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RESEARCH ARTICLE

Changes in plasma melatonin levels and pineal organ melatonin synthesis following acclimation of rainbow trout (*Oncorhynchus mykiss*) to different water salinities

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SUMMARY

Melatonin has been suggested to play a role in fish osmoregulation, and in salmonids has been related to the timing of adaptive mechanisms during smolting. It has been described that acclimation to different environmental salinities alters levels of circulating melatonin in a number of fish species, including rainbow trout. However, nothing is known regarding salinity effects on melatonin synthesis in the pineal organ, which is the main source of rhythmically produced and secreted melatonin in blood. In the present study we have evaluated, in rainbow trout, the effects of acclimation to different salinities on day and night plasma melatonin values and pineal organ melatonin synthesis. Groups of freshwater (FW)-adapted rainbow trout were placed in tanks with four different levels of water salinity (FW, 6, 12, 18 p.p.t.; parts per thousand) and maintained for 6 h or 5 days. Melatonin content in plasma and pineal organs, as well as the pineal content of serotonin (5-HT) and its main oxidative metabolite (5-hydroxyindole-3-acetic acid; 5-HIAA) were measured by high performance liquid chromatography. In addition, day–night changes in pineal organ arylalkylamine *N*-acetyltransferase (AANAT2) activity and *aanat2* gene expression were studied. Plasma osmolalities were found to be higher in rainbow trout exposed to all salinity levels compared with the control FW groups. A salinity-dependent increase in melatonin content was found in both plasma and pineal organs. This effect was observed during the night, and was related to an increase in *aanat2* mRNA abundance and AANAT2 enzyme activity, both of which also occurred during the day. Also, the levels of indoles (5-HT, 5-HIAA) in the pineal organ were negatively affected by increasing water salinity, which seems to be related to the higher recruitment of 5-HT as a substrate for the increased melatonin synthesis. A stimulatory effect of salinity on pineal *aanat2* mRNA expression was also identified. These results indicate that increased external salinity promotes melatonin synthesis in the pineal organ of rainbow trout by enhancing synthesis of AANAT protein independently of its regulation by light. The possibility that pineal melatonin is a target for hormones involved in the response of fish to osmotic challenge is discussed, as well as the potential role of melatonin in the timing of osmoregulatory processes.

Key words: melatonin, salinity, pineal organ, serotonin, osmoregulation, rainbow trout.

INTRODUCTION

The pineal organ of teleost fish is a photo-neuro-endocrine structure located on the roof of the brain and immediately below the skull. Pineal photoreceptor cells respond to environmental photoperiod by releasing neural or endocrine signals, the latter being involved in the synthesis of melatonin (Ekström and Meissl, 1997). This hormone is not stored and its plasma concentration reflects the synthesizing capacity of the gland, which is higher during darkness (Falcón, 1999). Daily rhythms of circulating melatonin are believed to entrain the temporal co-ordination of a number of physiological processes associated with daily and seasonal rhythms, including locomotor and feeding behaviours, reproduction and smoltification, among others (Porter et al., 1998; Bromage et al., 2001; López-Olmeda et al., 2006; Falcón et al., 2007a; Falcón et al., 2010).

In all classes of fish, rhythms of melatonin in the blood are primarily dependent on its formation in the pineal cells. The rhythm results from the daily variations in the activity of the second last enzyme of melatonin biosynthesis, arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), which controls the rate at which serotonin (5-HT) is converted into *N*-acetylserotonin and is thought to be the limiting step in the pathway (Klein et al., 1997). In most fish species there is a complete melatonin-rhythm-generating system in each individual photoreceptor cell in the pineal gland, so large changes in melatonin are typically endogenous and persist on a circadian basis when fish are exposed to constant darkness or when pineal glands are placed in culture (Bolliet et al., 1994; Falcón, 1999). However, there are exceptions in salmonids, which apparently lack a functional intrapineal circadian clock (Gern and Greenhouse, 1988; Iigo et al., 2007; Migaud et al., 2007), and large changes in melatonin production are associated with rapid non-clock-dependent light suppression of AANAT activity (Falcón et al., 2007b). Two AANAT genes, named *aanat1* and *aanat2*, have been described in teleosts, with *aanat2* being the dominant form expressed in the pineal organ (Falcón et al., 2007b). Regulation of AANAT2 protein varies among fish species, but in most cases changes in *aanat* mRNA abundance determines AANAT activity (Bégay et al., 1998; Benyassi et al., 2000). The importance of this mechanism is poorly understood and might vary among fish species. Thus, in rainbow trout the *aanat2* gene has been reported to be expressed constitutively, and melatonin synthesis is strongly affected by daily AANAT proteosomal proteolysis (Falcón et al., 2001).

Even though light is the principal regulator of melatonin production in the fish pineal organ, other fluctuating environmental factors, such as temperature, can also modulate melatonin secretion

in the pineal organ through the regulation of AANAT2 activity (Zachmann et al., 1992; Falcón et al., 1994). Recently, it has been reported that changes in environment salinity could also influence circulating melatonin levels in a number of euryhaline fish, with the results varying depending on the fish species studied or the water acclimation periods. Thus, Kleszcynska et al. have shown that acclimation of gilthead sea bream (*Sparus aurata*) to hypersaline water or seawater (SW) for 2 weeks results in decreased plasma melatonin levels compared with the levels in low salinity conditions (Kleszcynska et al., 2006). Similarly, López-Olmeda et al. reported decreased plasma melatonin in European sea bass (*Dicentrarchus labrax*) acclimated to SW, with respect to fish acclimated to freshwater (FW) or brackish water (BW) (López-Olmeda et al., 2009). In contrast, in rainbow trout, plasma melatonin levels have been shown to increase after 2 weeks exposure to BW (Kulczykowska, 1999), and to be unaltered after an osmotic stress (Kulczykowska, 2001).

Euryhaline fish are able of living in environments that are subjected to variations in salinity because they have developed physiological strategies to adapt to such changes. The osmoregulatory function involves different endocrine responses that directly or indirectly promote changes in target tissues involved in osmotic balance and mobility of metabolic resources (Sangiao-Alvarellos et al., 2003; McCormick and Bradshaw, 2006). Recently, melatonin has been proposed to play a role in osmoregulation (Kleszczynska et al., 2006; Sangiao-Alvarellos et al., 2007), as it has also been implicated in the timing of salmonid smolting (Porter et al., 1998; Iigo et al., 2005). Indeed, melatonin-binding sites have been found in fish osmoregulatory tissues such as the gills, small intestine and kidney (López-Patiño, 2004; Kulzczykowska et al., 2006), suggesting a possible role of melatonin in water–ion balance in fish. Therefore, there may be an increase in circulating melatonin levels once the fish encounter new saline environments, thus favouring environmental adaptation. Although the osmotic influence on blood melatonin levels is likely to derive from changes in the pineal melatonin production, no specific studies have been carried out at this level. However, López-Olmeda et al. have reported that, in the European sea bass, in addition to plasma melatonin changes, water salinity also affected melatonin content in intestine and gills, suggesting that extrapineal tissues could also contribute to an alteration in blood melatonin levels in response to osmotic changes (López-Olmeda et al., 2009). We therefore focused the present study on the evaluation of changes in melatonin synthesis in the pineal organ of the rainbow trout exposed to different salinity levels, and how it was reflected in plasma melatonin levels. It is known that exposing fish to new saline environments induces differential short- and long-term changes in their physiology (Leray et al., 1981; Sangiao-Alvarellos et al., 2003). Consequently, two acclimation periods (6 h and 5 days) were tested in order to assess the time-dependent melatonin response to changes in the osmotic environment.

MATERIALS AND METHODS

Animals

All experiments were designed according to the guidelines of the European Union Council (86/609/EU), and the Spanish Government (RD 1201/2005) legal requirements. Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) with a body mass of 101±22 g were provided by a commercial hatchery (Soutorredondo, A Coruña, Spain). Animals were kept in experimental 100-litre tanks with filtered and continuously replenished fresh water, under constant photoperiod (12 h:12 h light:dark) and temperature (14±1°C) for 15 days before any experimental procedure. Feeding time was scheduled at zeitgeber time (ZT) 3 (ZT0=lights on), and consisted of a commercial (Dibaq diproteg, Segovia, Spain) dry pellet diet (1% body mass).

Experiments and sampling

Two sets of experiments were designed in order to test the time-dependent effect of salinity on rainbow trout melatonin production. In the first experiment, fish were acutely exposed for 6 h to different water salinities – 6 p.p.t. (102 mOsm l^{-1}), 12 p.p.t. (205 mOsm l^{-1}) and 18 p.p.t. (308 mOsm l^{-1}) – whereas the control group was kept in FW. To achieve the required salinity, one half of the water was slowly removed from every tank and replaced with FW to which different concentrations of marine salts were added to achieve the final salinities, given above. Two 100-l tanks were used for each water condition. In the first tank, animals (N=8 per group) were exposed to FW or one of the different salinities for up to 6 h, starting at ZT2; fish were sacrificed and samples collected at ZT8. In the second tank the same protocol was performed but treatments were scheduled to start 1 h before the end of the light phase (ZT11); 6 h later, i.e. near to the middle of the scotophase (ZT17), animals (N=8 per treatment) were sacrificed and samples collected under dim red light.

A second experiment, with a similar experimental protocol involving two tanks of fish for each of the salinity condition tested (FW, 6, 12 and 18 p.p.t.) was performed, but in this case trout were allowed to acclimate for 5 days before sampling. After this time fish (N=8 per group) were sacrificed and sampled at either light phase (ZT8) or near to dark phase (ZT17), depending on the experimental groups.

At the sampling time fish were deeply anaesthetized with MS-222 (50 mg l^{-1}) buffered to pH 7.4 with sodium bicarbonate, and killed at either the day or night points assayed. Blood was collected separately from the caudal vein of each fish using 1 ml heparinized syringes. After that, each fish was immediately killed by decapitation and the pineal organ was removed with the aid of sterilized material and placed into RNase-free 1.5 ml Eppendorf tubes. Then, samples were immediately frozen in liquid nitrogen, and stored at –80°C until assayed. Plasma was obtained by centrifuging blood at 12,900 ***g*** for 10 min at 4°C. Aliquots were then frozen on dry ice and stored at –80°C until analysis.

Plasma Na^+ and osmolality assays

Plasma Na^+ content, and plasma osmolality were measured in order to determine the variations in both parameters after exposing the animals to different water salinities. Na^+ content was measured using an atomic absorption sprectrophotometer (Varian SpectrAA-250 Plus, Varian Medical Systems, Zug, Switzerland) and osmolality was measured with a Roebling (Berlin, Germany) 13DR Autocal osmometer.

Plasma and pineal gland melatonin quantification

Plasma melatonin was assayed according to Muñoz et al. (Muñoz et al., 2009), with modifications. Briefly, a 200 µl aliquot of plasma was mixed (1:1 v/v) with 0.1 mol l^{-1} acetic acetate buffer (pH 4.6) and 2 ml chloroform added. The mixture was mixed for 1 min, centrifuged (3800 ***g***, 10 min) and the aqueous phase aspirated. The organic layer was separated and 500 µl 0.1 mol l^{-1} NaOH were added. After stirring and centrifugation, the aqueous phase was aspirated and the organic layer was dried out under an air stream at room temperature. The residue was dissolved in 100 µl of mobile phase and filtered through a 0.5 µm filter. An aliquot (50 µl) of the filtrate

was injected into the HPLC system. Data from the analysis are expressed as pg ml^{-1} of plasma.

The chromatographic system consisted of a Gilson (Middleton, WI, USA) 321 solvent delivery pump equipped with a 50 µl Rheodyne (IDEX-HSS, Oak Harbor, WA, USA) injection valve, and a Jasco Analitica (Madrid, Spain) FP-1520 fluorescence detector set at 280/345 nm excitation/emission wavelengths. Melatonin was separated on a Beckmann Ultrasphere ODS column (3 µm particles, 75×4.6 mm i.d.; Fullerton, CA, USA). The mobile phase consisted of a solution of 85 mmol l^{-1} acetic acetate, 0.1 mmol l^{-1} Na_2-EDTA and acetonitrile (14% of final volume), with the pH adjusted to 4.7. All analyses were performed at room temperature at a flow rate of 1.0 ml min^{-1}.

For pineal melatonin quantification, each organ (N=8 per group) was homogenized by sonication in 100 µl of 0.2 mol l^{-1} phosphate buffer (pH 6.7) and centrifuged at 16000 ***g*** for 10 min. The supernatant was divided into two aliquots. A 60 µl aliquot was assayed for melatonin and pineal indole (see below). The second, a 40 µl aliquot, was immediately assayed for AANAT2 activity (see below). From the 60 µl aliquot, pineal melatonin content was measured following direct injection of a 20 µl volume into the HPLC system, which was similar to that described for plasma melatonin assays. Other conditions were as described by Ceinos et al. (Ceinos et al., 2008). Acquisition and integration of chromatograms were performed using the BiocromXP software (Micron Analítica, Madrid, Spain). Sample peak areas were compared with those of appropriate standards to estimate the quantities of compounds present.

Measurement of indole content in pineal organs

Pineal 5-HT and its acidic metabolite, 5-hydroxindole acetic acid (5-HIAA) were measured by HPLC according to Ceinos et al. (Ceinos et al., 2005). Briefly, 20 µl of a 1:40 dilution from the 60 µl original aliquot from each pineal sample were injected into the HPLC system, which consisted of a Jasco PU2080 pump and an ESA Coulochem detector (Bedford, MA, USA). The mobile phase was a solution of 85 mmol l^{-1} NaH_2PO_4, 0.72 mmol l^{-1} octanosulfonic acid, 18% methanol, which was adjusted to pH 3.0. All separations were performed at room temperature at a flow rate of 0.8 ml min^{-1}. The detection system consisted of a double analytical M5011 ESA cell with the electrode potentials set at +20 and +300 mV. Acquisition and integration of chromatograms were performed using the BiocromXP software.

Pineal AANAT activity assays

Pineal AANAT2 activity was assayed by *in vitro* incubation of sample homogenates with the substrate (tryptamine), and acetyl-CoA as cofactor, according to Ceinos et al. (Ceinos et al., 2008) with some modifications. Pineal organs were individually sonicated in 100 µl of 0.2 mol l^{-1} phosphate buffer (pH 6.2) on ice, and the homogenate was centrifuged at 16,000 ***g*** for 10 min. Then, 40 µl of the supernatant were mixed with 40 µl of 27 mmol l^{-1} tryptamine and 40 µl of 1.0 mmol l^{-1} acetyl-CoA (final concentrations in assay: 9 mmol l^{-1} tryptamine and 0.5 mmol l^{-1} acetyl-CoA) and incubated for 60 min at 16°C. The reaction was stopped by adding 1 ml of 4°C chloroform, and the samples were then vortexed (1 min) and centrifuged at 12,900 ***g*** for 10 min. The resulting aqueous supernatant was discarded and the organic layer was evaporated to dryness under an air stream, at room temperature. Then, the extracts were dissolved in 100 µl of mobile phase (65 mmol l^{-1} NaH_2PO_4, 0.123 mmol l^{-1} Na_2-EDTA and 15% acetonitrile; pH 4.7). 20 µl of this solution were directly injected into the fluorescence HPLC system in order to quantify the reaction product (*N*-acetyl tryptamine; NAT) formed. The system consisted of a HPLC pump (Gilson M101) with a Ultrasphere Beckman column (3 µm particles, 75 mm and 4.6 mm i.d.) and a Jasco FP-1520 fluorimetric detector set at 285/360 nm excitation/emission wavelengths. All analyses were performed at room temperature at a flow rate of 1 ml min^{-1}. Sample peak areas were quantified with appropriate standards using the Biochrom XP software.

Analysis of pineal organ *aanat2* mRNA

Immediately after dissection pineal organs from fish receiving the same treatment were pooled (N=2). Total mRNA was extracted from these pooled pineal organs using the TRIzol method (Gibco BRL, Gaithersburg, MD, USA) according to manufacturer's instructions. The isolated RNA quality and quantity was spectrophotometrically determined. From each sample, 2 µg RNA were transferred to a new vial and sterile nuclease-free H_2O was added to make it up to 10 µl. Then 1 µl random primers (C1181, Promega, Madison, WI, USA) was added, and vials were then incubated for up to 5 min at 70°C. After that, 9 µl of a mixture containing 0.2 µl RNAguard^{TM} RNase inhibitor (27-0816-01, Promega), 1 µl of 10 mmol l^{-1} dNTP Mix (U1511, Promega), 1 µl of M-MLV reverse transcriptase (M1701, Promega), 4 µl M-MLV RT 5× buffer (M531A, Promega) and 2.8 µl sterile nuclease-free H_2O, were added to each vial. Vials were then incubated at 37°C for 1 h and 65°C for 5 min. A negative control for each sample was assessed without reverse transcriptase in order to confirm the absence of any genomic contamination.

Real-time quantitative RT-PCR (qPCR) was performed using a Maxima^{TM} SYBR Green qPCR Master Mix (K0221, Fermentas, Burlington, ON, Canada) and a Bio-Rad (Hercules, CA, USA) MyIQ real-time PCR system. The primers and probes were based on previously reported sequences of rainbow trout genes and obtained from Sigma-Genosys (St Louis, MO, USA), including: *aanat2* (accession number AF106006.1) forward 5′-CATTCGTCTCT-GTGTCTGGT-3′, reverse 5′-TTTCTGGGATATGCTGGGT-3′; and β-actin gene (AJ438158) forward 5′-GATGGGCCAGAAA-GACAGCTA-3′, reverse 5′-TCGTCCCAGTTGGTGACGAT-3′. Gene expression for each sample was normalized to that of the β-actin gene. Relative mRNA expression was calculated by using the standard comparative ΔC_t method. For each gene, samples collected at the same time point were processed in parallel and the expression was measured in triplicate.

Statistical analysis

Comparisons between groups were performed at each time (6 h and 5 days) using two-way ANOVA with salinity degree and time of day as main factors. When a significant effect was identified within a factor, *post hoc* comparisons were carried out within that factor using a Student–Newman–Keuls test, and differences were considered statistically significant at P<0.05.

RESULTS

Plasma Na^+ content and osmolality

Plasma osmotic parameters are shown in Table 1. There was an apparent increase in plasma osmolality and Na^+ concentration with increasing water salinity after both 6 h and 5 days exposure. Significant differences in plasma Na^+ content were observed in the 12 p.p.t. salinity group (5 days) and 18 p.p.t. salinity group (6 h and 5 days) compared with their respective control (FW) groups. For plasma osmolality, there were significant increases in the 12 and

Table 1. Plasma Na^+ content ($mEq\,l^{-1}$) and osmolality ($mOsm\,l^{-1}$) under the different experimental conditions

Treatment	Plasma Na^+ content	Plasma osmolality
6 h		
Control	123±8	298±4
6 p.p.t.	132±8	311±4
12 p.p.t.	136±8	323±4*
18 p.p.t.	149±4*	359±3*
5 days		
Control	128±8	286±4
6 p.p.t.	140±8	293±2
12 p.p.t.	170±8*	322±2*
18 p.p.t.	191±8*	365±7*

Values are means ± s.e.m. (N=8 per group).
*P<0.05 *vs* the control group.

18 p.p.t. salinity groups (6-h and 5-day experiments) compared with their respective controls.

Plasma and pineal organ melatonin contents

There were significant day–night variations in plasma melatonin levels in all groups of fish after both 6 h and 5 days of exposure (Fig. 1A,B). In addition, animals acclimated to different salinities (6, 12 and 18 p.p.t.) did not show any significant change in daytime plasma melatonin concentrations after 6 h or 5 days, relative to their respective FW control group. In contrast, nocturnal circulating melatonin levels at both 6 h and 5 days were significantly increased by salinity. This effect was not dependent on the salinity concentration in the 6 h treatment but it was clearly salinity dependent after 5 days exposure because it was observed only in the samples from fish exposed to 12 and 18 p.p.t. salinity.

Similar to plasma, pineal organ melatonin content also showed day–night variations with the maximal levels observed during the dark phase in all the experimental groups (Fig. 1C,D). No significant differences were detected in the daytime pineal melatonin content for each salinity treatment at both 6 h and 5 days. However, nighttime pineal melatonin levels did show a salinity-dependent effect, with fish exposed to 18 p.p.t. for 6 h showing a significantly increase in melatonin content (P<0.05 *vs* control, 6 and 12 p.p.t.; Fig. 1C), and fish acclimated for 5 days to 12 and 18 p.p.t. also showed a significant nocturnal melatonin increase relative to control and 6 p.p.t. salinity (Fig. 1D).

5-HT and 5-HIAA levels in rainbow trout pineal organ

The effects of salinity on the day–night variation of indoles in the pineal gland are shown in Fig. 2. After both 6 h and 5 days, significant day–night variations were observed in the 5-HT content of fish exposed to FW and 6 and 12 p.p.t. water salinity (Fig. 2A,B). In all of them, levels of 5-HT were higher during the day than at night. However, fish acclimated for 6 h to 18 p.p.t. salinity showed lower pineal 5-HT content in the daytime relative to the other groups (P<0.05 *vs* control, 6 and 12 p.p.t.), and no significant day–night fluctuation was found in fish in this group (Fig. 2A). In addition, trout exposed for 6 h to 12 and 18 p.p.t. salinity showed significantly lower nocturnal levels of 5-HT in the pineal organ compared with the nocturnal values of the FW control group.

After 5 days acclimation, the day–night variation in 5-HT in the pineal organ was maintained in all groups exposed to the different treatments (Fig. 2B). A low level of 5-HT in the 18 p.p.t. salinity group in the daytime, which might have been expected from the 6-h samples, was not found; indeed the levels were higher in both the 12 and 18 p.p.t. groups than in the control group, although this was not significant. At night, there was a significant decline in pineal gland 5-HT content in all groups of fish acclimated to the different salinities (6, 12 and 18 p.p.t.) relative to the control FW group (P<0.01 in all cases).

The levels of the main 5-HT acid metabolite, 5-HIAA, had similar day–night variations to those of 5-HT (Fig. 2C,D). This fluctuation occurred in all experimental salinity groups. The main effect of water salinity on pineal 5-HIAA levels was a significant decrease in daytime 5-HIAA levels after exposing animals to 18 p.p.t. for 6 h (P<0.05 *vs* FW, 6 and 12 p.p.t. salinity). This effect was also observed after 5 days of acclimation to the different salinity conditions (P=0.05 *vs* FW, 6 and 12 p.p.t. salinity).

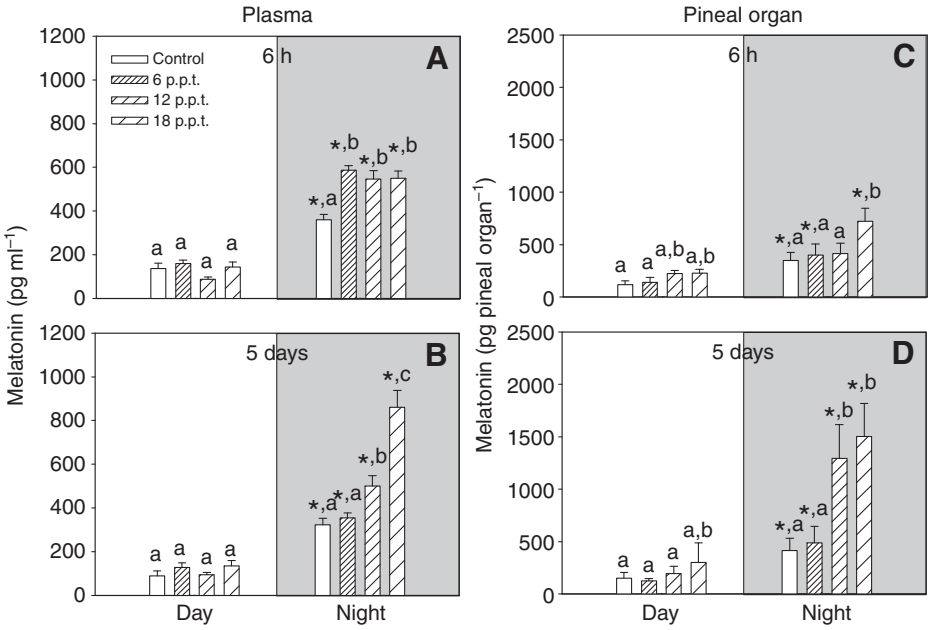

Fig. 1. Day–night changes of plasma (A,B) and pineal organ (C,D) melatonin levels in rainbow trout acclimated for 6 h (A,C) or 5 days (B,D) to freshwater (control) or several salinities (6, 12 and 18 p.p.t.). Data are presented as means ± s.e.m. (N=8 per group). *Significantly different from their respective treatment group during the daytime (P<0.05). Different letters above the bars indicate a significant difference (P<0.05) between treatment groups at the same time point.

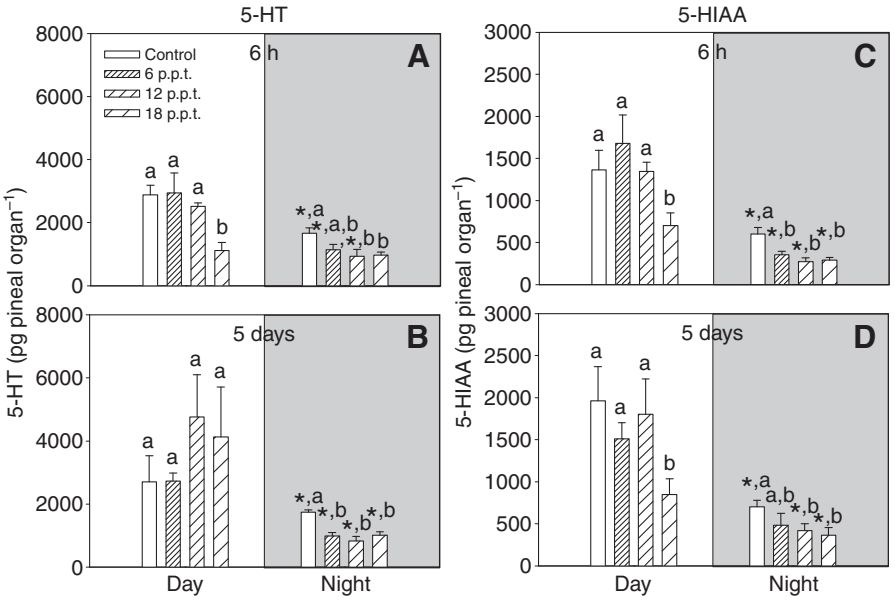

Fig. 2. Changes in the levels of serotonin (5-HT; A,B), and 5-hydroxindole acetic acid (5-HIAA; C,D) in the pineal organ of trout following 6 h (A,C) and 5 days (B,D) acclimation to freshwater (control) or increased water salinity (6, 12 and 18 p.p.t.). Data are presented as means ± s.e.m. (N=8 per group). *Significantly different from their respective treatment group during the daytime (P<0.05). Different letters above the bars indicate a significant difference (P<0.05) between treatment groups at the same time point.

Pineal organ AANAT2 activity and aanat2 mRNA expression

Day–night variations in rainbow trout pineal organ AANAT2 activity are shown in Fig. 3. In all the experimental groups, nocturnal AANAT2 activities were higher than the diurnal ones in both the 6-h and 5-day experiments. In addition, both diurnal and nocturnal pineal AANAT2 activities were progressively increased with increasing salinity in the 6 h experiment (Fig. 3A), in such a way that the enzyme activity of fish in all salinities was significantly higher that the respective day and night FW control groups.

Similar results were found after 5 days of treatment in both the day and night measurements, with pineal AANAT2 activity being significantly higher in trout exposed to the different salinities (P<0.05 *vs* respective control groups; Fig. 3B). In contrast, no meaningful differences in AANAT2 activity was found between salinities in both the day and the night samples.

mRNA levels of the *aanat2* gene were examined in the control and 12 p.p.t. salinity groups after 6 h and 5 days acclimation. Significant day–night variation in *aanat2* mRNA expression was found in both groups, with the highest activity being measured during the night phase (Fig. 4). Consistent with the AANAT2 activity, there was a significant increase in nocturnal *aanat2* mRNA expression after acute (6 h) exposure to 12 p.p.t. salinity, compared with its respective FW control group (P<0.01; Fig. 4A). In addition, trout acclimated for 5 days to 12 p.p.t. salinity showed significantly increased *aanat2* mRNA expression both during the day and at night (P<0.05 *vs* respective FW groups; Fig. 4B).

DISCUSSION

Rainbow trout are euryhaline teleosts so they can survive in a relatively wide range of salinities, which allows them to mate and spawn in freshwater and then the juveniles can migrate to the open sea for growth (Boeuf, 1993). Acclimation of rainbow trout to SW occurs in two distinct phases: an initial adjustment phase that is characterized by increasing plasma osmotic concentration, and a later regulative phase during which control is established over the osmotic concentration. The adjustment phase is crucial for acclimation to seawater when fish are abruptly exposed to an elevated water osmolality (420–450 mOsm l^{-1}), which could result in a general physiological failure. However, for salinities lower than 30% SW a slight osmotic shock occurs that permits the fish to balance between ion flux and efflux for 1 or 2 days after transfer (Jackson, 1981). Our results show a moderate increase in plasma osmolality in fish exposed for 6 h to low and moderate salinity (ranging from 103 to 308 mOsm l^{-1}), which persisted for 5 days (see Table 1). These data are in agreement with those previously reported for both short- and long-term exposure to different water salinities

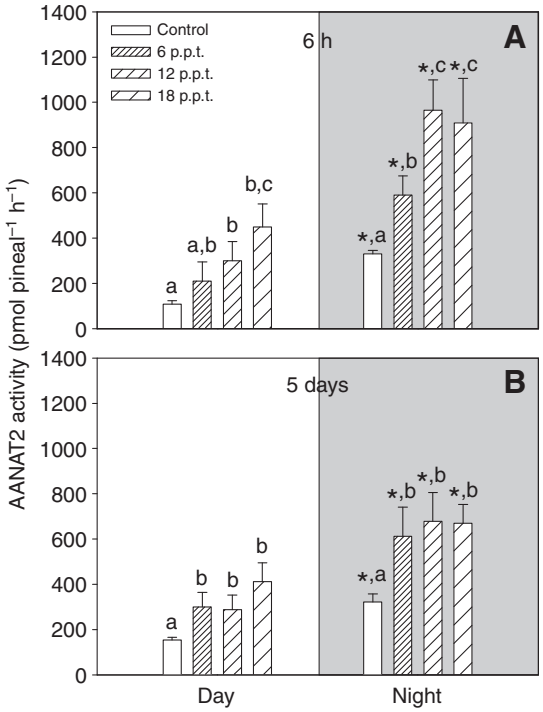

Fig. 3. AANAT2 activity during the day and night in pineal organs of rainbow trout acclimated for 6 h (A) or 5 days (B) to freshwater (control) or different environmental salinities. Data are presented as means ± s.e.m. (N=8 per group). *Significantly different from their respective treatment group during the daytime (P<0.05). Different letters above the bars indicate a significant difference (P<0.05) between treatment groups at the same time point.

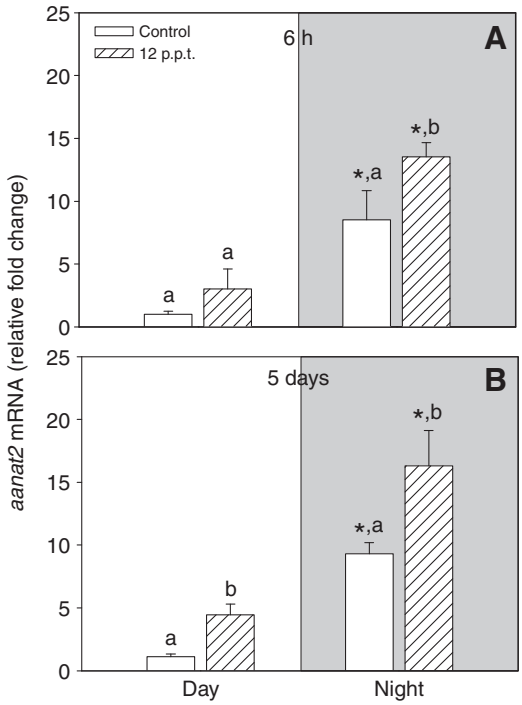

Fig. 4. Day–night variations in pineal organ *aanat2* mRNA expression in rainbow trout exposed for 6 h (A) and 5 days (B) to freshwater (control) or saline (12 p.p.t.) water. Relative fold change was estimated by taking as reference the daytime values of the control group after 6 h (gene expression was considered as 1). Data are presented as means ± s.e.m. (N=4 per group). *Significantly different from their respective treatment group during the daytime (P<0.05). Different letters above the bars indicate a significant difference (P<0.05) between treatment groups at the same time point.

(Jackson, 1981; Leray et al., 1981; Kulczykowska, 1997; Kulczykowska, 1999). It is interesting also that in the present study the two lowest salinities used (6 and 12 p.p.t.) were hypo-osmotic compared with plasma. Therefore, changes in osmolality of the plasma were probably due to increased ionic concentrations (i.e. Na^+ levels) rather than to an osmotic dehydration by reduction of plasma volume.

Circulating melatonin levels were affected by increased water salinity. Nocturnal melatonin levels increased after both 6 h (6, 12 and 18 p.p.t. salinities) and 5 days (12 and 18 p.p.t. salinities) of treatment, in agreement with previous studies in rainbow trout exposed for 2 weeks to BW (Kulczykowska, 1999). Data from other salmonid species are scarce, and are not in agreement with that herein reported. For example, a higher plasma melatonin concentration in parallel with high Na^+ and Cl^- values was demonstrated in coho salmon during SW adaptation (Folmar and Dickhoff, 1981), and in yearling coho salmon during a 1-day exposure to SW (Gern et al., 1984). Saito et al. also reported increased daytime plasma melatonin in SW-acclimated chum salmon compared with FW-captured fish (Saito et al., 2004). However, some major controversies exist in marine species. Thus, plasma melatonin levels were found to increase in the daytime (a night-time effect was not studied) in gilthead seam bream (*Sparus aurata*) during acclimation to low environmental salinity (Kleszczynska et al., 2006), whereas melatonin levels increased during the night (no changes occurred during the day) in the European sea bass (*Dicentrarchus labrax*) (López-Olmeda et al., 2009). All these data suggest that melatonin is only part of the process that enables fish to cope with osmotic challenge, and that responses of melatonin might vary in euryhaline teleosts depending on species-specific tolerances.

Furthermore, our results show that salinity-induced changes in plasma melatonin levels occurred predominantly during darkness, and no effect was observed during daytime in any of the salinity conditions assayed. Similarly, pineal organ melatonin content was affected by salinity only at night, with increased melatonin levels after 6 h (18 p.p.t. salinity) and 5 days (12 and 18 p.p.t. salinities). However, changes in AANAT2 activity, which were also enhanced after increasing water salinity, were found in fish sampled during the day and the night. An effect of salinity on AANAT2 enzyme activity was observed in fish exposed for 6 h and 5 days, but the effect of the latter exposure was not dependent on salinity concentration. Furthermore, a significant day–night difference in AANAT2 activity was still found in all groups of fish. Considering the small amount of melatonin produced in the pineal organ during the day (in comparison with that synthesized at night) it is plausible that changes in melatonin during acclimation to saline water were not so evident in the pineal organ during the daytime, in contrast to the changes observed in AANAT enzymatic activity. Also, melatonin is released from the pineal organ into a large volume of plasma. This would explain the clear effect of salinity on plasma melatonin levels at night (when pineal melatonin secretion is high), and its absence during daytime (when melatonin secretion from the pineal organ is low). Moreover, alterations in the plasmatic clearance of the hormone could contribute to the apparently different day–night effect of salinity on plasma and pineal melatonin content, compared with pineal AANAT activity.

In many fish species light inhibits melatonin synthesis in the pineal organ, thus modulating the daily pattern of several melatonin-related indoles (Iigo et al., 1991; Migaud et al., 2007). In teleosts, AANAT2 limits the rate at which 5-HT is converted into *N*-acetylserotonin, which probably limits the pineal organ melatonin synthesis, as long as a daily rhythmic pattern for AANAT2 activity parallels that of melatonin content (Falcón, 1999). Thus, AANAT2 activity is increased during the scotophase (Falcón et al., 1992; Ceinos et al., 2005), leading to a nocturnal increase in melatonin synthesis and its secretion into the blood. Our data clearly showed day–night variations in AANAT2 activity in trout kept in FW, and also in most groups of trout exposed for either 6 h or 5 days to any of the salinity conditions studied. These data indicate that increased salinity enhances melatonin synthesis by increasing AANAT activity, an effect that was independent of the environmental light phase. Moreover, decreased levels of 5-HT (the AANAT2 substrate) and its main oxidative metabolite (5-HIAA) were found under increased saline concentrations, both during the day (18 p.p.t. salinity) and at night (all salinities assayed). In FW control fish, a clear day–night rhythm was observed for 5-HT content, with lowest values at night due to its use as a substrate for the elevated melatonin synthesis (Ceinos et al., 2005). Accordingly, the observed decrease in 5-HT content in fish acclimated to the different salinity conditions are likely to derive from an activated melatonin synthesis in response to the saline-induced increase in AANAT activity. Also for 5-HIAA, decreases due to salinity exposure seem to be a consequence of the reduced availability of 5-HT because of its higher utilization to form melatonin. All these data allow us to clearly conclude that pineal organ function in trout is activated during exposure to saline environments, resulting in increased melatonin synthesis and release into the blood. The salinity effect seems to be mediated by enhancing daily AANAT2 activity and apparently did not disturb processes interpreting the lighting message in the pineal photoreceptor cells.

Teleost fish have two different genes encoding for AANAT: *aanat1* and *aanat2* (Bégay et al., 1998). However, at least in pike and rainbow trout only the *aanat2* gene is expressed in the pineal organ, with no orthologue in other vertebrate classes (Falcón et al., 2007a). The *aanat2* expression has been shown to follow daily variations, with the higher values occurring during the scotophase (Bégay et al., 1998), and presumably it is involved in pineal organ melatonin synthesis. However, the rainbow trout studies failed to demonstrate changes in the *aanat2* expression pattern in the pineal organ (Bégay et al., 1998; Coon et al., 1998; Falcón et al., 2001). In contrast, in the present study we observed a significant increase in nocturnal *aanat2* gene expression compared with that in the daytime, in correspondence with daily changes in AANAT enzyme activity. The reasons for these discrepancies are not known. In the rainbow trout studies, *aanat2* transcripts were quantified by conventional northern blot analysis (Bégay et al., 1998; Coon et al., 1998), which usually requires elevated total RNA amounts. Owing to the small size of pineal organ, several organs were pooled in order to obtain tissue enough for assays. Moreover, we used the more sensitive qPCR technique with RNA extracted from two pineal organs. Therefore, it is plausible that daily *aanat2* changes were not observed in other studies, in contrast to the clear day–night fluctuation observed in this study, because of the analytical techniques used. However, it is also possible that rainbow trout used in the different studies were of different strains or differentially affected by other environmental factors. Independently of that, our data clearly show that light is able to suppress *aanat2* gene expression and enzymatic activity, an effect that involves photoreceptor cell hyperpolarisation, leading to decreased intracellular calcium and cAMP levels, with the subsequent dephosphorylation of AANAT2 and its degradation through proteosomal proteolysis (Falcón et al., 2001; Ganguly et al., 2001). During the scotophase, the pineal photoreceptor cells are depolarized and then intracellular calcium levels are increased. This leads to the increase in the expression of several genes, including *aanat2*, with the subsequent increase in the enzyme synthesis and activity (Kroeber et al., 2000; Falcón et al., 2007b). According to our data, AANAT expression and activity changed when the fish were exposed to 12 p.p.t. salinity during both day and night for 6 h, with the effect being larger after 5 days. In addition, AANAT activity increased during the day after exposure to 18 p.p.t., reaching levels even higher than that observed in the FW control group during the night. This also suggests that melatonin synthesis might be influenced by other non-identified limiting factors (i.e. hydroxindole-O-methyltransferase activity). Whether these time-dependent changes in gene expression after salinity challenge are related to the two distinct phases affecting osmoregulatory capacity in trout deserves further investigation.

Osmoregulatory mechanisms are activated when fish are exposed to alterations in their saline environment, with the subsequent activation of a number of hormones and metabolic processes (Gern et al., 1984; McCormick and Bradshaw, 2006). It has been reported that exposure of rainbow trout to hypersaline environments induces a rapid and relatively persistent increase in catecholamines and cortisol secretion (Wendelaar-Bonga, 1997; Liebert and Schreck, 2006). This is known as the osmotic stress response, and helps fish to cope with the demands of the osmoregulatory response during the saline-adaptation process (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2007). A physiological role for catecholamines in the trout pineal organ has been suggested, but they have not been shown to control melatonin production, as they have in the pike pineal organ (Falcón et al., 1991). Cortisol, however, is a serious candidate for involvement in melatonin formation in fish. In fact, glucocorticoid receptors have been reported in the rainbow trout pineal organ, and dexamethasone, a glucocorticoid analogue, was observed to negatively influence melatonin synthesis in cultured pineal organs (Benyassi et al., 2001). Also cortisol was shown to inhibit *in vitro* nocturnal melatonin production in the tilapia pineal gland (Nikaido et al., 2010). This does not agree with the increases found in our study following saline acclimation of trout. Therefore, a role of elevated cortisol levels in the saline-induced increase in melatonin synthesis in trout is difficult to predict. Moreover, the results of studies on the effect of stress on fish melatonin levels vary. Thus, increased levels of plasma melatonin were found in gilthead sea bream subjected to high stocking density (Mancera et al., 2008), whereas disturbance stress reduced plasma melatonin levels in trout (Kulczykowska, 2001). Besides catecholamines and cortisol, other hormones could be mediating changes in melatonin synthesis during osmotic acclimation in trout. Thus, the neurohypophyseal hormone vasotocin, which is thought to play a role in adaptation of teleost fish to external salinity changes (Warne et al., 2005), has also been shown to interact with melatonin (Kulczykowska, 2001; Kulczykowska, 2002) to control the physiological adaptation of fish to daily and seasonal environmental changes. However, no specific studies evaluating the influence of vasotocin and other stress-related hormones in fish melatonin synthesis in the pineal organ have been done to date.

The physiological significance of osmotically triggered changes in melatonin synthesis is not known, but a role of melatonin in osmoregulation has been strongly suggested. Thus, melatonin treatments were shown to influence osmoregulatory processes in trout, including gill and kidney Na^+/K^+-ATPase activity and plasma ion balance (Sangiao-Alvarellos et al., 2007). Melatonin treatments also enhanced survival in SW tolerance tests in Atlantic salmon (*Salmo salar*) (Porter et al., 1998) and were suggested to affect timing of smoltification in masu salmon (*Oncorhynchus masou*) (Iigo et al., 2005). The existence of melatonin-binding sites in osmoregulatory tissues of several teleost species, i.e. flounder, trout and sea bream (Kulczykowska et al., 2006) also pointed to these organs as potential target sites for melatonin action in ion water–ion balance. Our present results, showing that circulating melatonin levels in rainbow trout are altered after external osmotic changes, also reinforce the idea that melatonin is one more of the endocrine substance involved in acclimation and homeostasis in different salinity conditions. Although melatonin might modulate processes in fish osmoregulatory organs, the hormone is frequently linked to timing of physiological functions (Falcón et al., 2010). Therefore, it would be of interest to evaluate how other rhythmic functions of fish are influenced by environmental osmotic changes, as well as the potential of melatonin to temporally adjust metabolic, hormonal and behavioural processes during fish osmotic acclimation.

To summarize, we demonstrate that salinity increases trout melatonin synthesis and secretion. The pineal organ is a major target for the effects of salinity since osmotically related increases in pineal organ melatonin synthesis were found and they paralleled those observed in plasma after short- (6 h) and long-term (5 days) exposure of rainbow trout to saline water. We also show that the effects of salinity on pineal melatonin synthesis are mediated by higher *aanat2* mRNA expression and AANAT2 enzyme activity. Thus, the enzymatic action that typically controls light-induced daily changes in melatonin synthesis also appears to integrate information about the saline environment. Moreover, increased pineal organ melatonin synthesis in response to elevated external salinity was found during both day and night, suggesting that there is no interference with

cellular mechanisms mediating the cyclical light–dark, inhibition–activation of melatonin formation in pineal cells. More research is needed to clarify the role of the pineal gland in osmoregulatory and/or stress hormones that are mobilized during fish acclimation to changes in environmental salinity.

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	5-hydroxytryptamine. Serotonin
AANAT	arylalkylamine *N*-acetyltransferase
BW	brackish water
FW	freshwater
HPLC	high performance liquid chromatography
SW	seawater

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