EVIDENCE FOR DIFFERENTIAL PHOTIC REGULATION OF PINEAL MELATONIN SYNTHESIS IN TELEOSTS

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ABSTRACT

The aim of this study was to compare the circadian control of melatonin production in teleosts. To do so, the effects of ophthalmectomy on circulating melatonin rhythms were studied along with *ex vivo* pineal culture in six different teleosts. Results strongly suggested that the circadian control of melatonin production could have dramatically changed with at least three different systems being present in teleosts when one considers the photic regulation of pineal melatonin production. Firstly, salmonids presented a decentralized system in which the pineal gland responds directly to light independently of the eyes. Then, in seabass and cod both the eyes and the pineal gland are required to sustain full night-time melatonin production. Finally, a third type of circadian control of melatonin production is proposed in tilapia and catfish in which the pineal gland would not be light sensitive (or only slightly) and required the eyes to perceive light and inhibit melatonin synthesis. Further studies (anatomical, ultrastructural, retinal projections) are needed to confirm these results. *Ex vivo* experiments indirectly confirmed these results, as while the pineal gland responded normally to day-night rhythms in salmonids, seabass and cod, only very low levels were obtained at night in tilapia and no melatonin could be measured from isolated pineal glands in catfish. Together, these findings suggest that mechanisms involved in the perception of light and the transduction of this signal through the circadian axis has changed in teleosts possibly as a reflection of the photic environment in which they have evolved in.

INTRODUCTION

Photoperiodism in all vertebrates relies upon a "central circadian axis" comprising the retina, suprachiasmatic nucleus of the hypothalamus (or comparable brain region) and pineal complex, which have been shown to be involved in the control and regulation of circadian and circannual rhythms [1-3]. There is extensive literature describing the gross structure and examining the potential role performed by these individual components, in particular the pineal, in non-mammalian vertebrates; however, there is limited work considering the system as a whole and discussing its interaction [4]. Common to all vertebrates is the fact that the circadian axis is based around a circadian pacemaker mechanism fed entraining light signals from photoreceptors that are then turned into neuroendocrinological signals that subsequently transmit this information to target tissues that then determine the physiological response [5-7]. In mammals the indoleamine melatonin released into the plasma by the pineal gland, accurately reflects night period and it is shown to regulate many of the above-mentioned rhythms by targeting receptors in the hypothalamic region of the brain [7-9]. In non-mammalian vertebrates it has been suggested that circulating melatonin can be produced by solely the pineal, or the retina can provide a contribution [4, 6, 10-11]. Although there has been much work focused on melatonin as it is the main endocrine signal shown to be regulated by photoperiod [12-15], its role in regulation of physiological rhythms such as reproduction remains unclear in teleosts [4, 16].

Importantly, there is a strong indication that the control of pineal activity has changed dramatically during phylogeny, as a response to 500 million years of evolution to the diverse environments occupied by vertebrates during that time [3, 17]. In mammals, previous studies have demonstrated through ophthalmectomy [18-22] that photoentrainment is exclusively mediated by retinal photoreceptors and as such pineal photoreceptors have lost their direct light

sensory abilities in comparison to lower vertebrates, reducing their role solely to a melatonin secretory gland [6]. However, unlike mammals, in all teleosts species studied so far, to our knowledge, as in birds, *in vitro* studies have shown that the pineal gland was directly photosensitive [12-15, 23-28]. Such reports came from studies mainly performed in temperate fish species but also two tropical species, the goldfish *Carassius auratus* [29] and zebrafish *Danio rerio* [30]. In summary two forms of circadian organization have been previously suggested in fish relating to melatonin secretion by the pineal gland [3, 4, 6, 9]: a) salmonids, a group of fish characterized by a directly light sensitive pineal, without pacemaker activity (no melatonin rhythm appears under constant darkness and a light entrained rhythm is observed under LD) , and b) all other fish studied, in which the pineal organ is a true circadian light sensitive pacemaker (melatonin displays a free running circadian rhythm under DD and a light entrained rhythm under LD). However, these two models are only based on the pineal gland and do not consider the potential integrated role of the retinas as is seen in higher vertebrates. Because there is a natural tendency to generalise results that one finds in a given fish species to the whole teleost phylogenic class [9], the hypothesis tested in the present study was that due to the variety of environments inhabited by fish, from temperate to tropical or freshwater to deep seawater, and high divergence demonstrated in fish physiology regarding biological rhythms in terms of feeding behavior and locomotor activity (diurnal *vs.* nocturnal) and reproductive strategies (iteropare *vs.* continuous spawner), it is unlikely that one unique circadian organization (retina-pineal gland network) exist in fish. The objective of this study was, therefore, firstly to compare the effects of ophthalmectomy on melatonin production in a diverse range of teleosts from temperate to tropical latitudes. Secondly, these results were confirmed through *ex vivo* pineal gland culture. Finally a comparison of light transmission through the cranium was measured in all species studied.

MATERIALS AND METHODS

Fish and facilities

Fish species, origin and mean weight of populations used during the experiments are presented in Table 1. Experiments have been performed in a number of rearing systems depending on the species. Three main facilities owned by IoA were used: flow through freshwater rearing tanks at Niall Bromage Freshwater Research Facility (NBFRF) for Rainbow trout (*Onchorhynchus mykiss*), flow through sea water tanks at Machrihanish Marine Environmental Research Laboratories (MERL) for Atlantic salmon (*Salmo salar*), European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) and recirculating tank systems at the Tropical Research Facilities for Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarius gariepinus*). All fish populations were reared under ambient temperature and photoperiodic regimes (simulated natural photoperiod, 56°N 3°W, range 6-18hr photophase) except for tilapia and catfish which were held at constant temperature (27 ± 1^{o}C) and photoperiod (12L:12D). All experiments were carried in accordance with the Animal (Scientific Procedures) Act 1986, UK.

Experiment 1*: in vivo* ophthalmectomy

Fish were acclimated to a 12L:12D photoperiod for at least 2 weeks before surgery. The fish were anaesthesised using a 1:20,000 concentration of 2-phenoxyethanol solution (0.2mL/L, SIGMA). The membrane around the eye was cut out, the eye lifted and the optic nerve sectioned. A drop of a 3/1 w/w mix of Orahesive powder (ConvaTec, Ref 25535, Squibb & Sons Ltd., UK) and cicatrin antibiotic (The Wellcome Foundation Ltd., London) was applied to the eye socket. No mortalities were observed. Two days following the ophthalmectomy procedure, fish were captured and immediately killed by lethal anaesthesia in a 2-phenoxyethanol solution (1mL/L,

SIGMA) and then blood sampled at day (12.00h) and night (04.00h) over two consecutive days (n=4-8 depending on species and sampling). No sham operation could be performed due to limitations in fish number and restrictions placed by our local ethical review committee. Nocturnal blood samples were taken in red dim light with the head of the fish covered.

Experiment 2: *Ex vivo* pineal gland culture

Fish from same origin than used for *in vivo* experiments (Table 1) were acclimatised to a 12L: 12D photoperiod and standard rearing temperature (10 ± 1°C for salmon, trout, cod and sea bass and 27 ± 1°C for tilapia and catfish) during a 2 week period. 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 a fraction collector automatically collecting samples every hour after passing through the culture chambers [28]. The culture media (Sigma, ref: R8755) was supplemented with HEPES sodium salt (Sigma, ref: H3784, 4.77g/l) as a pH regulator with the pH adjusted to 7.4 and penicillin-streptomycin (10mg/l) and Fungizone (5mg/ml) to avoid bacterial and fungal development. Medium was replaced every day. Immediately after their capture, fish were killed by lethal anaesthesia in a 2-phenoxyethanol solution (1mL/L, SIGMA). Fish were sampled during the day period and pineal glands removed using a dissecting microscope, washed with culture medium, placed in incubating chambers and then exposed *ex vivo* to the same photoperiod and temperature regime. Dissection of the pineal glands was adapted for each species depending on size, skull thickness, exact location and overall ease to sample. In trout and salmon, due to the relative large size of the pineal, the fish head was sectioned laterally below the brain which was then lifted to access the pineal gland. Whereas in cod, tilapia, catfish and sea bass the pineal gland was accessed dorsally by opening the skull

around the pineal window. Once in the culture system, pineal glands were maintained for two complete LD cycles. Pineal glands were illuminated by custom made light boxes with dichroic halogen bulbs characterized by an emission spectrum equivalent to a 4700ºK Black Body radiator (Solux, 4700K CRI 99, 10° spread, USA) providing a light intensity of approximately 12watts/m^2 at the pineal level during the day (measured by a single channel light sensor, Skye instruments, UK). Only selected media samples were analysed (2-3/day-night periods depending on species) for melatonin levels corresponding to 4, 8 and 12hrs of each day or night period. At the end of the culture period the pineal glands were removed from the culture chambers and cells viability was checked. To do so, the pineals were stained with 0.2% trypan blue (BDH Merck Ltd. UK.) in phosphate buffer and observed under x100 magnification using an Olympus CH light microscope (Olympus Optical Co., London, UK.).

Experiment 3: Cranial light transmission

Fish origin and mean weight are presented in Table 1. Results obtained for salmon and sea bass were previously published [28]. All fish were killed by a lethal dose of anaesthesic and then decapited. The cranium was dissected and tissue underneath the skull removed to access the pineal window (the overlying dermal tissue was left intact) and transmission measurements performed immediately. The same lighting system as that used in the *ex vivo* experiment was used in this study. The light box was placed at a standardised distance (26cm) from the dissected cranium. Light intensity from the light source was checked prior to measurement for all species. Light measurements were carried out using a spectroradiometer equipped with a fiber optic cable and cosine corrector (EPP2000c Stellarnet Inc., USA, calibrated to National Physics Laboratory UK standard light sources) placed directly behind the pineal window. To study the differential

penetration of light of different spectrum, visible spectrum was divided in seven equal narrow bandwidths using bandpass interference filters (Melles Griot Photonics Component Group) characterised by a FWHM (Full Width Haft Maximum) of 80nm (centre wavelengths: 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61nm). Differences in relative transmittance between filters were corrected by the use of neutral density filters in order to balance light intensity at 5 watts/m^2, $1.6x10^{15}$photons/sec/cm^2. Readings were recorded in watts/m^2 (400-740nm) and transformed into a percentage of full relative illumination passing through the pineal window.

Melatonin assay

Blood and *ex vivo* media samples were stored at - 70°C until assayed for melatonin using a commercially available ELISA kit (IBL, Hamburg, Germany). All standards and samples were assayed in duplicate. Intra-assay coefficient of variation were 5.5% (n=4) and inter-assay coefficient of variation were 9.4% (n=3). The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard was 3pg/ml. Pooled rainbow trout plasma with a melatonin content of approximately 250 pg/ml, sampled during the night, was used to check the reproducibility of measurements between assays, i.e. for quality control.

Statistical analysis

In vivo data (experiment 1) were analysed by a nested ANOVA using a General Linear Model (GLM) with treatment and time as tested factors (replicate nested within treatment). When comparing mean melatonin levels *ex vivo* (experiment 2, 4-6 pineal glands/species, 2-3 day-night

periods, 2-3 samples analysed/period) and penetration of the light through the pineal window (experiment 3), statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by Tukeys multiple comparison test. Data are expressed as mean + SEM. No replicate effects were observed and as such data were pooled. All statistical tests were carried out with Minitab v14.1. The minimum level of significance was set at $P \leq 0.05$.

RESULTS

No significant differences in melatonin profile and levels were observed in ophthalmectomised fish as compared to intact fish in both Atlantic salmon and rainbow trout (Fig. 1a-b). However, in ophthalmectomised sea bass and cod melatonin levels were significantly lower at night as compared to intact fish except in sea bass during the second night (Fig. 1 c-d). With regards to Nile tilapia and African catfish, night plasma melatonin increase was suppressed in ophthalmectomised fish with levels remaining comparable to basal day levels (Fig. 1e-f). Relative to night levels in controls, plasma melatonin in ophthalmectomised trout and salmon was unchanged (≥100%), reduced to 40-60% in sea bass and cod and below 20% in tilapia and catfish (Fig 2).

When trout, salmon, sea bass or cod pineal glands were exposed to a 12L:12D cycle, rhythmic melatonin production were observed with low day levels (below 100pg/ml in rainbow trout and Atlantic cod and below 500pg/ml in Atlantic salmon and sea bass) and high night-time levels (mean levels from 2500 to 3700pg/ml in trout, salmon and cod and 1200pg/ml in sea bass, Table 2). Melatonin synthesis and release from Nile tilapia pineal glands was very low at night (15.9 ± 2.8pg/ml), however, a day night rhythm was still observed although levels were below the assay sensitivity threshold (day levels of 0.6 ± 0.4pg/ml). Numerous attempts to culture catfish pineal

glands were performed under various conditions (fish history, pineal removal, medium, temperature) but no melatonin production above threshold of assay sensitivity was measured in response to a LD cycle. When comparing all species for the relative melatonin synthesis and release in the culture medium at night by the pineal gland expressed as a percentage of plasma melatonin, a clear difference was observed in tilapia (plasma melatonin equivalent to 660% of *ex vivo* melatonin released by a pineal gland) as compared to the other species (between 3 and 11%). Similarly, when considering day levels, tilapia plasma melatonin concentrations were equivalent to 1522% of what is produced by a pineal gland as opposed to <32% in all the other species.

Light penetration through the pineal window in the 6 species was studied (Fig. 3). A significantly higher percentage of light (ambient spectrum recreated by the use of day light bulbs) penetrated the tilapia pineal window (8.23 ± 0.58%) relative to sea bass (4.28 ± 0.26%), cod (3.21 ± 0.12%), trout (2.57 ± 0.10%), salmon (2.23 ± 0.16%) and catfish (1.05 ± 0.09%) (Fig. 3a). Penetration was directly related to wavelength with longer wavelengths having a greater penetrative ability (Fig. 3b). Penetration of light in tilapia remained significantly higher than all other species at wavelength >550nm.

DISCUSSION

The rhythmic melatonin signal remains a highly conserved circadian output across all vertebrates and reflects the perception of the prevailing photoperiod. However, the circadian control of melatonin production by the pineal gland has considerably evolved. In higher vertebrates this system is highly compartmentalised [31] which contrasts with that of lower vertebrates and invertebrates that possess a network of independent oscillatory components [32]. Many studies have focused on characterising the function of the pineal organ in fishes [e.g. 3, 12,

13, 25-30]. However, research into circadian biology to study the pineal gland as part of an entire system/network within the lower vertebrates has been sparse by comparison with that in mammalian and invertebrate models.

The current results bring further evidence from melatonin studies that suggest mechanisms involved in the light perception and transduction through the central circadian axis would have radically changed in teleosts species probably reflecting the environment in which they have evolved in. To date, only two kinds of circadian organization have been proposed *i.e.* salmon *vs.* other teleosts [6, 9]. It is presently suggested that a third organization could be at work in teleosts based on the photic control of melatonin production by the eyes and pineal gland. First, in salmonids, represented by salmon and trout in this study, the circadian melatonin rhythms and amplitude of the levels produced were not affected by the ophthalmectomy. A similar bilateral ophthalmectomy operation in goldfish [33] did not significantly affect plasma melatonin levels as well. This confirms in these species the pineal gland is light sensitive and does not require input from the eyes to control rhythmic melatonin production [4, 33]. Such a system could be considered as not specialized with pineal cells both perceiving light and producing melatonin. This also confirms that melatonin produced by the eyes in such species would not contribute to plasma levels. In fact, melatonin synthesis by fish retina was shown in certain cases (species and season dependent) to be high during the photophase [18, 34-36] as opposed to higher vertebrates where retinal melatonin synthesis is enhanced in the scotophase as in the pineal gland [7, 37]. Such phase shift differences between pineal and retinal melatonin production could be due to different functional roles with melatonin from the pineal gland providing a reliable endocrine indicator of the day/night cycle [9] while melatonin from the eyes could be involved in the paracrine protection and adaptation of the retina [34, 36, 38].

A different circadian system could be at work in seabass and cod as ophthalmectomy resulted in a significant decrease of night time production of melatonin. Such results are in accordance with previous reports in seabass [40] as well as birds [41-42] and amphibians [11]. In all these species, findings suggest that both the eyes and the pineal gland are required to sustain full amplitude melatonin rhythms meaning that light perceived by the eyes could regulate melatonin synthesis by the pineal gland probably through neural projections into the brain [42-43]. In fish, studies have shown that three different types of pinealocytes (true and modified photoreceptors and pinealocytes) co-exist in the lamprey or pike [6], although it is thought that pinealocytes are the evolved form of the true pineal photoreceptors; in mammals only pinealocytes remain [6, 8]. It is not known whether these different forms co-exist in both sea bass and cod, but if this were the case it could explain how light perceived by the retina may influence pineal activity. Further studies are clearly needed to characterize this network.

The situation in tilapia and catfish appeared very different from all other teleosts studied and suggests, for the first time, the existence of a possible third kind of circadian system in which the pineal gland would not be light sensitive or far less sensitive than previously studied teleost species. Furthermore, the results suggest these species would also not contain an independent circadian pacemaker as following bilateral ophthalmectomy, night-time melatonin rise was shown to be fully abolished with basal levels maintained as during the day. *Ex vivo*, the tilapia pineal gland displayed rhythmic melatonin production. It is very unlikely however that the levels recorded (20pg/ml/h) could explain blood levels observed in the species, especially as it has been shown in higher vertebrates that melatonin produced by the pineal gland is also directly released in the cerebrospinal fluid (CSF) through the pineal recess [44] resulting in levels twenty times as high in the CSF as in blood [45]. Although no *ex vivo* melatonin production was observed in catfish after many attempts and cell viability confirmed, no definitive conclusions can be made as

such results could still relate to the difficulty of extracting the gland in this species. As such, these results would suggest for the first time a mammal-like circadian organization in terms of the photic control of melatonin production in at least two teleost species in which the system would be more specialized, with the eyes involved in light perception and the pineal gland reduced to a slaved secretory gland. However, one fundamental difference with mammals remains, that being the lack of an apparent independent circadian pacemaker which would drive the melatonin production in the absence of the eyes. Interestingly, another circadian organisation also relying on retinal photoreception has been suggested in a more primitive fish species, the hagfish, *Eptatretus burgeri* [46, 47]. Further studies are clearly needed to confirm the existence of such systems with especially the characterization of the anatomy and ultrastructure of the pineal gland in relation to retinal neural projections. The same is true for the sea bass and cod as the present results clearly imply that in all four species retinal and/or deep brain photoreception may contribute, i*n vivo,* to the control of melatonin production. But, to date, to our knowledge, no direct connection between the retina and the pineal gland has been clearly identified in teleosts.

It is recognised that the effects of post-surgery stress on melatonin synthesis following ophthalmectomy may raise concerns. However, the present results obtained in salmonids and sea bass match the findings of previous studies [17, 39] where in some cases [39], samples were taken two weeks post-surgery as opposed to 48 hours in the present study. This could thus confirm that post-surgical stress would not affect melatonin production and secretion. It appears then unlikely that results obtained in the remaining species studied could have been influenced by post-surgery stress while results in salmon and sea bass appeared not to be.

To understand whether the circadian system at work could be related to the perception of light by the pineal gland, light transmittance through the cranium was investigated. Clear differences were observed between species with the lowest overall light transmittance in catfish

(1%) and the highest in tilapia (>8%). Furthermore, light transmittance is clearly dependent on the spectral content of the light with longer wavelength penetrating the cranium more efficiently. Interestingly, irrespective of pigmentation, trout, salmon and catfish showed a similar profile of transmittance across the visible spectrum but tilapia was characterized by a much higher penetration than the other species for spectra ≥ 650nm (>14% *vs*. <6% in the other species). Together, these results are surprising as both tilapia and catfish would appear to have similar circadian control of melatonin production. It has been suggested [46] that the inherent advantage of localised (decentralised) photoreception (and regulation) as seen in salmonids, sea bass and cod in the present study, is lost with evolution in an environmental niche with weak environmental entraining signals. In such a habitat multiple oscillators bring the risk of generating conflicting messages, and a more centralised system is favoured, such as that in mammals. In fact, only the most sensitive photoreceptors in the most exposed tissues that can receive enough light to generate a response would remain during evolution [46]. Such a hypothesis is further strengthened by the apparent lack of photic sensitivity in catfish pineal gland, which could be an adaptation to the very low light transmittance of this species chosen habitat. However, it is difficult at this stage to explain how and why the pineal gland in tilapia, although exposed to more light than all the other teleost species studied, would not directly respond to light or only slightly. It is possible however that the ancestral line was earlier subjected to such a selection pressure (e.g. nocturnal existence) which forced the circadian adaptation apparent today, as has been proposed for mammals.

The circadian axis in fish thus appears to be a very interesting system to study evolution within a single vertebrate class. While some teleosts have a fully integrated “circadian axis” without pacemaker activity within the pineal gland (salmonids, Fig. 4a), in others the light

sensitive pineal gland has become increasingly dependent on retinal (and possibly deep brain) photoreception (sea bass and cod, Fig. 4b) to such an extend that in some cases (tilapia and catfish, Fig. 4c) the pineal gland could have lost its light sensitivity and become reliant on retinal (and possibly deep brain) photoreception alone. This would clearly suggest that a shift has occurred within teleosts towards a compartmentalized "circadian system", similar to what is seen in mammals (Fig. 4). Importantly, the location and role of circadian pacemakers within these systems has yet to be characterized. As previously stated [48], the differences in circadian organisation that one finds among the vertebrates are to a large extent the consequence of rapid adaptation to particular photic niches into which groups have been pushed by a variety of unrelated selection pressures. Fish have undoubtedly evolved during a very long period to very diverse environments. And importantly, if these adaptations have been dictated by numerous factors (e.g. temperature, water level, food availability, predation…) it can be suggested that the circadian systems have been mainly shaped by the light signal [46]. The diversity of circadian system suggested in the present study is at first glance closely related to the phylogeny of the fish species studied. However, findings in catfish clearly showed that phylogeny may be a little too simplistic as although catfish could be considered as primitive as salmonids (subdivision of the Ostariophysi) [49], a comparable circadian system to tilapia was suggested by the present data. Further studies on species across the animal kingdom will certainly help to understand the evolution of the circadian control of melatonin and particular attention should be paid to the environmental history in which species have evolved to better define the role this has played in shaping this key regulatory system.

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Table 1. Origin and mean weight of each fish species used in the three experiments.

Species		Origin	Experiment 1: Ophthalmectomy	Experiment 2: Pineal *in vitro*	Experiment 3: Light transmission
Common	**Latin name**				
Rainbow trout	*Oncorhynchus mykiss*	▪ Glen Wyllin, all female population (spring 05) ▪ NBFRF[1]	150 ± 19 g	86 ± 13 g	600 ± 151 g
Atlantic salmon	*Salmo salar*	▪ Howietown Fisheries (March 05) ▪ MERL[2]	114 ± 12 g	193 ± 37 g	1850 ± 250[4]
European sea bass	*Dicentrarchus labrax*	▪ Llyn Aquaculture (spring 03) ▪ MERL	660 ± 123 g	152 ± 22 g	609 ± 100 g[4]
Atlantic cod	*Gadus morhua*	▪ MMF[3] (spring 05) ▪ MERL	63 ± 8 g	140 ± 42 g	932 ± 129 g
Nile tilapia	*Oreochromis niloticus*	▪ Tropical Facilities at IOA (2005) ▪ Red strain from the tilapia Reference Collection	208 ± 56.1 g	216 ± 71.0 g	523 ± 150 g
African catfish	*Clarias gariepinus*	▪ Tropical Facilities at IOA (spring 2005)	160 ± 27 g	420 ± 85 g	

[1] Niall Bromage Freshwater Research Facilities, IoA; [2] Machihanish Environmental Research Laboratories, IoA; [3] Machrihanish Marine Farms (cod hatchery); [4] salmon and sea bass used for the light transmission experiments were respectively originated from Marine Harvest Lochairlort Research Station and the Instituto de Acuicultura de Torre de la Sal in Spain.

Table 2. Comparisons between melatonin levels in the plasma and produced by pineal glands in culture. Levels are expressed as mean ± SEM with n representing the number of animals sampled and number of pineal glands, respectively, for *in vivo* (plasma) and *in vitro* (pineal culture) experiments.

Species	**Plasma (pg/ml)**		**Pineal culture (pg/ml/hour)**		**Relative melatonin production in plasma /pineal**	
	Night-time	**Day-time**	**Night-time**	**Day-time**	**Night**	**Day**
Rainbow trout	116.4 ± 9.5 n = 12	19.1 ± 2.1 n = 12	3706.7 ± 69.3 n = 4	60.0 ± 4.6 n = 4	3.14%	31.9%
Atlantic salmon	291.6 ± 10.7 n = 10	11.8 ± 2.5 n = 10	2536.2 ± 53.3 n = 6	405.0 ± 71.6 n = 6	11.5%	2.9%
European sea bass	43.8 ± 2.6 n = 10	5.9 ± 1.1 n = 10	1207.1 ± 46.6 n = 4	383.8 ± 31.7 n = 4	3.6%	1.5%
Atlantic cod	112.0 ± 11.4 n = 12	9.6 ± 1.3 n = 12	2563.4 ± 99.5 n = 6	86.2 ± 4.1 n = 6	4.4%	11.4%
Nile tilapia	105.1 ± 8.2 n = 8	8.8 ± 1.5 n = 8	15.9 ± 2.8 n = 6	0.6 ± 0.4 n = 6	660.5%	1522.4%
African catfish	47.0 ± 2.9 n = 12	5.6 ± 0.5 n = 12	-	-	-	-

Fig. 1. Effect of ophthalmectomy (Eye X) on *in vivo* plasma melatonin levels in comparison to intact fish (control) in rainbow trout (a), Atlantic salmon (b), sea bass (c), Atlantic cod (d), Nile tilapia (e) and African catfish (f). Values are expressed as mean ± SEM (3-7 individuals/sampling point). Superscripts denote significant differences (GLM, $p<0.05$) and numbers sampling size.

Fig. 2. Summary of the relative percentage of night time melatonin levels in ophthalmectomised fish as compared to control fish in all species studied. Values are expressed as mean of n=3-7 individuals over two night periods.

Fig. 3. Percentage of white artificial light (a, Solux bulb) and narrow bandwidth light at 411.9, 472.28, 510.43, 555.20, 613.17, 661.22 and 704.61nm (centre wavelengths)(b, Solux bulb + bandpass interference filters) through rainbow trout (n=4), Atlantic salmon (n=7), sea bass (n=6), Atlantic cod (n=4), Nile tilapia (n=6) and African catfish (n=6) pineal windows. Superscripts denote significant differences between species for a given light treatment.

Figure 4. Suggested evolution of the regulation of pineal melatonin synthesis by the circadian axis in teleosts. In addition to the two types of circadian organisation already proposed in fish (a and b), a third type could exist where pineal light sensitivity would be dramatically reduced (c). The regulation of pineal activity would have thus evolved from an independent light sensitive pineal gland, without pacemaker activity, as seen in salmonids (a); to an intermediary state where the pineal gland remains light sensitive and could possess a circadian pacemaker, but is also regulated by photic information perceived by the retina as seen in seabass and cod (b); to reach a more advanced system closer to higher vertebrates where light sensitivity of the pineal gland would be significantly reduced and its melatonin synthesis activity primarily regulated by a circadian pacemaker (unknown location) entrained by photic information perceived by the retina (c).

Fig. 1

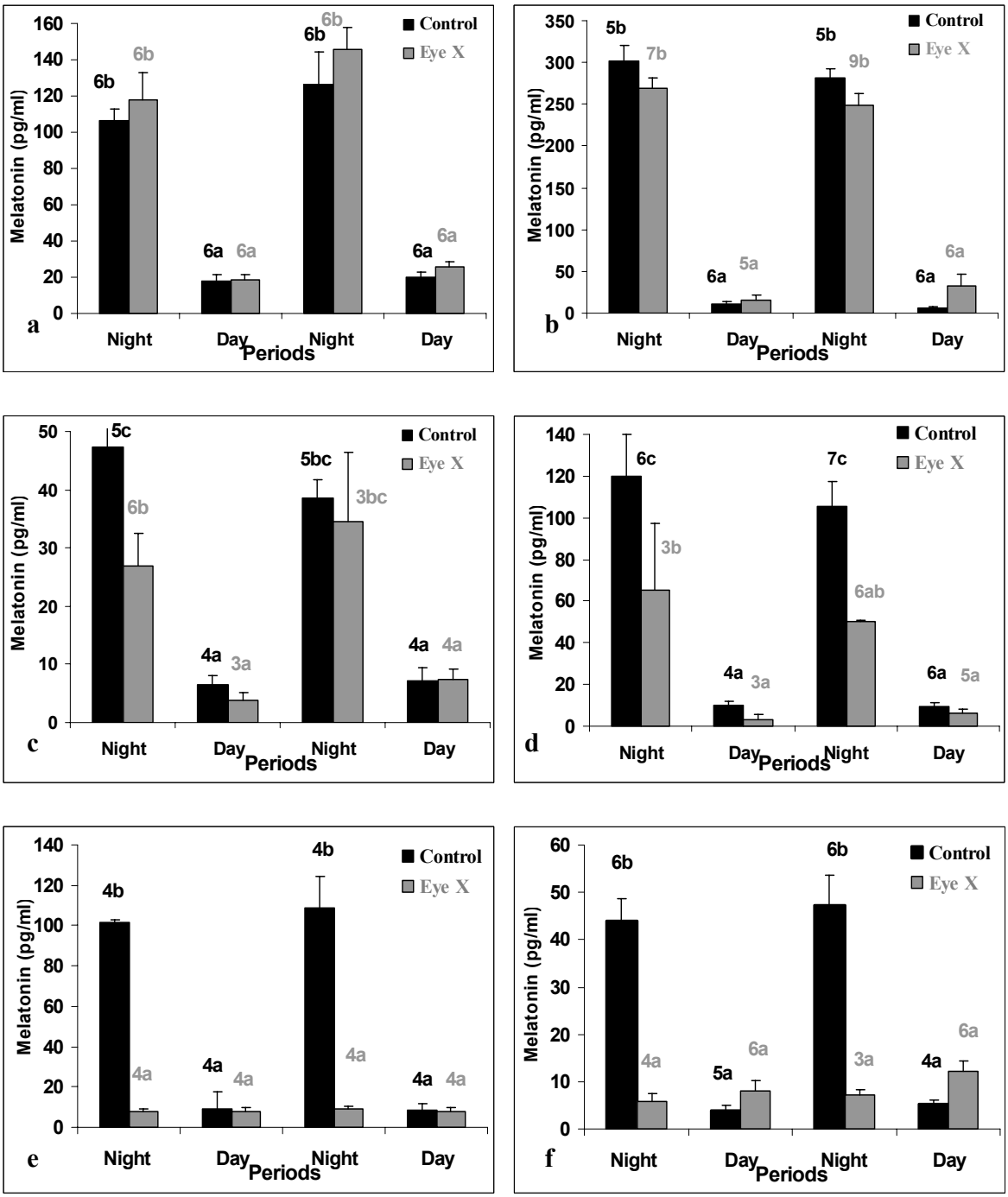


Fig. 2

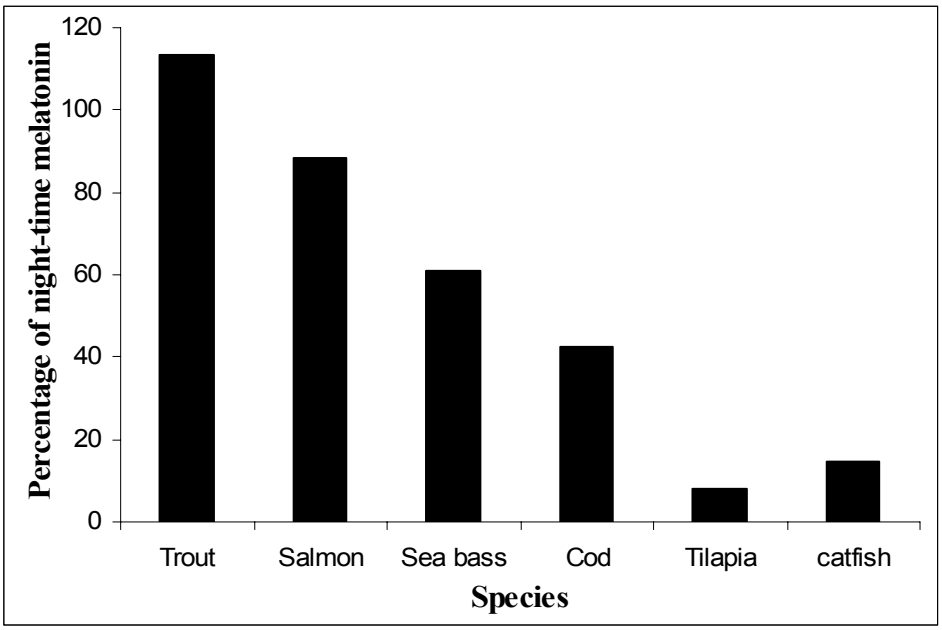

Percentage of night-time melatonin
120
100
80
60
40
20
0
Trout
Salmon
Sea bass
Cod
Tilapia
catfish
Species

Fig. 3

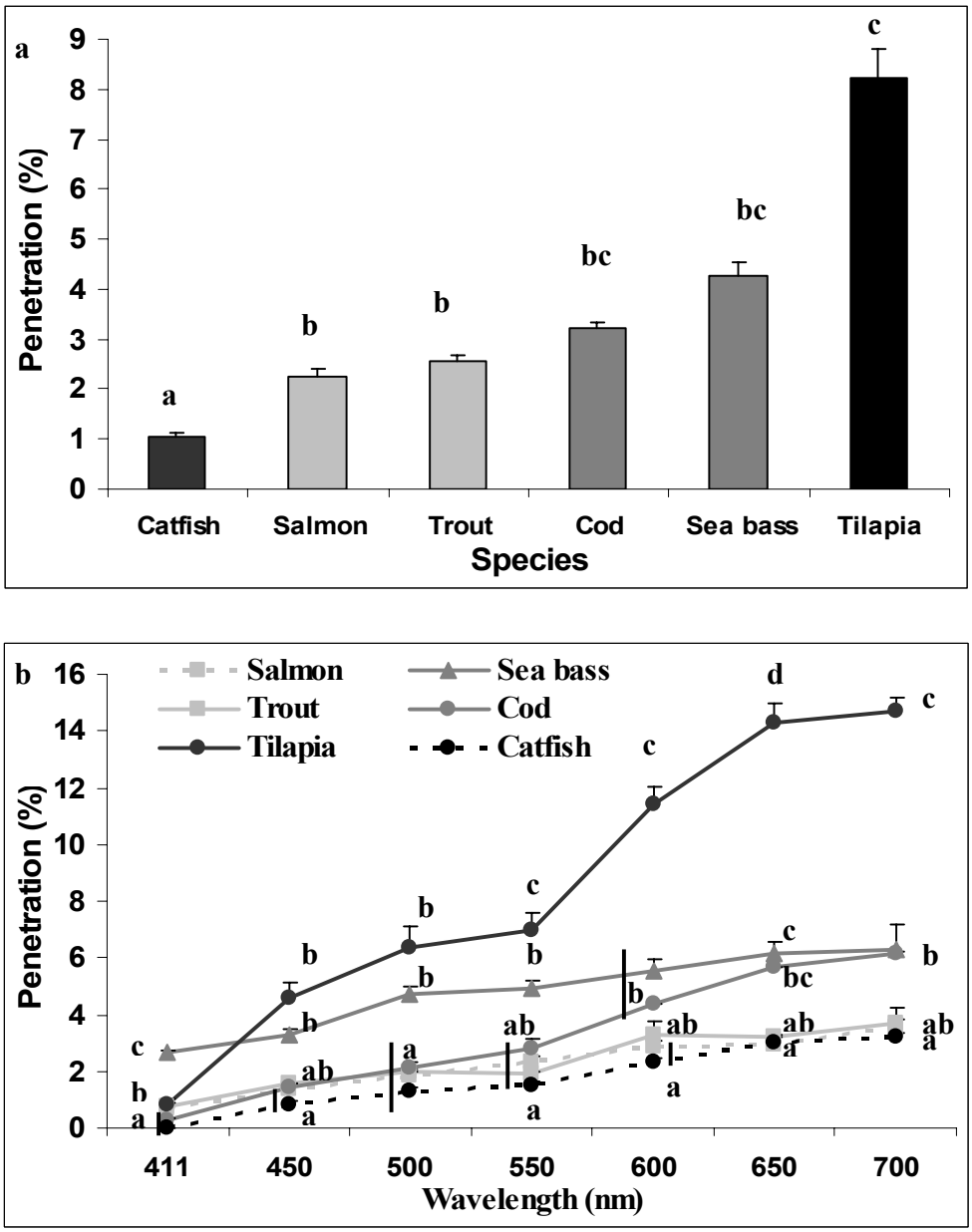
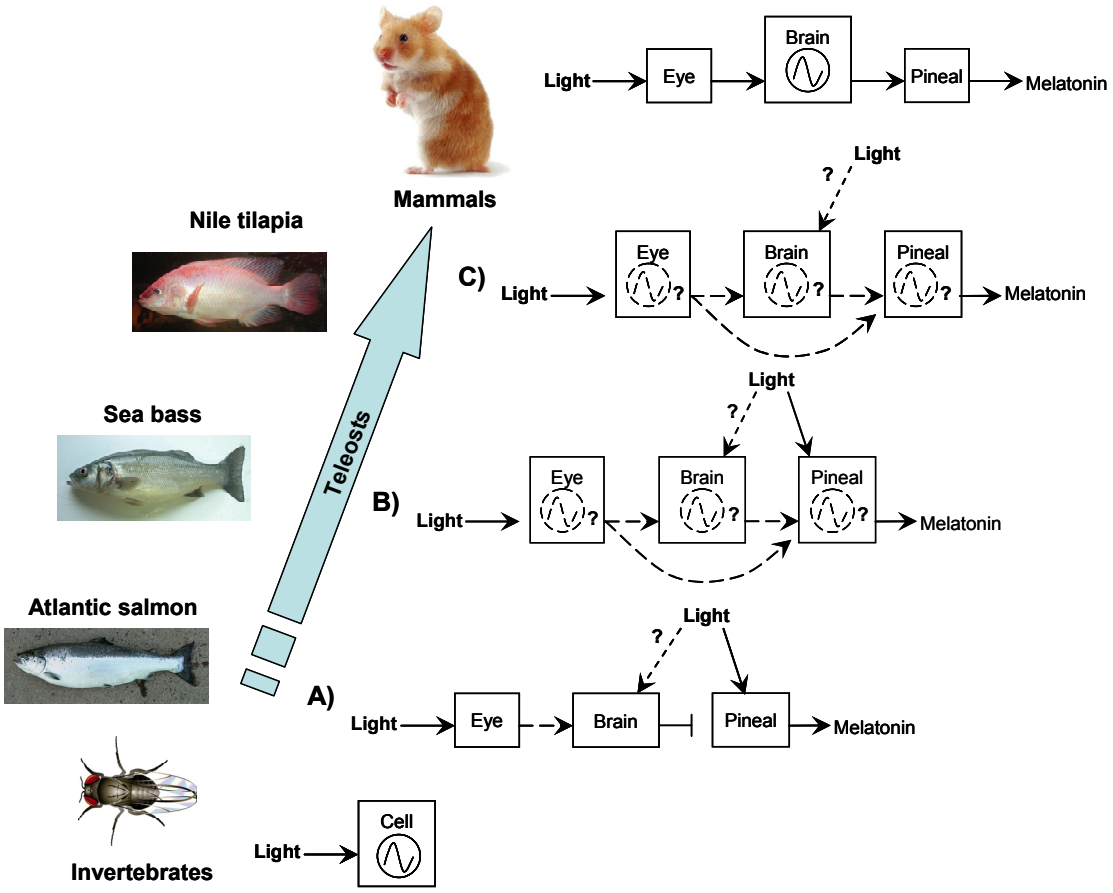

a
b
b
bc
bc
c
Penetration (%)
0
1
2
3
4
5
6
7
8
9
Catfish
Salmon
Trout
Cod
Sea bass
Tilapia
Species
b
Salmon
Sea bass
Trout
Cod
Tilapia
Catfish
Penetration (%)
0
2
4
6
8
10
12
14
16
411
450
500
550
600
650
700
Wavelength (nm)

Fig. 4


Mammals
Light
Eye
Brain
Pineal
Melatonin
Nile tilapia
Sea bass
Atlantic salmon
Teleosts
C)
B)
A)
?
Light
Eye
Brain
Pineal
Melatonin
Invertebrates
Cell
Light