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Comparative study of pineal clock gene and AANAT2 expression in relation to melatonin synthesis in Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*)

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ABSTRACT

The photoreceptive teleost pineal is considered to be essential to the generation, synchronisation and maintenance of biological rhythms, primarily via melatonin release. The role of internal (circadian clock) and external (light) signals controlling melatonin production in the fish pineal differs between species, yet the reasons underpinning this remain largely unknown. Whilst in salmonids, pineal melatonin is apparently regulated directly by light, in all other studied teleosts, rhythmic melatonin production persists endogenously under the regulation of clock gene expression. To better understand the role of clocks in teleost pineals, this study aimed to characterise the expression of selected clock genes in vitro under different photoperiodic conditions in comparison to in vivo in both Atlantic salmon (*Salmo salar*) and in European seabass (*Dicentrarchus labrax*) (in vitro 12L:12D), a species known to display endogenous rhythmic melatonin synthesis. Results revealed no rhythmic clock gene (*Clock*, *Period 1* & *2*) expression in Atlantic salmon or European seabass (*Clock* and *Period 1*) pineal in vitro. However rhythmic expression of *Cryptochrome 2* and *Period 1* in the Atlantic salmon pineal was observed in vivo, which infers extra-pineal regulation of clocks in this species. No rhythmic arylalkylamine N-acetyltransferase 2 (*Aanat2*) expression was observed in the Atlantic salmon yet in the European seabass, circadian *Aanat2* expression was observed. Subsequent in silico analysis of available *Aanat2* genomic sequences reveals that Atlantic salmon *Aanat2* promoter sequences do not contain similar regulatory architecture as present in European seabass, and previously described in other teleosts which alludes to a loss in functional connection in the pathway.

1. Introduction

The pineal is considered to be fundamental in the generation and maintenance of biological rhythms in vertebrates. In non-mammalian vertebrates the pineal, as in mammals, is the primary source of melatonin however in addition it is directly photosensitive and capable of independent entrainment of melatonin production (Migaud et al., 2006; Falcon et al., 2010). Melatonin biosynthesis begins with the uptake of tryptophan by the pineal and through a series of enzymatic reactions involving tryptophan hydroxylase (TPH, EC 1.14.16.4), aromatic amino-acid decarboxylase (AAAD, EC 4.1.1.28), arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) and acetylserotonin *O*-methyltransferase (HIOMT, EC 2.1.1.4) finally produces melatonin (Falcon et al., 2010, 2011). Of all the above enzymes involved in the melatonin synthesis pathway, the expression of the *Aanat* genes and activity of AANAT enzymes in the pineal is closely mirrored by the profile of circulating melatonin and is commonly described as the rate limiting enzyme for melatonin synthesis (Falcon et al., 2010). Whilst mammals have one unique form of AANAT, teleosts have at least two forms following the teleost wide genome duplication. AANAT1 is preferentially expressed in the retina whilst AANAT2 in the pineal. However, expression of both forms is not limited to these tissues and is also observed in other locations such as the brain. In addition to this, following the identification of two forms of *Aanat1* (a & b) genes in some teleosts it has been proposed that, at least within teleosts, *Aanat* genes can serve multiple functions in addition to melatonin synthesis (Coon and Klein, 2006).

Rhythmic melatonin synthesis in teleosts is driven by the daily rhythm in AANAT2 activity and is up-regulated in the dark in most teleost species. In non-salmonid teleosts, *Aanat2* mRNA transcription mirrors enzymatic activity (Falcon et al., 2011, 2003; Ganguly et al., 2002; Gothilf et al., 1999; Klein et al., 1997). This rhythmic abundance/activity is regulated in two ways: directly via the 24 h light/dark cycle as well as endogenously via circadian clocks. In dark conditions, photoreceptors become depolarised, intracellular calcium (Ca^{2+}) is accumulated, which then regulates the light dependent action of AANAT2 by increasing the efficiency of β 1-adrenergic receptor activation of adenylyl cyclase (Klein, 2007). This consequently results in an elevation of cAMP which in turn results in the formation of the AANAT/14-3-3 complex (Klein, 2007). When light is present, Ca^{2+} is depleted, AANAT2 is degraded and melatonin synthesis ceases (Falcon et al., 2011). The endogenous

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regulation by the circadian clock occurs at a transcriptomic level; the CLOCK:BMAL heterodimer, a component of the core molecular clock, up-regulates *Aanat2* mRNA transcription by binding to E-box elements in the promoter region of the *Aanat2* gene (Appelbaum et al., 2004, 2006; Appelbaum and Gothilf, 2006). Melatonin is highly lipophilic and therefore released continuously into circulation when synthesised. With the onset of light both AANAT2 activity and melatonin are rapidly degraded (halving time of approximately 3.5 min) (Falcon, 2007; Klein, 2007). Melatonin therefore has a low residency time within the blood and levels of circulating melatonin are directly reflective of AANAT2 activity and melatonin synthesis.

In fish, the regulation of melatonin synthesis by internal and external signals is complicated with no clear generalist model fitting the range of species studied. Endogenous regulation of AANAT2 and therefore melatonin, by clocks, is evident in the majority of teleost species. In vivo and in vitro studies have shown that melatonin day/night cycling can persist under constant darkness in most teleosts studied (Bolliet et al., 1996; Cahill, 1997; Kazimi and Cahill, 1999; Iigo et al., 2007a; Martinez-Chavez et al., 2008) including European seabass (*Dicentrarchus labrax*) (Bayarri et al., 2004). Moreover, pineal melatonin synthesis is independently entrainable by light; when exposed to altered light:dark cycles the pineal can re-entrain the melatonin rhythm to the external conditions (Falcon, 1999). However, amongst teleosts it appears that salmonids are the exception. Under continuous darkness melatonin production does not follow an endogenous circadian profile, instead levels of melatonin are consistently high throughout as shown in both in vivo and in vitro conditions (Gern and Greenhouse, 1988; Randall et al., 1991; Iigo et al., 2007a,b). Following a comparison of salmonids and closely related osmerids and pike species, Iigo et al. (2007a) proposed that the pineal of ancestral protacanthopterygians harbours the circadian clock but ancestral salmonids have lost the circadian regulation of melatonin production in the pineal during evolution after the divergence from Osmeriformes/Esociformes. Although it is widely recognised that the salmonid pineal has lost endogenous clock regulation of melatonin synthesis, it remains unclear whether clock gene cycling has become decoupled from melatonin synthesis or if in fact functional circadian clocks are no longer present in the salmonid pineal (Bégay et al., 1998; Coon et al., 1998; Falcon et al., 2003, 2010).

In order to better understand the role of clock genes and their interaction with melatonin synthesis in the teleost pineal the current research aimed to compare the differential expression pattern of these genes in the Atlantic salmon (*Salmo salar*) pineal both in vivo and in vitro and with European seabass. Finally, post-hoc in-silico analyses of Atlantic salmon and European seabass *Aanat2* genomic sequences were carried out to explore how the transcriptional regulation may have been altered in the context of endogenous melatonin synthesis.

2. Materials and methods

2.1. Animals, housing and tissue sampling

2.1.1. Atlantic salmon in vivo and in vitro studies

Fish used were a standard farmed stock origin (mixed sex), housed at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling, UK, 56.04 N, −4.00E) under ambient conditions (4.6 ± 0.2 °C and 2.2 ± 0.1 °C for the in vivo and in vitro studies, respectively) and were fed in excess throughout the daylight hours with the use of automated feeders.

For the in vivo experiment seventy parr (64.0 ± 2.3 g) were acclimated to a 12L:12D photoperiod with the light phase extending from 07:00 h to 19:00 h. After 4 week acclimation, ten fish were sacrificed by lethal anaesthesia (2-phenoxyethanol, 1 mL/L, Sigma) before 1 mL of blood was withdrawn from the caudal peduncle using a heparinised syringe, starting at 09:00 h and then every 4 h thereafter until 09:00 h the following day. Fish were then decapitated and a section of cranial cap removed which encompassed the pineal window and was then stored in a RNA stabilisation solution (RNAlater®, Applied Biosystems). Within 1 h of removing the blood samples, plasma was separated by centrifugation (15 min at 1500 *g*). Plasma aliquots were frozen in liquid nitrogen vapour prior to storage at −70 °C. The cranial caps were stored in the RNA stabilisation solution for 24 h at 4 °C and then removed from the cranial cap and stored at −70 °C. Nocturnal sampling was performed under the illumination of a dim red light.

For the in vitro study, Atlantic salmon parr (46.1 ± 2.7 g) were acclimated as described above. After 4 weeks, Atlantic salmon parr (n = 70 per treatment) were sacrificed by lethal anaesthesia followed by decapitation. Pineals were dissected out by exposing the dorsal surface of the brain by making a rostro-caudal incision in the horizontal plane extending from the eyes to the end of the cranium. During this incision the pineal stalk was severed and the pineal was found resting in the pineal window beneath the liberated inverted cranial cap. Once isolated the pineals were placed into fresh culture medium maintained at 8 °C for a maximum of 3 h before being placed under experimental culture conditions (see below). Pineal harvesting was repeated on two subsequent occasions for the reversed photoperiod (12D:12L) and 24D:00L experiments.

2.1.2. European seabass in vitro study

European seabass used for in vitro experiment were housed at the University of Murcia, chronobiology laboratory facility at the Algameca naval station (37.6 N, −0.98333 W) in Spain. Seventy European seabass (169.9 ± 10.6 g) were acclimated for 2 weeks to 12L:12D (lights on 06:00 h, lights off 18:00 h) at an ambient constant temperature of 16 °C. After the acclimation period all fish were sacrificed via lethal anaesthesia using clove oil, eugenol (Guinama, Valencia, Spain) dissolved in 10 mL of ethanol at a final concentration of 50 μL/L. Lethal anaesthesia was rapidly followed by decapitation. Pineals were removed dorsally by thinning the tissue and bone around the pineal window, then carefully removing the whole pineal according to Migaud et al. (2006). The pineal itself was then removed by carefully cutting the pineal stalk close to its origin and placing it into fresh culture medium (see below) and maintained at 17 °C in groups (35 per 100 mL) until culture conditions were established (see below).

2.2. Pineal culture

2.2.1. Atlantic salmon pineal culture

All Atlantic salmon pineal cultures were carried out at 8 °C in a light and temperature controlled chamber using RPMI-1640 culture medium (Sigma-Aldrich, R8755-10X1L, Gillingham, UK) according to Migaud et al. (2006). Medium was supplemented with 4.8 g/L Hepes Sodium salt (Sigma-Aldrich), 10 mg/L Penicillin–streptomycin solution, 5 mg/L amphotericin B (Sigma-Aldrich) and then pH was adjusted to 7.4 by adding 2 M HCl before 0.2 μm filtration to sterilise and then it was placed in chilled storage (4 °C) for no more than 72 h before use. Aliquots of media were pre-warmed to the culture temperature prior to use. In all Atlantic salmon in vitro experiments pineals were maintained in 20 mL glass vials (10 pineals/20 mL culture medium) with a fine nylon mesh to prevent the pineals from floating. Every 4 h, 15 mL of medium was removed from the culture vial and replaced with fresh, temperature equilibrated, medium.

Three different experimental photoperiods were tested, 12L:12D, reversed photoperiod (12D:12L) and 24D:00L. For the 12L:12D experiment, pineals were harvested between 09:00 h and 12:00 h on Day 0 and placed in culture vials by 13:00 h and subjected to a 12L:12D photoperiod in synchrony with that experienced prior to harvest. Pineals were left in culture overnight undergoing regular medium changes and then from 13:00 h on Day 1, 10 pineals were removed every 4 h until 13:00 h on Day 2 and instantly frozen over liquid nitrogen vapour and then samples were stored at −70 °C for later RNA extraction. At the same time three aliquots of culture media per time point were frozen for melatonin analyses. For the reversed photoperiod (12D:12L) trial, at the