

Keywords: pineal gland, light intensity, spectrum, melatonin, teleosts fish.

INTRODUCTION

The pineal gland of teleost fish is an evagination of the diencephalon containing cone-like photoreceptors that are functional light sensors (Falcón, 1999). In most vertebrates, this gland is described as a photoneuroendocrine system composed of photoreceptor cells, self-sustained oscillators and neuroendocrine effectors (Ekström & Meissl, 1997; Korf et al., 1998) that synthesizes the indolamine melatonin in response to the ambient illumination. Highest melatonin levels are produced during the dark phase and circadian rhythms are self-sustained under continuous darkness (Falcón et al., 1989; Iigo et al., 1994; Masuda et al., 2003). However, within teleosts, salmonids appear to be exceptions to this generalized system as melatonin rhythms are not sustained under prolonged dark periods suggesting that they have lost the clock regulation of rhythmic melatonin release (Iigo et al., 2007). The photic regulation of the melatonin production is complex and involves photoreceptors in the eyes, pineal gland and possibly deep brain with divergent circadian organizations found within teleosts (Migaud et al, 2007). It is likely that the light sensitivity of these different systems will differ possibly due to the type of photoreceptors involved, their location but also the range of photic environments inhabited by teleosts. However, to date, very few comparative studies have been performed despite the significant implications for the control and management of fish physiology (i.e. reproduction, migration, feeding, locomotor activity…..). This would certainly help to better characterise and understand local adaptations to specific environments.

Previous studies have shown that light sensitivity of the melatonin cascade greatly differ between teleost species. The lowest light intensity to suppress melatonin production *in vivo* in sea bass *Dicentrarchus labrax* was 6.0 $\mu W/cm^2$ (equivalent to 1.92 x 10^{13} photons$\cdot s^{-1}\cdot cm^2$) (Bayarri et al., 2002) whereas in Senegal sole it was 5.3 $\mu W/cm^2$ (1.70 x 10^{13} photons$\cdot s^{-1}\cdot cm^2$) (Oliveira et al., 2007) and in tench 3.3 $\mu W/cm^2$ (1.10 x 10^{13} photons$\cdot s^{-1}\cdot cm^2$)(Vera et al.,

2005) when 1 hour light pulse was tested in the middle of the night. However, results largely differed in other *ex vivo* studies with theoretical *in vivo* threshold of light intensity (taking into account the light penetration through the skull), determined between 3.8 x 10^{-5} – 3.8 x 10^{-6} W/m^2 (equivalent to 1.22 x 10^{10} and 1.22 x 10^9 photons·s^{-1}·cm^2) in sea bass (Migaud et al., 2006). These discrepancies could be explained by experimental differences between both studies as light tested in the later was continuously applied throughout the dark phase (12 hours period) which might explain the increased photic sensitivity as compared to light pulses. This is known as dark adaptation and well documented in the mammalian visual system (Refinetti, 2001). Atlantic salmon, *Salmo salar* appeared to have much lower light sensitivity of the pineal gland than sea bass with a threshold of day and night melatonin levels found between 3.8 x 10^{-4} and 3.8 x 10^{-5} W/m^2 (equivalent to 1.22 x 10^{11} and 1.22 x 10^{10} photons·s^{-1}·cm^2 (Migaud et al., 2006). The photic sensitivity of the melatonin system will also depend on the spectral properties of the light. Several photopigments have been identified in the outer segment of the pineal photoreceptors in fish. In rainbow trout, *Oncorhynchus mykiss*, it was reported the existence of two populations of photoreceptors with two different action spectra peaking in the blue and in the green region of the visible spectrum (Marchiafava & Kusmic, 1992). In mesopelagic fish the pineal morphological organization is similar to that observed in shallow-water species with photopigments that have a λ_{max} between 485 and 503 nm whereas in the deep demersal eel, *Synaphobranchus kaupi*, the pineal photopigment has a λ_{max} at 515 nm (Bownaker & Wagner, 2004). Several *in vivo* and *ex vivo* experiments have tested the efficiency of different wavelengths to reduce nocturnal melatonin and have shown that in sea bass the blue end of the visible spectrum (blue, 434-477 nm) was more effective than longer green and red wavelengths (Bayarri et al., 2002). In zebrafish, however, green light (512 nm) was shown to be the most efficient in suppressing melatonin production by pineal glands in culture (Ziv et al., 2007). While there are many indirect

evidences of differential photic sensitivities in temperate fish species through assessment of the effects of artificial lighting regimes on growth (Boeuf & Le Bail, 1999; Ruchin, 2004), reproduction (Popek at al., 1992; Randall at al., 1995), behaviour such as light attraction, feeding and locomotor activity (Ballagh et al., 2008; Giménez & Esteve, 2008; Kavaliers, 1981; Underwood, 1989) and embryo/larvae development and performances (Downing & Litvak, 2002; Monk et al., 2006), there is still a lack of clear demonstration of these at the pineal level, especially with regards to spectrum.

The objective of our study was therefore to 1) determine the light intensity threshold of melatonin production by Atlantic cod (*Gadus morhua*) pineal gland cultured *ex-vivo* and compare results with previously published data on Atlantic salmon and European sea bass (Migaud et al., 2006), 2) compare spectral sensitivity of Atlantic salmon, European sea bass and Atlantic cod pineal glands *ex vivo* and 3) investigate relative pineal sensitivity in Atlantic cod.

MATERIALS AND METHODS

Animals

Atlantic salmon (body weight: 92.9 ± 4.0 g, total length: 20.7 ± 0.2 cm), European sea bass (body weight: 368.3 ± 15.6 g, total length: 30.8 ± 0.5 cm) and Atlantic cod (body weight: 256.6 ± 16.6 g, total length: 28.6 ± 0.7 cm) were obtained from the Machrihanish Environmental Research Laboratories of the Institute of Aquaculture (Scotland). Fish were reared and acclimated to a constant 12L: 12D artificial photoperiod (lights on at 08:00, lights off at 20:00) and a temperature of 14 ± 1ºC for a period of at least 2 weeks prior to the start of the experiments. Fish were killed by a lethal dose of 2-phenoxyethanol solution (1 $mL.L^{-1}$, SIGMA, Ref. P 1126). All experiments were carried in accordance with the Animal (Scientific Procedures) Act 1986, UK.

Experiment 1: *Ex vivo* pineal light intensity sensitivity in Atlantic cod

Fish were culled between 15:00 hrs and 16:00 hrs and pineal glands dissected by opening the skull dorsally around the pineal window and extracting the intact gland. After removal, pineal glands (n = 4) were washed with culture medium and then placed individually in the culture chambers. The pineal culture system consisted of a continuous flow through system regulated by a peristaltic pump at a flow rate of 1.5 ml of culture medium/hour and samples were collected every hour by an automatic fraction collector as previously described by Migaud et al. (2006). The culture media (ref. RPMI 1640; Sigma), which was changed daily, was supplemented with HEPES sodium salt (ref. H3784, 4.77 g/L; Sigma) to buffer the pH adjusted to 7.4 and penicillin-streptomycin (10 mg/L) and Fungizone (5 g/mL) to avoid bacterial and fungal development. Samples were removed from the culture system daily and stored at -70º C prior to analysis. The pineal glands were subjected to a matching photoperiod regime *ex-vivo* as they had previously been acclimated to *in-vivo* with culture media samples being collected from 17:00 on the day of pineal removal. Each *ex vivo* trial started and ended by a 12L: 12D cycle to which fish were acclimatized to, serving as controls for normal melatonin production by the pineal glands. On the second and third night of culture (subjective night, SN), pineal glands were subjected to one of a range of 7 different intensities from 3.85×10^{6} to 3.85×10^{12} photons.s^{-1}.cm^{-2} (Table 1). Illumination was supplied by dichroic halogen bulbs with an emission spectrum equivalent to a 4700 K Black Body Radiator (Solux, 4700 K CRI 99, 10º spread, USA) artificially recreating ambient daylight (Fig. 1). Day light intensity was set at 5.8 W/m^2 (1.92×10^{15} photons.s^{-1}.cm^{-2}). Light spectrum was analysed using a portable spectroradiometer with a fibre optic umbilical (EPP2000c Stellarnet Inc., USA) and light intensity (W/m^2 and photons/sec/cm^2, 400-740nm) was measured using a single channel light sensor (Skye instruments, UK). Both systems were calibrated to National Physics Laboratory UK standard light sources.

Experiment 2: *Ex vivo* pineal spectral sensitivities in salmon, sea bass and cod

Dissection of the pineal glands was adapted for each species. In salmon the skull was cut under the brain from the rostral to the caudal region, then the brain was removed and the pineal gland exposed. In sea bass and cod pineal removal was done as described in exp. 1. In this experiment, three different intensities were tested during the subjective night for each species depending on previously demonstrated light thresholds (Migaud et al., 2006; exp. 1 for cod) with one above, one under and one at threshold levels (Table 1). Day light intensity was set at 5.8 W/m^2 (1.92 x 10^{15} photons.s^{-1}.cm^{-2}). For every intensity, three different narrow bandwidth "colour" lights were tested (Full width at half maximum, FWHM = 80 nm) with λ max = 472, 555 and 661 nm corresponding to blue, green and red, respectively. Light treatments were achieved using combinations of neutral density (to normalize light intensities across spectra) and bandpass interference filters (Melles Griot Photonics Component Group). Spectral profiles are given in Figure 1.

Experiment 3: Relative pineal sensitivity in Atlantic cod

As described above pineal glands were dissected from cod and placed in continuous flow through culture for four days. Day light intensity was set at either 1.92×10^{16}, or 1.92×10^{14} photons.s^{-1}.cm^{-2} with the pineals (n=4) being cultured under LD for the first cycle then they were illuminated in the subjective dark of the second and third night at 3.85×10^{7}, 1.22×10^{9} or 1.22×10^{11} photons.s^{-1}.cm^{-2} (Table 1). Thereafter they were returned to an LD cycle to control for normal melatonin production. Media samples were removed from the culture system daily and stored at -70º C prior to analysis. These data were compared to those generated in experiment 1.

Melatonin assay

Melatonin was determined by a direct radioimmunoassay (Fraser et al., 1983). Due to the large number of samples generated and the high costs of the analyses only every fourth hour samples have been analysed and presented for all trials performed (i.e. 3 time points assayed per dark, light or filter period tested for four pineals per treatment and species). In all cases, the first night samples were taken at 22.00 (2hrs after dusk) on the day of setup. Samples were thawed, placed in polypropylene tubes and 250 µl of assay buffer (Tricine 12.9 g/L, NaCl 9g/L, gelatine 1g/L) was added. After mixing, 200 µl of sheep anti melatonin antiserum (Stockgrand Ltd, ref. AB/S/01) was added to all tubes except to the non specific binding (NSB) and 100 µl of (*O-methyl-*^{3}*H*) melatonin (Amersham, ref. TRK-798) was added to all tubes and the mixture was incubated overnight at 4 ºC. After adding 500 µl of dextran coated charcoal (Sigma, ref. C6241) suspended in assay buffer (9.6g/L) the tubes were incubated for 15 min at 4 ºC and centrifuged. Finally, 1 mL of supernatant was transferred to scintillation vials that were filled up with 4 mL of scintillation cocktail (Ultima Gold, Perkin Elmer Life Sciences, ref. 6013329) and radioactivity was measured in a liquid scintillation analyzer (Packard, model 1900 TR). Prior to the analyses, the RIA assay has been validated by confirming the parallelism between serial dilutions of night-time pooled medium for each species to the standard curve (data not presented). The intra-and inter-assay coefficients of variation were 4.9% and 8.3%, respectively.

Data analysis

Data are expressed as mean ± S.E.M. Statistical differences between mean melatonin concentrations were analyzed by one-way analysis of variance (ANOVA) followed by a Duncan's test, with $P < 0.05$ taken as the statistically significant threshold. All statistical tests were carried out with the program SPSS v16.0 (SPSS Inc., USA).

Percentages of melatonin produced during the subjective night (SN) relative to the normal night production (% SN/N). Furthermore, results were also presented as percentage of the normal day melatonin production relative to the SN concentrations (% D/SN). For comparative purposes the % SN/N and % D/SN data for cod from experiment 1 were fitted with a four parameter logistic model (Zeitzer et al., 2000) using Sigmaplot Ver. 10 (Systat Software, Inc, USA) as follows:

$$f(\chi) = \frac{a-d}{1+(\chi/b)^c} + d$$

Where a and d represent maximum and minimum values respectively (for both %SN/N and % D/SN data a was fixed to 100%), b represents the intensity at which 50% of the maximal effect is observed and c is a measure of the steepness of the rising portion of the curve. Residuals analysis revealed a normal distribution of data and that there was no autocorrelation.

RESULTS

Experiment 1: *Ex vivo* pineal light intensity sensitivity in cod

Under a light dark cycle (control LD cycle), melatonin production in *ex vivo* pineal culture was reduced from > 2500 $pg.ml^{-1}$ during the night phase to <100 $pg.ml^{-1}$ during the day phase in Atlantic cod (Fig. 2). When pineals were illuminated during the subjective night, melatonin synthesis was inversely related to the intensity of the illumination applied. Melatonin released during the subjective night expressed either with respect to normal night (% SN/N) or day (% D/SN) levels were plotted against the light intensity to which the pineals were exposed during the SN (Fig 3). This data showed a good fit to a four parameter logistic model (Table II) which predicts that the half maximal response to dark phase levels is $8.95 \times 10^7 \pm 1.3$ $photons.s^{-1}.cm^{-2}$ and day levels is $1.86 \times 10^{10} \pm 1.3$ $photons.s^{-1}.cm^{-2}$.

Experiment 2: *Ex vivo* pineal spectral sensitivities in salmon, sea bass and cod

In Atlantic salmon, melatonin production by pineals in culture was reduced from 7715.5 ± 243.2 $pg.mL^{-1}$ during the night to < 400 $pg.mL^{-1}$ during the day, whereas values during the subjective night varied with the different light intensities and spectra tested. When pineal glands were exposed to light intensities of 1.22 x 10^{13} and 1.22 x 10^{12} $photons.s^{-1}.cm^{-2}$ and blue or green wavelengths, melatonin concentrations remained basal as diurnal levels (Fig. 4A-B). However, under a red light melatonin production was statistically higher than that observed during the day, with levels of 2691 ± 292 and 5338 ± 720 $pg.mL^{-1}$ observed for intensities of 1.22 x 10^{13} and 1.22 x 10^{12} $photons.s^{-1}.cm^{-2}$ respectively, although these concentrations still remained statistically lower than the nocturnal ones (Fig.4, A-B). When pineal glands were exposed to a lower light intensity during the SN (1.22 x 10^{11} $photons.s^{-1}.cm^{-2}$) melatonin production increased to values comparable to those obtained during the night when the light spectrum tested was green or red (except for red light which remained significantly lower than levels during the second night) (Fig. 4C). Melatonin levels in pineals exposed to blue light was however still significantly lower than during both night periods. These results are corroborated by significantly higher % SN/N and lower % D/SN ratios under red versus blue spectrum at both 1.22 x 10^{12} and 1.22 x 10^{13} $photons.s^{-1}.cm^{-2}$.

In European sea bass, maximum nocturnal melatonin levels were 4124.0 ± 128.2 $pg.mL^{-1}$ and they were reduced down to <1400 $pg.mL^{-1}$ during the day. During the SN phase, melatonin was suppressed when lights of 1.22 x 10^{12} $photons.s^{-1}.cm^{-2}$ were applied, irrespective of the spectrum (Fig. 5A). When a light intensity of 1.22 x 10^{11} $photons.s^{-1}.cm^{-2}$ was applied, an increase of melatonin levels during the SN phase was observed for all spectrum, especially for blue and red lights, although these concentrations remained significantly lower than nocturnal values (Fig. 5B). Finally, green and red lights of 1.22 x 10^{10} $photons.s^{-1}.cm^{-2}$ did not suppress melatonin production with concentrations comparable to night-time (Fig. 5C). However,

while under blue light the pineals increased their melatonin production in comparison with previous light intensities tested, it did not reach values statistically comparable to those seen during the night phase. The % SN/N was significantly higher for red spectrum at the lowest light intensity (1.22 x 10^{10} photons.s^{-1}.cm^{-2}) and except a significant difference at the lower intensity with green showing a higher % D/SN, no further differences were observed for this ratio between spectra.

As for cod, nocturnal melatonin concentrations were 2300.2 ± 154.4 pg.mL^{-1} whereas during the day these levels were reduced down to <142 pg.mL^{-1}. However, large variability in peak nocturnal levels was observed between pineal glands. Lights of 1.22 x 10^{10} photons.s^{-1}.cm^{-2} inhibited the production of melatonin during the SN phase for all spectra (Fig. 6A). When light intensity was reduced to 1.22 x 10^{9} photons.s^{-1}.cm^{-2}, melatonin concentrations increased irrespective of the spectrum (Fig. 6B). Finally, blue, green and red lights of 1.22 x 10^{8} photons.s^{-1}.cm^{-2} did not inhibit the synthesis of melatonin during the subjective night as we recorded concentrations comparable to the night period (Fig. 6C). No differences were observed in the % SN/N ratios between spectra. As for % D/SN, a spectral effect was apparent at the highest intensity (blue > green > red), although these differences were not statistically significant (p=0.06).

Experiment 3: Relative pineal sensitivity in cod

Melatonin expressed during the SN was influenced by the day intensity to which the pineal glands were acclimated (Fig 7). In comparison to when pineals were illuminated at 1.9 ×10^{15} photons.s^{-1}.cm^{-2} during the day (Exp 1), an order of magnitude increase in day intensity elicited a significantly elevated % SN/N level when illuminated at 1.22 ×10^{9} photons.s^{-1}.cm^{-2} and 1.22 ×10^{11} photons.s^{-1}.cm^{-2} during the SN and a significantly reduced % D/SN when illuminated at 3.85 ×10^{7} photons.s^{-1}.cm^{-2} and 1.22 ×10^{9} photons.s^{-1}.cm^{-2} during the SN.

Furthermore an order of magnitude reduction in day intensity significantly reduced % SN/N and increased % D/SN values when illuminated at 1.22 $\times 10^9$ photons.s^{-1}.cm^{-2} and 1.22 $\times 10^{11}$ photons.s^{-1}.cm^{-2} during the SN. Although the limited samples preclude the possibility to fit mathematical models overall this data suggests that illuminance response curves for both % SN/N and % D/SN could be phase shifted in direct response to day light intensity.

DISCUSION

Our results showed clear species differences in the pineal illuminance and spectral sensitivities with regards to the melatonin production by the pineal gland. The first *ex vivo* pineal trials performed in this study aimed at determining the threshold of light perception of Atlantic cod pineal gland and comparing to other previously studied species (Migaud et al., 2006). The cod study revealed the difference between intensities which do not suppress nocturnal melatonin synthesis ($\leq 3.2 \times 10^7$ photons.s^{-1}.cm^{-2}) and fully suppress it ($\geq 3.2 \times 10^{12}$ photons.s^{-1}.cm^{-2}) is a range of 1×10^5 photons.s^{-1}.cm^{-2}. When the light transmission through cod cranial bones is considered, 3.2% according to previous findings (Migaud et al., 2007), *in vivo* illuminance threshold would thus be around 5.8×10^{11} photons.s^{-1}.cm^{-2} (equivalent to 1.8×10^{-3} watt/m^2). When the response of cod is compared to the results obtained in salmon and seabass (Migaud et al., 2006) cod pineal glands are much more sensitive than the other two species. When data is modelled using a four parameter logistic regression (Table II), the b value (which represents the intensity at which 50% of the maximal effect is observed) for the %SN/N data can be used for comparative purposes. Light intensity sensitivity of cod pineal glands (b = 8.9 x 10^7 photons.s^{-1}.cm^{-2}) would therefore be in the region of 100 to 10000 times higher than in sea bass (b = 5.62 x 10^9 photons.s^{-1}.cm^{-2}) and salmon (b = 1.15 x 10^{11} photons.s^{-1}.cm^{-2}) respectively. The current reports on the lack of physiological effects of additional artificial light in cod farming (Taranger et al., 2006), in contrast to previous

findings obtained in enclosed tank systems (Davie et al., 2007) suggests too low light intensities are being applied to create appropriate light fields throughout open cage systems. However, intensities tested so far are clearly above the theoretical thresholds defined in the present study (Migaud et al., unpublished). This suggests that the biological system is not as simple as first thought. Indeed, these results are specific to the pineal gland and do not take into consideration the possible role of the eyes in the pineal melatonin regulation. Differences in the photic sensitivities of the melatonin production between cod, sea bass and salmon might depend on the organization of the circadian axis (which consists of the retina, supachiasmatic nucleus of the hypothalamus or comparable brain region in fish and pineal complex). Recent studies have suggested the existence of three different modes of photoentrainment of the pineal gland through either light exclusively perceived by pineal photoreceptors (salmonids) (Ekström & Meissl, 1997; Migaud et al., 2007) or retinal photoreceptors (tilapia *Oreochromis niloticus*) (Migaud et al., 2007) or both (sea bass and cod) (Bayarri et al., 2003; Migaud et al., 2007). Therefore, the extrapolation of current results on pineal sensitivities to the whole animal and species comparisons are not straightforward as light perceived by both the eyes and the pineal gland regulate melatonin production in sea bass and cod as opposed to salmon in which the eyes have no direct involvement. Importantly, retinal and pineal photoreceptors might have different intensity and spectral sensitivities depending on their type, location and levels of illuminance experienced, retinal cells being more exposed to photic stimuli than pineal cells. On the other hand, retinal light perception and regulation of the melatonin produced by the pineal gland could overrun the photic control at the pineal level. Moreover, the existence of deep brain photoreceptors in fish has been documented (Alvarez-Viejo et al., 2004; Masuda et al., 2005). These photoreceptors could also be involved in non visual light perception (Foster and Hankins, 2002). Finally,

extrapineal and extraocular source of melatonin have been found in fish (e.g. intestine, Bubenik and Pang, 1997) but to date its regulation is unknown.

The spectra sensitivity tested in the present *ex vivo* trials clearly confirmed the light intensity thresholds previously published in salmon and sea bass (Migaud et al., 2006) and reported in the present study (exp. 1). In salmon, a clear spectral effect was shown with the red wavelength (650 nm) being far less effective in suppressing nocturnal melatonin than shorter wavelengths (blue, 450 nm and green, 550 nm), as melatonin levels increased under red light even at higher intensity reaching 40% of night levels. In sea bass, no significant differences between spectra were observed at the threshold of light sensitivity although green wavelengths appeared to suppress melatonin more efficiently than blue and red. This could confirm previous results obtained in zebrafish according to which wavelengths in the region of 512 nm are the most efficient at suppressing melatonin (Ziv et al., 2007). However, the present results are contrasting with previous *ex vivo* findings showing a more acute sensitivity of the melatonin production to blue rather than red wavelength in sea bass (Bayarri et al., 2002). A direct comparison is difficult as fish were only subjected to 1 hour pulses of light as compared to the exposition of the pineal glands for 12 hours in the present study. Furthermore, given the role of the eyes in the photic regulation of the melatonin production (Migaud et al., 2007), the current results may only define the pineal spectral sensitivity and not the animals sensitivity overall. It must be acknowledged that variability in night time melatonin production by cod pineal glands (from ~1500 to 3500 $pg.ml^{-1}$) was observed, which might be due to genetic effects as shown in mammals (Coon et al., 1999).

The current results clearly highlight species differences. Wavelengths are differentially absorbed in the water column with, in general, penetration being inversely related to the wavelength in marine environment. It is therefore perhaps not surprising that pineal glands of fish species inhabiting deep marine environments are less sensitive to red light as an

adaptation to the absence of these wavelengths in their natural habitat. In this case maximal sensitivity would be in synergy with the predominant spectral bandwidth experienced in the natural habitat (Lythgoe, 1980). Of the three species studied in the current work it might be assumed that Atlantic cod, a benthic marine species, would show the greatest spectral refinement however this does not appear to be the case. This is perhaps because, while cod are commonly considered to be a benthic marine species they do perform significant vertical migrations into the upper pelagic zones (Strand & Huse, 2007). This would mean that cod are naturally exposed to different wavelengths at different depths in the water column which could explain why its pineal showed the predominately similar sensitivity to all the different spectra tested. It is also important to consider the potential for change in the spectral sensitivity of the pineal organ. For example Atlantic salmon are capable of shifting the vitamin A_1/A_2 pigment ratio in the retina depending on the aquatic environment they inhabit (Dartnall, 1962; Wald, 1941), equally in Pacific salmon the retina of ocean-caught pacific individuals are dominated by rhodopsin, but as the fish move into coastal water, there is gradual switch towards porphyropsin. At the spawning site, in the upper, shallow, reaches of freshwater catchments, porphyropsin often accounts for over than 90% of the visual pigment content (Beatty, 1966). Several pineal photopigments have been identified to date in fish: rod-like and cone-like opsins, VA opsin (Foster & Hankins, 2002; Kojima et al., 2000; Moutsaki et al., 2000; Soni & Foster, 1997), parapinopsin (Blackshaw & Snyder, 1997) and extra-retinal rod-like opsin (ERrod-like opsin), which belongs to the rod opsin family but is not expressed in the retina (Bellingham et al., 2003). These pineal pigments are spectrally distinct from the retinal rods (Bowmaker & Wagner, 2004). The possibility that the relative expression/density/prevalence of pineal pigments, which in itself defines the light sensitivity of an individual, could change both in relation to the environment (Shand et al., 2008) but also the developmental stage (Beatty, 1966) needs to be further explored.

While this work has aimed at defining pineal light perception thresholds it is important to understand the plasticity of these definitions. In the case of cod the results show clearly that a single order of magnitude increase or decrease in day intensity elicits a significant shift in the predicted intensity response curve and hence the corresponding definitions of melatonin suppression. This suggests that the illuminance thresholds are dependent on the previously experienced day intensity. While this appears to make sense for species which can move rapidly between a wide range of photic environments (Strand & Huse, 2007), this is the first demonstration in any teleost, that such adaptive photoreception is present. Comparable studies are extremely limited but it appears while similar adaptive photoreception was not evident in the domestic pig (*Sus domestica*) (Tast et al., 2001) it has been reported in humans (Smith et al., 2004). Importantly for the aquaculture industry where the photoperiod management of physiology is essential for profitability, these results highlight the importance of more accurate management of the entire photic environment to enhance the efficiency of management practices. This study clearly demonstrates differential light intensity and spectral sensitivities in three temperate marine teleost species with regards to the melatonin synthesis. These differences could result from adaptations to specific photic environments although further studies are needed to confirm such a hypothesis. A better characterization of pineal photoreceptors and photopigments utilising techniques like microspectrophotometry would help to better describe and understand these different species specific light sensitivities according to the type of organization of the circadian axis.

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FIGURE LEGENDS

Fig.1. Normalized spectral profiles for Solux bulb (solid line) and three narrow bandwidth filtered light tested (λ max indicated above).

Fig.2. *Ex vivo* melatonin production ($pg.mL^{-1}$) by Atlantic cod pineal glands (n = 4) exposed to a 12L:12D photoperiod cycle with either night (black), day (light grey) or different light intensities tested during the subjective night (dark grey): (A) 3.85×10^7 $photons.s^{-1}.cm^{-2}$, (B) 1.22×10^8 $photons.s^{-1}.cm^{-2}$, (C) 1.22×10^9 $photons.s^{-1}.cm^{-2}$, (D) 3.85×10^9 $photons.s^{-1}.cm^{-2}$, (E) 1.22×10^{10} $photons.s^{-1}.cm^{-2}$ and (F) 1.22×10^{11} $photons.s^{-1}.cm^{-2}$. Horizontal bars represent day (white), night (black) and subjective night (grey). Data correspond to 4 pineals x 3 time point/period (day/night or subjective night) x 6 light intensity tested.

Fig. 3 Dose-response relationship between illuminance during the subjective night (SN) and melatonin produced during this time with respect to natural night levels (% SN/N) (black circle) or natural day levels (% D/SN) (grey circles). Data points represent mean ± SEM, n = 4 pineal glands. Broken lines are the best fit four parameter logistic models described in table 3.

Fig. 4 Mean melatonin production ($pg.mL^{-1}$) by Atlantic salmon pineal glands in culture exposed to either day, night or subjective night (12L:12D photoperiod). Data are expressed as mean ± SEM of three time points/period (day, night or filter) for n=4 pineals/intensity/spectrum. Subjective night consisted in testing three different spectra (blue, black bars; green, dark grey bars and red, light grey bars) at three different light intensities, (A), 1.22×10^{13} (B) 1.22×10^{12} and (C) 1.22×10^{11} $photons.s^{-1}.cm^{-2}$. Superscripts indicate significant differences between phases for each spectrum tested.

Fig.5. Mean melatonin production ($pg.mL^{-1}$) by Atlantic salmon pineal glands in culture exposed to either day, night or subjective night (12L:12D photoperiod). Data are expressed as mean ± SEM of three time points/period (day, night or filter) for n=4

pineals/intensity/spectrum. Subjective night consisted in testing three different spectra (blue, black bars; green, dark grey bars and red, light grey bars) at three different light intensities, (A) 1.22 x 10^{12}, (B) 1.22 x 10^{11} and (C) 1.22 x 10^{10} photons.s^{-1}.cm^{-2}. Superscripts indicate significant differences between phases for each spectrum tested.

Fig.6. Mean melatonin production (pg.mL^{-1}) by Atlantic cod pineal glands in culture exposed to either day, night or subjective night (12L:12D photoperiod). Data are expressed as mean ± SEM of three time points/period (day, night or filter) for n=4 pineals/intensity/spectrum. Subjective night consisted in testing three different spectra (blue, black bars; green, dark grey bars and red, light grey bars) at three different light intensities, (A) 1.22 x 10^{10}, (B) 1.22 x 10^{9} and (C) 1.22 x 10^{8} photons.s^{-1}.cm^{-2}. Superscripts indicate significant differences between phases for each spectrum tested.

Fig.7 Mean percentage of melatonin levels produced during the SN relative to the night levels (% SN/N) (A) and percentage of melatonin produced during the day relative to the SN levels (%D/SN) (B) in Atlantic cod pineal glands subjected to different day intensities. Each data point represents the mean ± SEM for 4 pineals. Solid line represents four parameter logistic model fitted to 1.9 ×10^{15} photons.s^{-1}.cm^{-2} dataset (A: p = 0.0025 *adj* r^2 = 0.94, and B: p<0.0001, *adj* r^2 = 0.99) while broken lines represent the same model phase advanced or delayed to align with 1.9 ×10^{16} photons.s^{-1}.cm^{-2} or 1.9 ×10^{14} photons.s^{-1}.cm^{-2} datasets respectively. Superscripts indicate significant differences between day intensities for a given SN intensity.

Table 1: Light intensity treatments expressed in Lux, Watts/m^2 and photons flux (photons/sec/cm^2). Transmittance, light intensity ratio between day and night and nominal density filters used are also described. Ambient day lighting was recreated by the use of a solux bulb (4700K CRI 99, 10° spread) which deliver a similar spectrum than in ambient natural conditions. Light intensity provided by one solux bulb was 22000 lux, 116 watts/m^2 equivalent to $3.72x10^{16}$ photons/sec/cm^2. Transmittance (%) related to the max bulb intensity. C, S, SB and X refer to treatments applied in cod, salmon, sea bass and the three studied species, respectively.

Periods	Transmittance (%)	Light intensity ratio at subjective night/day	Nominal density of Neutral density filters used	**Lux**	**Watts/m^2**	**Photons/sec/cm^2**	**Exp 1**	**Exp 2**	**Exp 3**
Daylight	50	n/a	0.3	11026	58	$1.92\ 10^{16}$			X
	5 (reference)	LL 100	1.3	1102	5.8	$1.92\ 10^{15}$	X	X	
	0.5	n/a	2.3	110	0.58	$1.92\ 10^{14}$			X
Light treatment during subjective night	0.032	0.6	2.5	7	$3.8\ 10^{-2}$	$1.22\ 10^{13}$		S	
	0.01	0.2	4	2.2	$1.2\ 10^{-2}$	$3.85\ 10^{12}$	X		
	0.0032	0.06	3.5	0.7	$3.8\ 10^{-3}$	$1.22\ 10^{12}$		S, SB	
	0.00032	0.006	5.5	0.07	$3.8\ 10^{-4}$	$1.22\ 10^{11}$	X	S, SB	X
	0.000032	0.0006	6.5	0.007	$3.8\ 10^{-5}$	$1.22\ 10^{10}$	X	C, SB	
	0.00001	0.00019	7	0.0022	$1.2\ 10^{-5}$	$3.85\ 10^{9}$	X		
	0.0000031	0.00006	7.5	0.0007	$3.8\ 10^{-6}$	$1.22\ 10^{9}$	X	C	X
	0.00000031	0.000006	8.5	$7\ 10^{-5}$	$3.8\ 10^{-7}$	$1.22\ 10^{8}$	X	C	
	0.0000001	0.0000019	9.0	$2\ 10^{-5}$	1.2 10-^{7}	$3.85\ 10^{7}$	X		X
	0.00000001	0.00000019	10.0	$2\ 10^{-6}$	$1.2\ 10^{-8}$	$3.85\ 10^{6}$	X		

Table 2: Parameter estimates ± 1 S.E. and adjusted r^2 of the four parameter logistic model: $f(\chi) = \frac{a-d}{1+(\chi/b)^c} + d$ for the variables % SN/N and % D/SN of Atlantic cod and % SN/N for Atlantic salmon and European Sea bass.

Variable	*a*	*b* (log photon.sec^{-1}.cm^{-2})	*c*	*d*	*adj r^2*
% SN/N Cod	100 ± 9.58	7.95 ± 0.11	46.52 ± 24.97	10.25 ± 4.28	0.94
% D/SN Cod	100 ± 4.99	10.27 ± 0.12	-14.2 ± 1.95	1.76 ± 2.11	0.99
% SN/N Salmon	100 ± 16.3	11.06 ± 0.35	21.23 ± 14.27	2.96 ± 12.46	0.89
% SN/N Seabass	100 ± 10.26	9.75 ± 0.22	47.36 ± 27.27	-0.64 ± 2.47	0.97

Figure 1

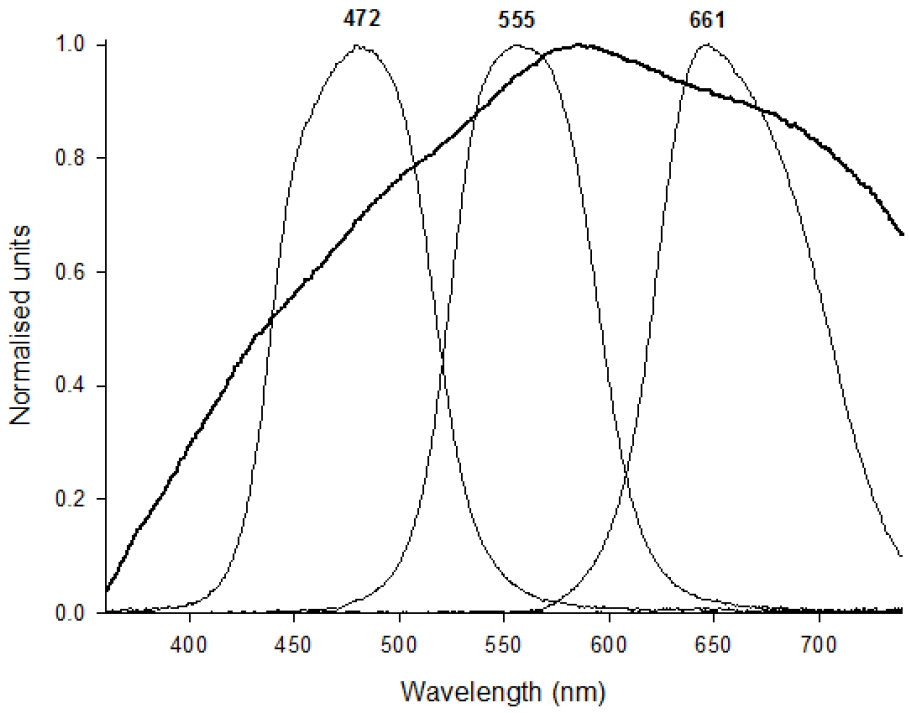

472
555
661
1.0
0.8
0.6
0.4
0.2
0.0
Normalised units
400
450
500
550
600
650
700
Wavelength (nm)

Figure 2

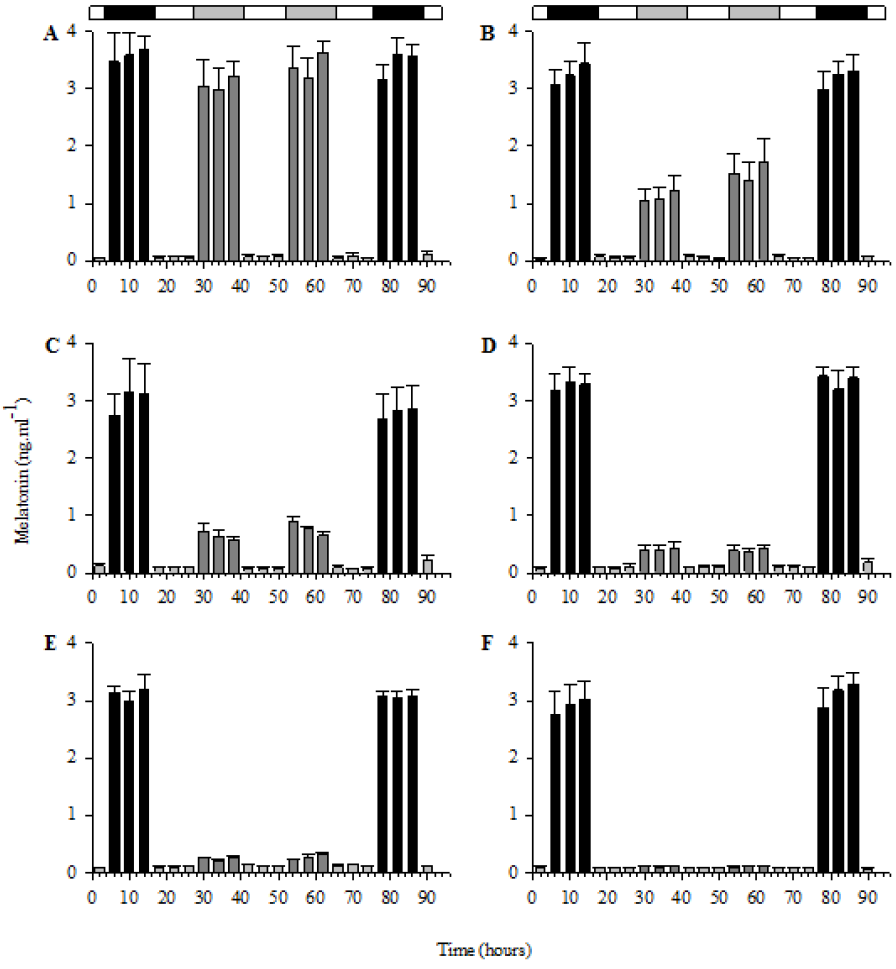

A
B
C
D
E
F
Melatonin (ng.ml-1)
Time (hours)
0
1
2
3
4
0 10 20 30 40 50 60 70 80 90

Figure 3

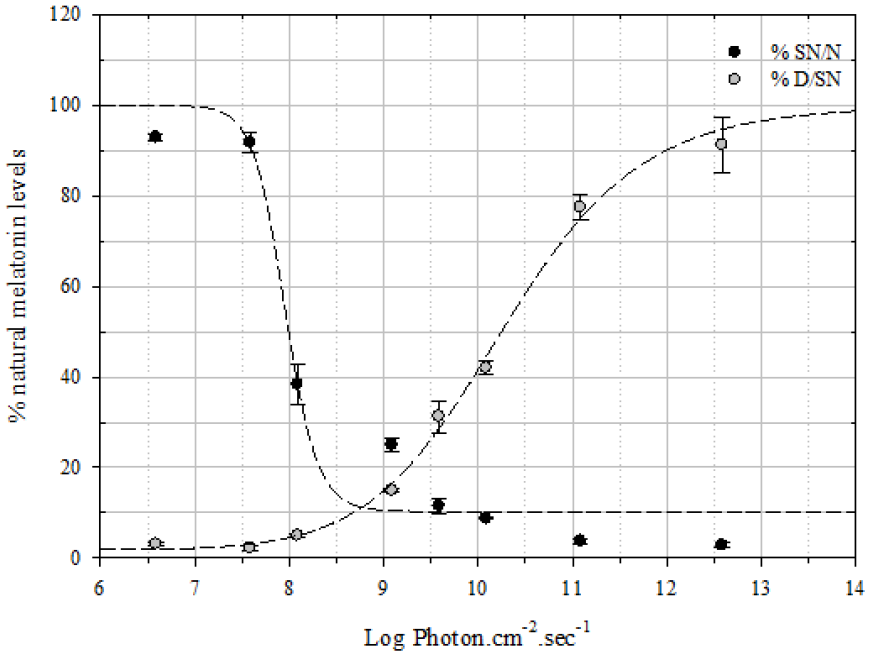

% SN/N
% D/SN
% natural melatonin levels
Log Photon.cm-2.sec-1
0
20
40
60
80
100
120
6
7
8
9
10
11
12
13
14

Figure 4

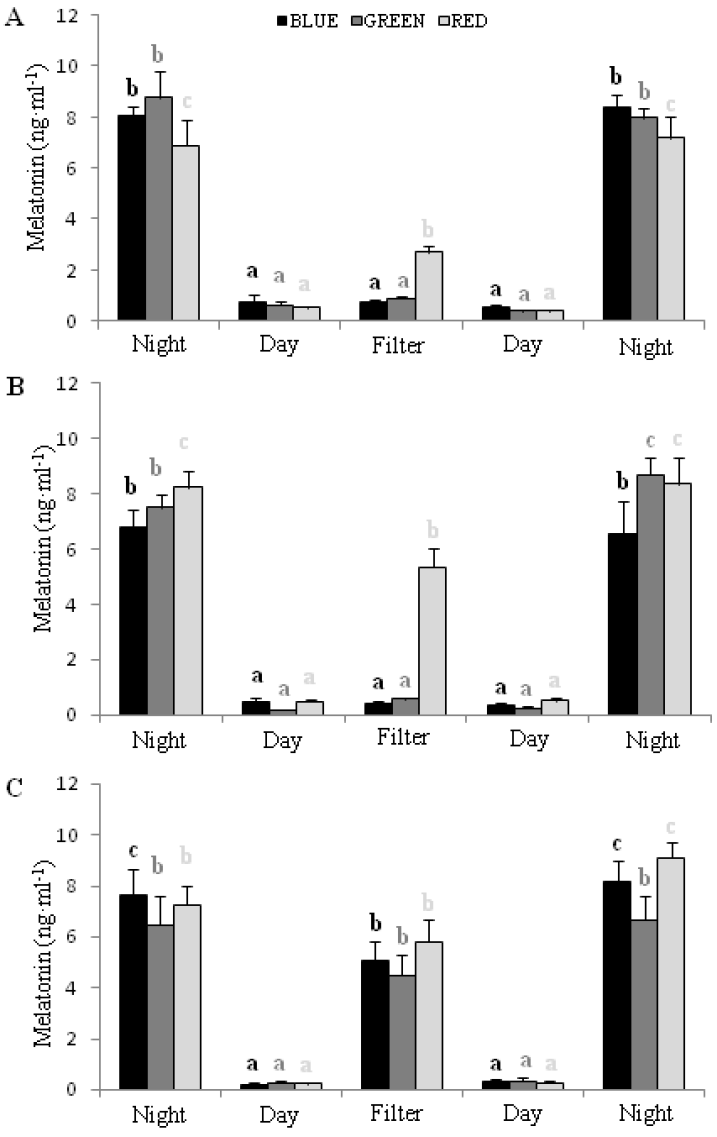

A
B
C
BLUE
GREEN
RED
Melatonin (ng·ml-1)
Night
Day
Filter
Day
Night

Figure 5

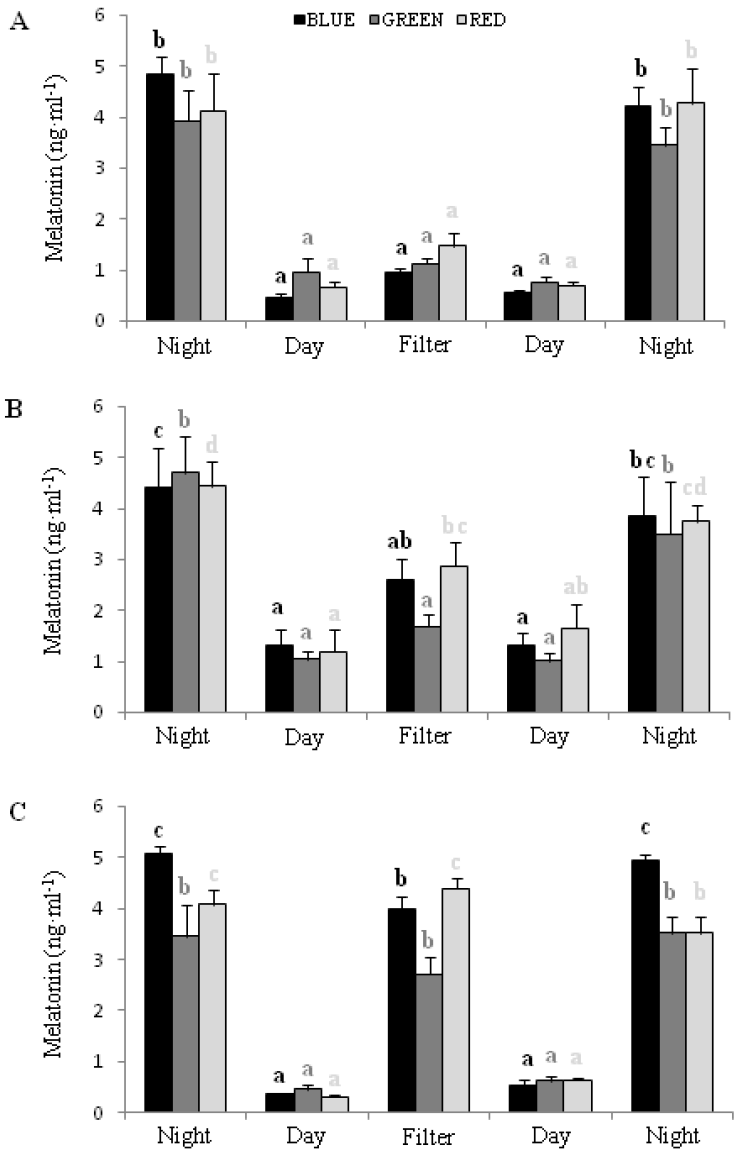

A
BLUE GREEN RED
Melatonin (ng·ml-1)
Night
Day
Filter
Day
Night
B
Melatonin (ng·ml-1)
Night
Day
Filter
Day
Night
C
Melatonin (ng·ml-1)
Night
Day
Filter
Day
Night

Figure 6

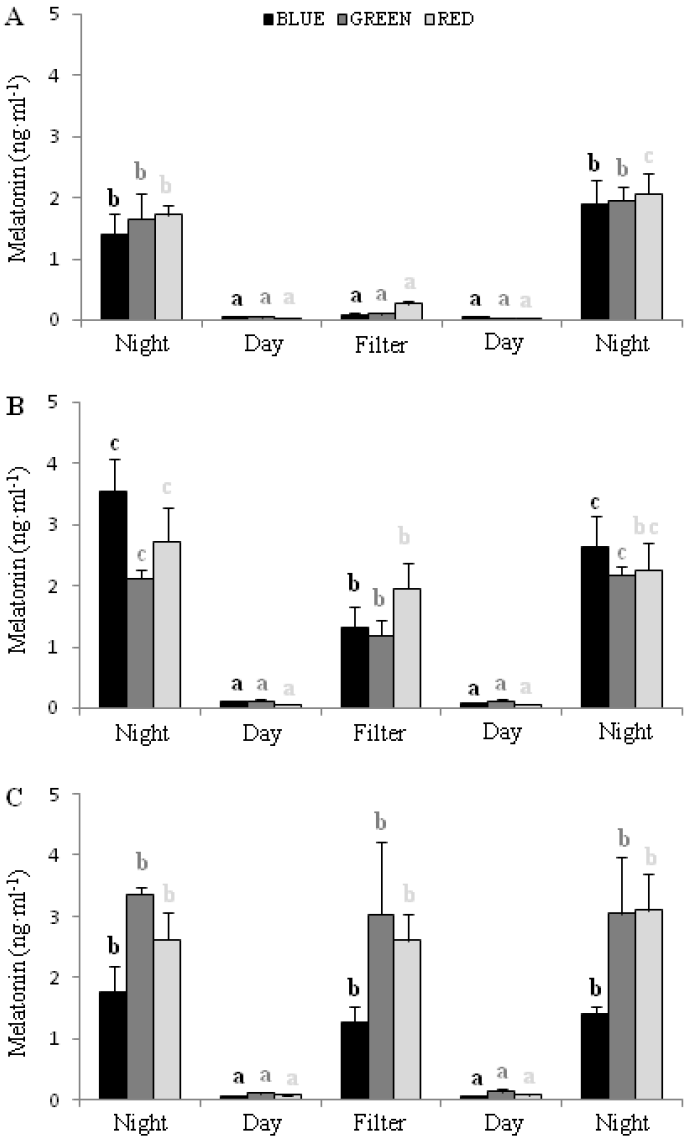
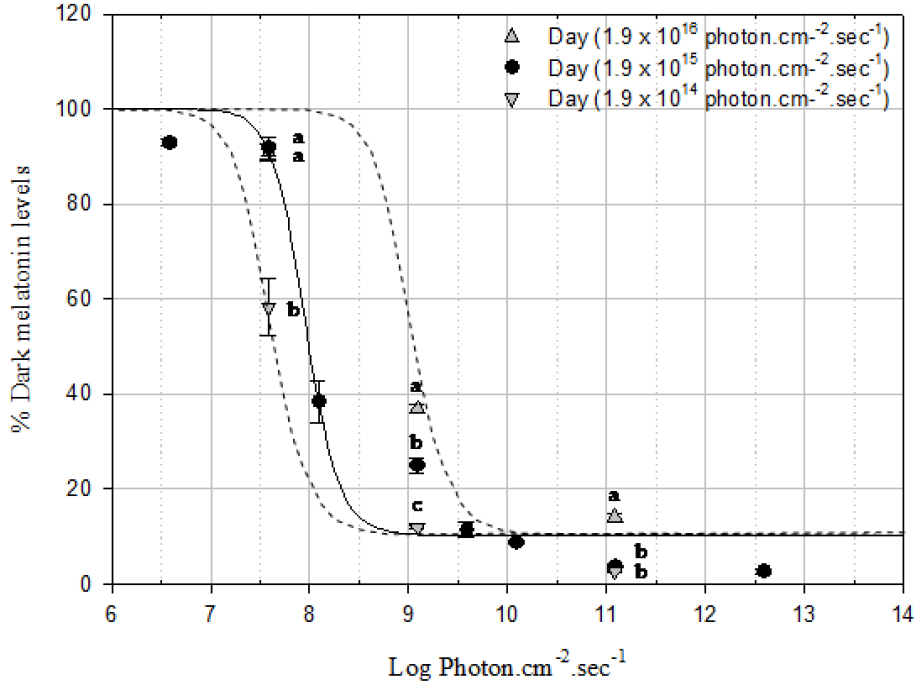
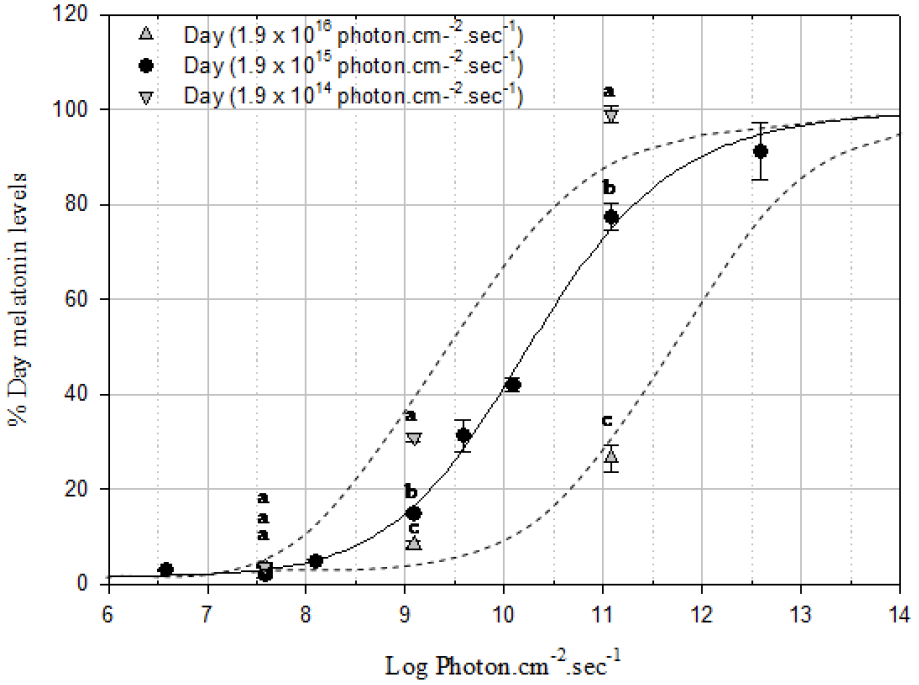

A
BLUE GREEN RED
Melatonin (ng·ml-1)
Night
Day
Filter
Day
Night
B
C

Figure 7


Day (1.9 x 10^16 photon.cm-^2.sec^-1)
Day (1.9 x 10^15 photon.cm-^2.sec^-1)
Day (1.9 x 10^14 photon.cm-^2.sec^-1)
% Dark melatonin levels
Log Photon.cm^-2.sec^-1


Day (1.9 x 10^16 photon.cm-^2.sec^-1)
Day (1.9 x 10^15 photon.cm-^2.sec^-1)
Day (1.9 x 10^14 photon.cm-^2.sec^-1)
% Day melatonin levels
Log Photon.cm^-2.sec^-1