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ORIGINAL PAPER

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Day–night specific binding of 2-[^{125}I]Iodomelatonin and melatonin content in gill, small intestine and kidney of three fish species

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Abstract Some of melatonin's (Mel) well-established physiological effects are mediated via high-affinity cell-membrane receptors belonging to the superfamily of G-protein-coupled receptors. Specific binding of ligand 2-[^{125}I]iodomelatonin, using membrane preparations from osmoregulatory tissues of flounder, rainbow trout and sea bream, together with Mel concentrations in the tissues and plasma were studied. The kidney, gill and small intestine samples were collected during the day and at night. The dissociation constants (K_d) and maximal binding densities (B_{max}) were calculated for each tissue at 11:00 and 23:00 h. The binding sites with K_d values in the tissues in the picomolar range indicated the high affinity. K_d and B_{max} values were tissue- and species-dependent. The GTP analogue [Guanosine 5′-*O*-(3-thiotriphosphate)] treatment significantly reduced the B_{max} value, indicating that the 2-[^{125}I]iodomelatonin-binding sites are probably coupled to a G-protein. No daily variations in K_d and B_{max} values were observed. These are the first studies of the presence of 2-[^{125}I]iodomelatonin-binding sites in the small intestine, kidney tubule and gill of fish. The data strongly suggest new potential targets for Mel action and the influence of Mel on water/ion balance in fish. The intestine seems to be a site of Mel synthesis and/or an active accumulation of the hormone.

Keywords Melatonin · Osmoregulation · Fish · [^{125}I]Iodomelatonin binding · Osmoregulatory tissues

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Abbreviations Mel: Melatonin · SPE: Solid-phase extraction · K_d: Dissociation constant · B_{max}: Maximal binding density · HPLC: High-performance liquid chromatography · GTPγS: Guanosine 5′-*O*-(3-thiotriphosphate) · GIT: Gastrointestinal tract · RIA: Radioimmunoassay · SB: Specific binding · SEM: Standard error of the mean

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine; Mel) synthesized in the pineal gland and retina is a potent regulator of circadian and seasonal rhythms in vertebrates, including fish (Ekström and Meissl 1997; Falcon 1999; Reiter 1993). In all species examined, plasma Mel concentration shows a diurnal rhythm, with the higher levels during the dark period (Binkley 1988; Reiter 1991, 1993). This small molecule can penetrate all tissues easily, thus its presence in many organs is expected. However, the presence of Mel in tissues at higher concentrations than would be expected as a result of normal distribution, may suggest an active uptake and/or a local synthesis of this indoleamine and its action here. In fact, Mel seems to be a multifaceted molecule influencing many physiological functions and behaviors (Binkley 1988; Reiter 1991; Vanecek 1998). There are growing data on new Mel functions and new potential targets of Mel action in organisms (Macchi and Bruce 2004; Barrenetxe et al. 2004). Some of the well-established physiological effects are mediated via high-affinity cell-membrane receptors belonging to the superfamily of G-protein-coupled receptors (Stankov et al. 1993; Niles 1997; Reppert 1997). These receptors have been identified and characterized in several tissues and organs by in vitro autoradiography and conventional binding assays using [^{125}I]iodomelatonin as a ligand. High-affinity 2-[^{125}I]iodomelatonin-binding sites were detected at first in the central nervous system and shortly afterward also in several peripheral mammalian and avian tissues, such as lymphocytes,

caudal artery, Harderian gland, adrenal gland, spleen, gut, gonads and kidney (Stankov et al. 1993).

In a previous work by Kulczykowska (2001), the first suggestion for the presence of 2-[^{125}I]iodomelatonin-binding sites in fish gill and kidney was provided and the potential action of Mel in the major organs responsible for osmoregulation in fish was discussed. In this study, we have examined the distribution of the specific binding (SB) of ligand 2-[^{125}I]iodomelatonin in the membranes of kidney, gill and small intestine, together with Mel concentration in these osmoregulatory organs. The experiments were carried out in freshwater and seawater fish species, i.e., rainbow trout (*Oncorhynchus mykiss*), flounder (*Platichthys flesus*) and sea bream (*Sparus aurata*) during the day and at night.

Materials and methods

Animals and sampling

Rainbow trout (*O. mykiss*) (250–400 g) of both sexes were the progeny of single-parent spawning. Two weeks prior to the experiment, the acclimation procedure was carried out. Fish were kept in freshwater-holding tanks at 10–14°C under natural photoperiod of 10 L:14 D. Three days before experimentation, the photoperiod regime was switched to the controlled illumination of 12 L:12 D (lights on at 08:00, lights off at 20:00). Blood and tissues samples were taken at the time of sacrifice twice a day: at 11:00 h ($n=6$) and 23:00 h ($n=6$). Blood samples for Mel were collected from the dorsal aorta of decapitated, unanesthetized fish. Plasma was separated by centrifugation at 10,000*g* for 5 min and stored at −70°C prior to analysis.

Flounder (*P. flesus*) (300–550 g) of both sexes were kept in seawater-holding tanks at the University of Manchester at 7–10°C under the natural photoperiod of 11 L:13 D. The duration of the acclimation period in the laboratory was approximately 1 month. For at least a period of 3 days before experimentation, fishes were held under controlled illumination of 12 L:12 D (lights on at 08:00, lights off at 20:00). Blood and tissues were sampled at time of sacrifice twice a day at 11:00 and 23:00 h. Blood samples for Mel were collected by a needle puncture of the unanesthetized fish. After blood centrifugation at 13,000*g* for 5 min, the plasma was rapidly frozen and stored at −70°C prior to analysis.

Sea bream (*S. aurata*) (300–500 g) were kept in seawater-holding tanks at Marine Station of the University of Algarve at 19°C under controlled illumination of 12 L:12 D (lights on at 08:00, lights off at 20:00) for at least 2 weeks prior to the experiment. Blood and tissues were sampled at the time of sacrifice twice a day at 11:00 and 23:00 h. Blood samples for Mel were collected by a needle puncture of the fish anesthetized in a seawater solution of MS-222. After blood centrifugation at 10,000*g* for 5 min, the plasma was rapidly frozen and stored at −70°C prior to analysis.

Tissue samples (gill, kidney and small intestine) for Mel content and SB of the ligand 2-[^{125}I]iodomelatonin were rapidly frozen in liquid nitrogen and stored at −70°C prior to homogenization/sonification and analysis. In flounder and rainbow trout, the kidney tubules were prepared. The homogenization (small intestine, gill) or sonification (kidney, kidney tubule) were performed in 0.05 M phosphate buffer containing 0.01% Thimerosal (Sigma, St Louis, MO, USA). After homogenate centrifugation at 15,000*g* for 20 min, the supernatants were collected and assayed for Mel and protein.

Melatonin analysis

Plasma Mel in rainbow trout was measured by high-performance liquid chromatography (HPLC). Mel was extracted from plasma (1 ml) by solid-phase extraction (SPE) using the C_{18} Bakerbond cartridges (J.T. Baker, Phillipsburg, NJ, USA; pore size: 60 Å, particle diameter: 40 μm). The sample was eluted with methanol. The separation and detection were performed with the Beckman modular HPLC system (Beckman Instruments, San Ramon, CA, USA), connected to a Shimadzu spectrofluorometric detector RF-551. Chromatographic isocratic separations were carried out on the same Ultrasphere C_{18} column (ID: 250×4.6 mm; particle diameter: 5 μm, pore size: 80 Å) connected to a precolumn (45×4.6 mm I.D.) filled with the same material, both obtained from Beckman Instruments. The mobile phase was 60% HPLC-grade methanol. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. The detection limit was 3 pg/ml of plasma. The inter- and intra-assay coefficients of variation were 14% ($n=9$) and 10% ($n=9$), respectively (see: Kulczykowska and Iuvone 1998). Synthetic Mel (mol. wt. 232.3) for calibration curve was obtained from Sigma Chemical (St Louis, MO, USA).

Plasma Mel in sea bream and flounder was assayed using total Mel radioimmunoassay (RIA) kit (IBL, Hamburg), without the preceding extraction procedure, but with enzymatical pre-treatment. Samples were assayed in duplicate. The detection limit was 3 pg/ml of plasma. The intra- and inter-assay coefficients of variation for Mel were 8.0 and 15.0%, respectively. The assay was validated by HPLC assay (Kulczykowska and Iuvone 1998).

Mel concentrations in tissues were determined by RIA using total Mel kit with preceding extraction procedure modified for tissues. SPE of Mel from the supernatant (100 μl) was carried out on an Octadecyl C_{18} Speedisk Column, 10 μm (J.T. Baker, Phillipsburg, NJ, USA). Samples were eluted with methanol according to a previous procedure described for Mel extraction. After extraction, dried samples were resuspended in Dulbecco's phosphate buffered saline containing 0.01% Thimerosal and assayed using the RIA kit without enzymatic pre-treatment. All samples in duplicate were counted in a Wallac Wizard γ-counter. The detection limit was 3.5 pg/ml of the extract. The intra- and

inter-assay coefficients of variation for Mel were 8.5 and 14.8%, respectively. Mel concentration was expressed as a femtomole per gram of wet tissue (small intestine, gill and kidney) or femtomole per milligram of protein (kidney tubule). Protein was determined by the Lowry method with Peterson's (1977) modification using Total Protein kit (Sigma, St Louis, MO, USA).

Binding of ligand 2-[^{125}I]Iodomelatonin in tissues

The tissues (about 10 mg of wet weight) were homogenized or sonicated in 2 ml of ice-cold 50-mM Tris–HCl of pH 7.4 with 4 nM $CaCl_2$ buffer. The homogenate was centrifuged at 10,000–15,000*g* for 20 min at 4°C. The resulting pellet was rehomogenized in the same buffer and centrifuged again. For the assay, 100-µl aliquots of the membrane preparations (final pellets resuspended in the buffer) were incubated with various concentrations of 2-[^{125}I]iodomelatonin (3–250 pM; 2,000 Ci/mmol, Amersham, UK) in the presence or absence of 50 µM non-labeled Mel as a competing agent. The binding was measured in triplicate after incubation on ice (at about 4°C) for 60 min. In kinetic studies, incubation times varied from 5 to 90 min. Membranes were collected by centrifugation. The tip of the microcentrifuge tube with membrane preparation was cut off, then placed in the vial and the radioactivity was measured in a Wallac Wizard γ-counter. SB was calculated as the difference between total binding and non-specific binding (not displaced in the presence of 50 µM non-labeled Mel). SB reached a plateau at approximately 100–120 pM. Thus, 2-[^{125}I]iodomelatonin-binding sites were saturated at 150 pM.

The binding affinities (K_d) and binding densities (B_{max}) were calculated according to a Scatchard analysis. The values of correlation coefficient r for Scatchard plot and Hill coefficient for Hill plot were determined.

Statistical analysis

The obtained values are presented as means ± SEM. For multiple comparisons, the analysis of variance was used. Significant differences between means for paired sample studies were identified using Student's paired t-test. Significance was taken at $P < 0.05$. Significant differences between means for non-paired sample studies were identified using Tukey's test. Significance was taken at $P < 0.05$.

Results

Plasma-Mel concentrations measured in three fish species at 11:00 and 23:00 h are presented in Fig. 1. Mel levels at night were significantly higher than those during the day in all fish ($P < 0.001$).

Mel concentrations in gill, small intestine and kidney (or kidney tubules) in the three fish species at 11:00 and 23:00 h are presented in Fig. 2a–c. No significant day–night differences in Mel concentrations in any tissues were observed except for the gill of sea bream ($P < 0.05$). The highest Mel concentrations were measured in the small intestine in all fish. Mel concentrations in the gill and small intestine in sea bream were significantly higher compared to the other two species ($P < 0.001$).

The binding affinities (K_d) and maximal binding densities (B_{max}) in the tissues at 11:00 and 23:00 h are presented in Figs. 3a–c and 4a–c, respectively. There were no daily variations in the K_d and B_{max} values. The linearity of Scatchard plots (r-values ranging from 0.89 to 0.97) and the unity of Hill coefficients (ranging from 0.91 to 1.10) for all the tissues tested, strongly suggested presence of a single class of binding sites (Table 1). K_d and B_{max} values were evidently tissue- and species-dependent.

To check the potential interaction of 2-[^{125}I]iodomelatonin-binding sites with G-protein, the effect of the non-hydrolyzable GTP analogue [(GTPγS—guanosine 5′-*O*-(3-thiotriphosphate); Sigma, St Louis, MO, USA] on ligand binding was examined. The 5×10^{-4} M GTPγS added to the incubation medium reduced significantly the B_{max} of 2-[^{125}I]iodomelatonin in the tissues studied by 30–50% ($P < 0.001$; Table 2), though it was ineffective in the case of K_d (data not shown).

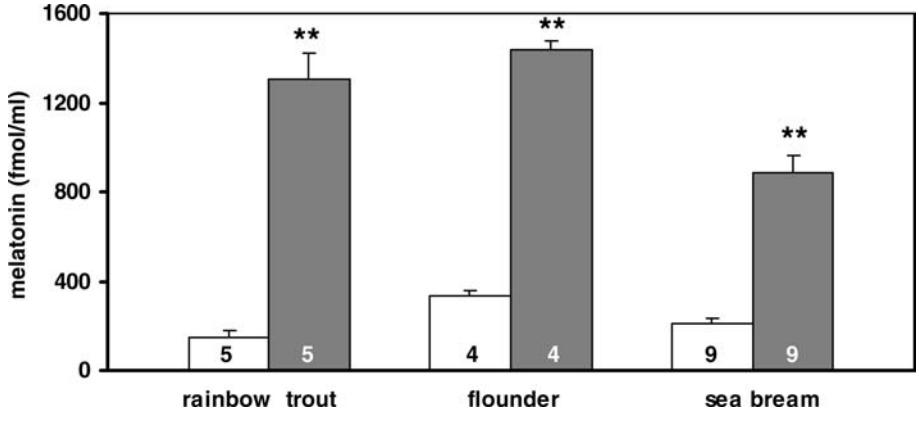

Fig. 1 Plasma-melatonin concentrations in samples taken at 11:00 and 23:00 h in three fish species. *Dark bars* indicate night (23:00 h). Number of fish is given in *bars*. **$P < 0.001$, for comparison of light and dark measures (Tukey's test)

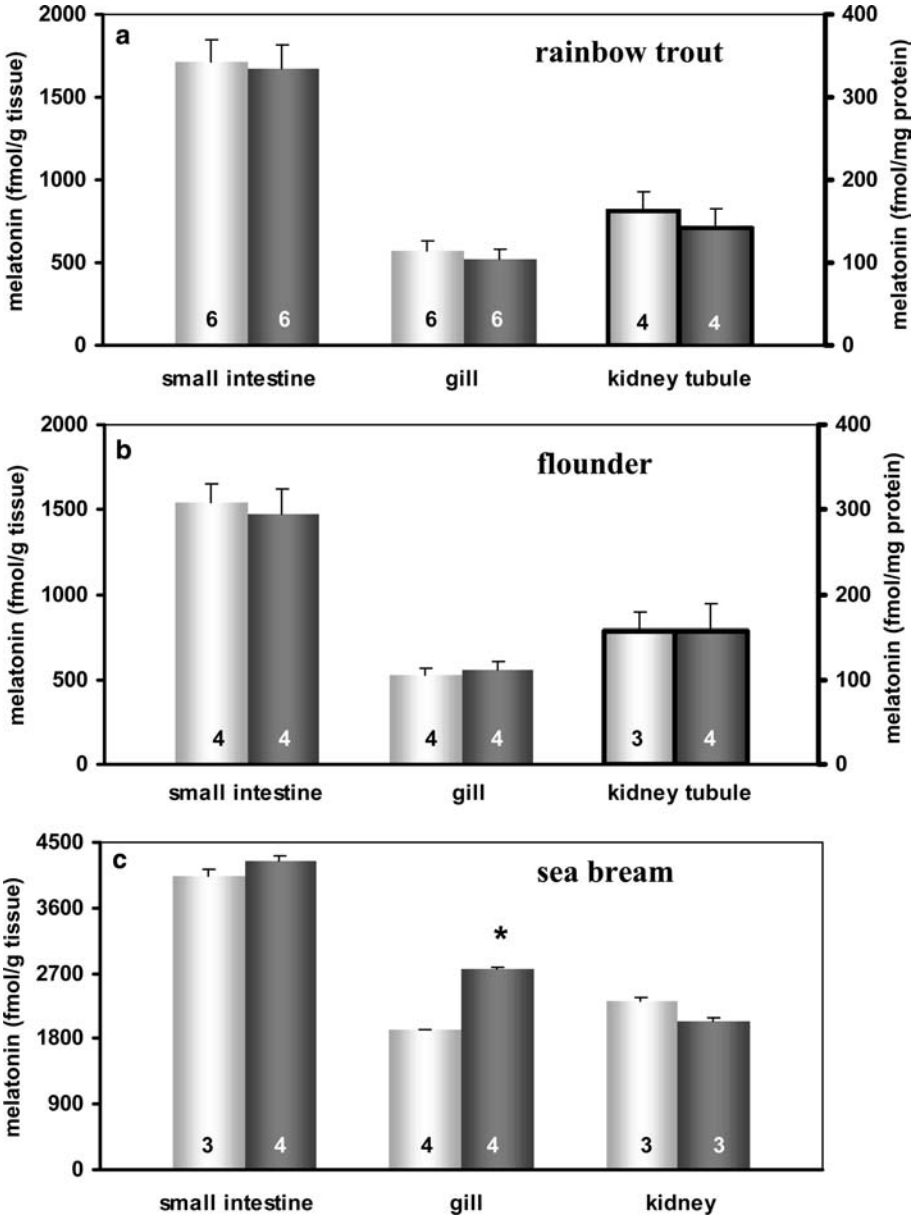

Fig. 2 Melatonin concentrations in tissues taken at 11:00 and 23:00 h from rainbow trout (**a**), flounder (**b**) and sea bream (**c**). *Dark bars* indicate night (23:00 h). Number of fish is given in *bars*. $*P < 0.01$, for comparison of light and dark measures (Tukey's test)

Discussion

This study provides the first evidence for the presence of 2-[^{125}I]iodomelatonin binding to membrane preparations from fish osmoregulatory tissues: gill, small intestine and kidney or kidney tubules. The possibility of Mel action in the fish gill and kidney has been already discussed (Kulczykowska 2001). The Mel-binding sites in the peripheral organs are reported in mammals and birds (Naji et al. 2004; Song et al. 1996; Witt-Enderby et al. 2003; Drew et al. 2001; Liu and Pang 1993). However, the reports of indoleamine-binding sites in fish are limited to the brain (Iigo et al. 1994, 1997; Gaildrat et al. 1998; Amano et al. 2003; Bayarri et al. 2004; Ekström and Vanecek 1992; Falcon et al. 1996), except one, to the authors knowledge, which concerns the fish heart (Pang et al. 1994).

To gain more information on the peripheral action of Mel in fish, we have measured for the first time both 2-[^{125}I]iodomelatonin binding and Mel concentrations during the night and day in three tissues known to play an active role in osmoregulation, namely the gill, small intestine and kidney. The K_d and B_{max} values obtained herein in the three fish species are within the range of [^{125}I]iodomelatonin binding shown in many mammalian and avian tissues (Song and Pang 1992; Stankov et al. 1993; Song et al. 1993, 1995, 1997). The differences in K_d and B_{max} between various tissues and species, a well-known phenomenon in mammals and birds (Song and Pang 1992; Stankov et al. 1993), has also been reported in this study for fish. The K_d values, within the

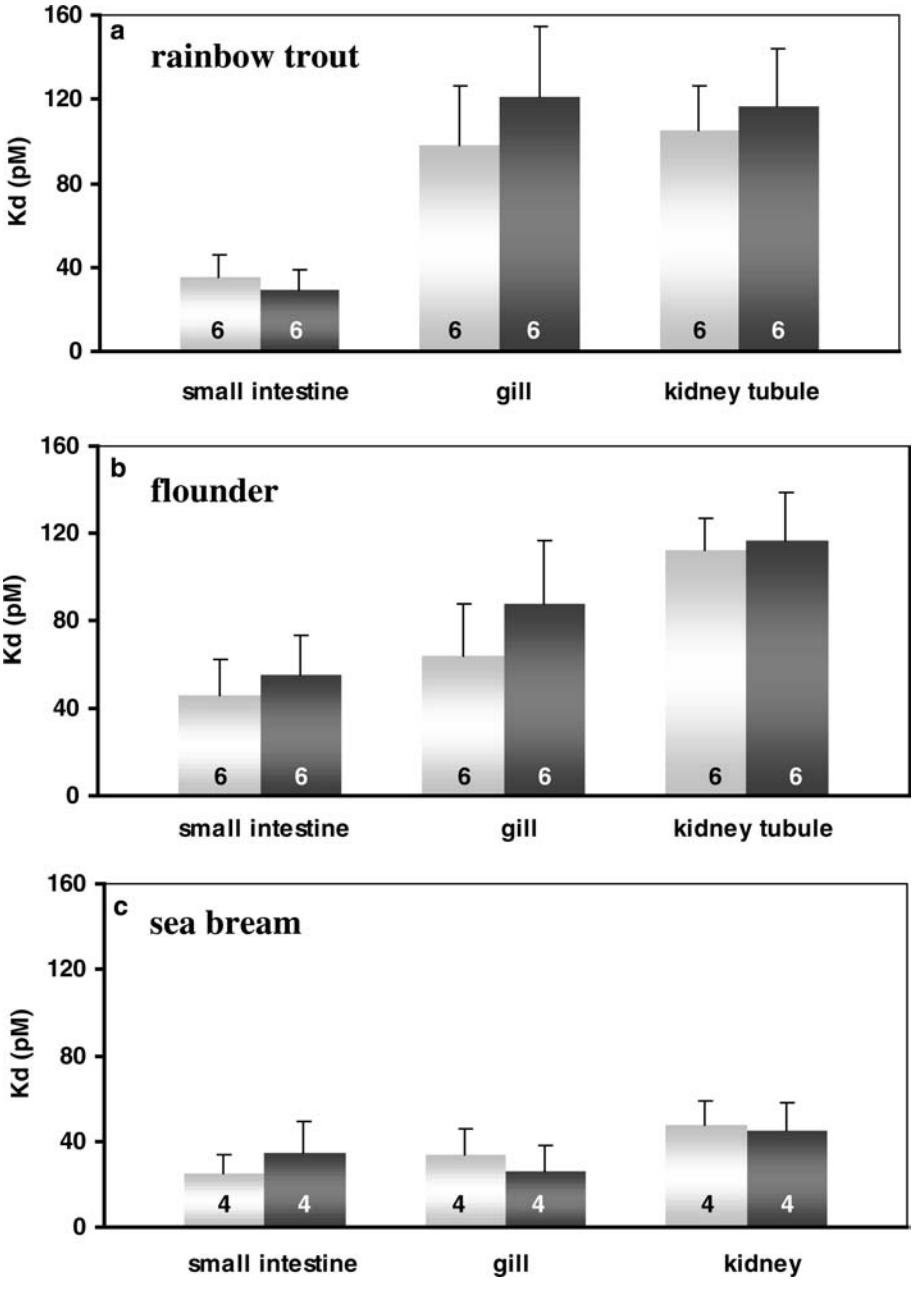

Fig. 3 The dissociation constants (K_d) of 2-[^{125}I]iodomelatonin in tissues taken at 11:00 and 23:00 h from rainbow trout (**a**), flounder (**b**) and sea bream (**c**). *Dark bars* indicate night (23:00 h). Number of fish is given in *bars*

picomolar range, indicated the high affinity of 2-[^{125}I]iodomelatonin bindings in all organs examined. The observation that GTPγS significantly reduces the specific binding of 2-[^{125}I]Iodomelatonin to the receptors strongly suggests that the 2-[^{125}I]iodomelatonin-binding sites are associated with a G-protein (at least in the two tissues examined, i.e., gill and small intestine in rainbow trout and flounder). The linearity of Scatchard plots and the unity of Hill coefficients for all the tissues examined strongly suggested presence of a single class of binding sites. In all the tissues, the day- and night-time concentrations of Mel which appear to be higher than K_d values, indicates that Mel is potentially able to exert an influence there.

The presence of Mel in tissues at higher concentrations than would be expected as a result of normal distribution, which is shown in this study, suggests a local production of this indoleamine. The remarkably high Mel concentrations in the tissues of sea bream, in particular, point to this possibility. In our studies, rainbow trout, flounder and sea bream were kept under the same lighting regime, but at different water temperatures, ranging from 7 to 19°C, with sea bream at the highest. It is well-established that in addition to light, temperature is also involved in the control of Mel production in poikilotherms. Mel is derived from serotonin by two enzymatic reactions: *N*-acetylation of serotonin catalyzed by serotonin-*N*-acetyltransferase (arylalkylamine-*N*-acetyl-

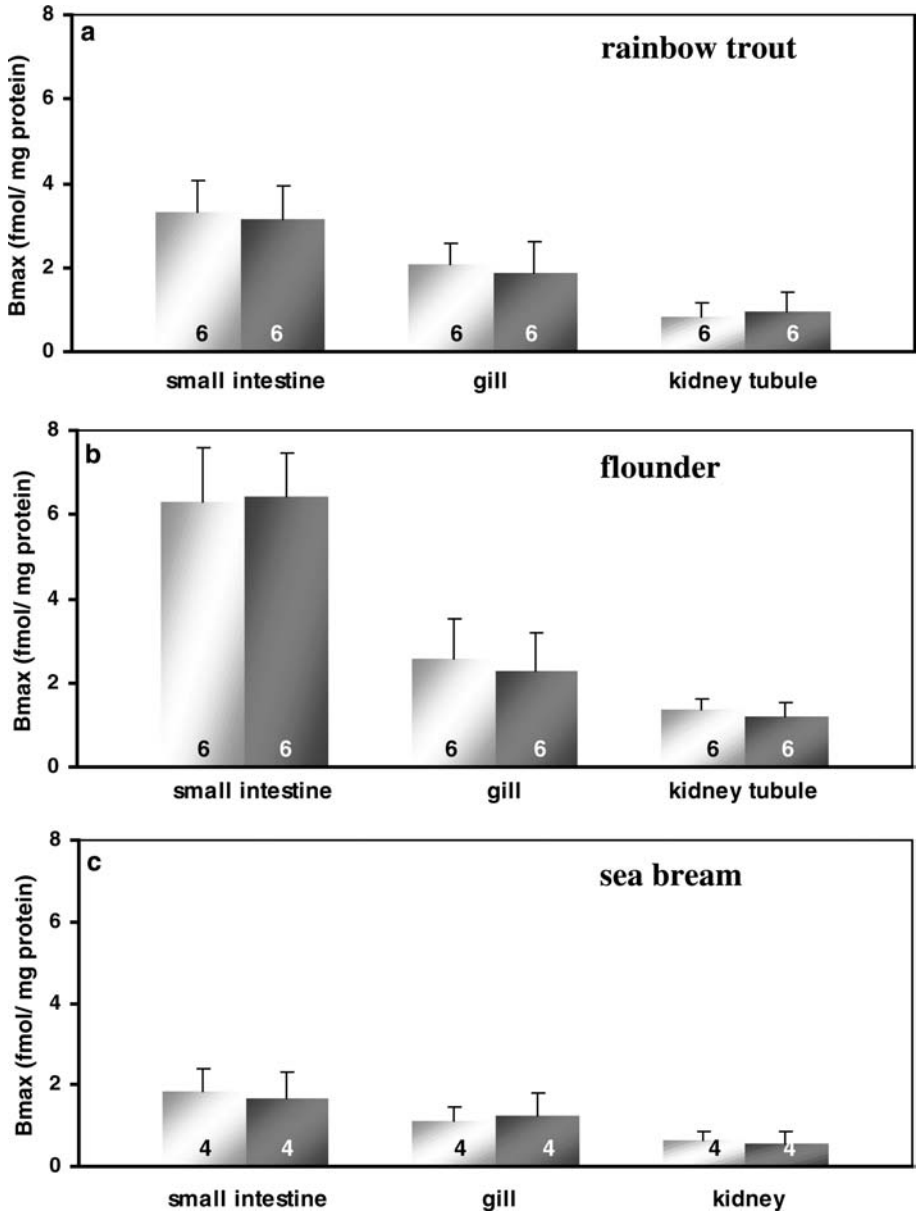

Fig. 4 The maximal binding densities (B_{max}) of 2-[^{125}I]iodomelatonin in tissues taken at 11:00 and 23:00 h from rainbow trout (**a**), flounder (**b**) and sea bream (**c**). *Dark bars* indicate night (23:00 h). Number of fish is given in *bars*

Table 1 Correlation coefficients (*r*-value) to Scatchard plots and Hill coefficients to Hill plots calculated for the tissues taken at 11:00 h (D) and 23:00 h (N) from rainbow trout, flounder and sea bream

	Rainbow trout		Flounder		Sea bream	
	D	N	D	N	D	N
Small intestine						
Hill coefficient	1.02	1.00	0.99	0.95	0.98	0.99
r-Value	0.95	0.96	0.96	0.94	0.94	0.90
Gill						
Hill coefficient	1.03	1.04	1.10	1.08	1.06	1.10
r-Value	0.93	0.95	0.93	0.91	0.90	0.89
Kidney						
Hill coefficient	1.00	0.97	1.04	1.01	0.91	0.93
r-Value	0.95	0.97	0.93	0.97	0.95	0.93

transferase, AANAT) and O-methylation of *N*-acetylserotonin catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT). In several fish species, including rainbow trout and sea bream, the AANATs demonstrate temperature–activity relationships (Coon et al. 1999; Zilberman-Peled et al. 2004). The maximal activities

Table 2 The effect of the GTP analogue [GTPγS—guanosine 5′-*O*-(3-thiotriphosphate)] on specific binding of ligand 2-[^{125}I]iodomelatonin in selected fish tissues collected at 11:00 h

Tissue	B_{max} (fmol/mg protein)	
	Control ($n=5$)	$+5\times10^{-4}$ M GTPγS ($n=4$)
Rainbow trout small intestine	3.3 ± 0.7	1.7 ± 0.4**
Rainbow trout gill	2.1 ± 0.5	1.3 ± 0.4*
Flounder small intestine	6.3 ± 1.3	3.3 ± 0.5**
Flounder gill	2.6 ± 0.9	1.8 ± 0.4*

Values are means ± SEM
*$P<0.01$ (Student's paired *t*-test)
**$P<0.001$ (Student's paired *t*-test)

occurred at 18–37°C. Moreover, the activity of HIOMT, the second important enzyme in the Mel synthesis pathway, increases along with an increase of incubation temperature from 5 to 40°C in rainbow trout (Morton and Forbes 1989). Therefore, the high Mel concentrations observed in sea bream in this study are probably a function of temperature.

In all three fish species, the Mel concentrations in the small intestine were significantly higher than those in plasma, most of all during the day. This is likely explained by the local production of the indoleamine. This fact is known in gastrointestinal tract (GIT) in mammals and birds (Van't Hof and Gwinner 1999; Kvetnoy et al. 2002; Martin et al. 1998; Messner et al. 2001; Bubenik 2002) and probably, also in reptiles, amphibians and fish (Bubenik and Pang 1997). Moreover, in the mammalian GIT, an additional accumulation of Mel from the circulation is postulated (Messner et al. 1998). That may be the case also in fish. The Mel concentration in the small intestine does not display any rhythmicity in fish, being in accordance with the data in other vertebrates (Van't Hof and Gwinner 1999; Bubenik and Pang 1997; Bubenik 2002). This indicates that any Mel production in the intestinal tissue is independent of the time of the day. The small intestine is probably an important extrapineal and extraretinal source of indoleamine, which is present in plasma also during the day when its synthesis in the pineal organ and retina is inhibited. The high Mel content together with the highest binding densities observed in the small intestine, strongly suggest that at this site, Mel plays an important role in fish. It is in accordance with many records on Mel activity in mammalian GIT (Kvetnoy et al. 2002; Martin et al. 1998; Bubenik 2002).

In this study, we have also reported the SB of 2-[^{125}I]iodomelatonin in fish gill and kidney or kidney tubule membranes. The SB of [^{125}I]iodomelatonin to kidney and kidney tubules in fish is in agreement with the results from studies in mammals and birds (Song et al. 1993, 1995, 1996, 1997; Song and Pang 1992). Mel concentrations in the gill and kidney of sea bream are above the highest plasma level of Mel at night. This may suggest an active uptake of the indoleamine from the circulation, probably for clearance. However, in higher vertebrates, about 90% of Mel released into the blood is thought to be enzymatically converted to 6-OH-melatonin and subjected to renal clearance as sulfated or glucuronidated conjugates (Binkley 1988). We can only speculate that in fish, Mel is accumulated and then excreted in the non-converted form in gill and kidney. The gill-Mel content in sea bream shows a significant day–night difference, thus, the rates of the gill active uptake and clearance of Mel would be proportional to the plasma-hormone concentration. This is not the case in rainbow trout and flounder gills, where Mel concentration does not change during the day and Mel night level is lower than that in plasma. In the kidney, however, an uptake of Mel by renal tubules, evident in the rainbow trout and flounder tubules preparations, seems to be intense during the whole day.

It is generally accepted that Mel conveys photoperiodic information to the peripheral organs affecting their daily rhythms, thus, a defined Mel profile is important for mediating these signals. In our study, in spite of the marked diurnal changes in plasma-Mel, observed in all fish, there are no day–night changes in Mel concentrations in the tissues, except the gill in sea bream. The density of Mel binding is known to fluctuate during the day in the brains of fish, i.e., pike, goldfish, sea bream, catfish and salmon (Iigo et al. 1994, 1997; Gaildrat et al. 1998; Amano et al. 2003; Bayarri et al. 2004), but there are no published data on day–night Mel binding in the peripheral organs in fish. In all the tissues examined in our study, neither the binding affinity nor the binding density exhibited any day–night changes. This would indicate that the 2-[^{125}I]iodomelatonin-binding sites are not under circadian control and consequently, the potential Mel action in the osmoregulatory tissues is not photoperiod-dependent. This finding differs from that reported in many mammals and birds (Rafii-El-Idrissi et al. 1996; Song and Pang 1992; Liu and Pang 1993), which may suggest a distinct character of Mel action in the peripheral tissues of fish.

Taken together, our findings of the presence of the specific 2-[^{125}I]iodomelatonin binding in the gill, small intestine and kidney (kidney tubules) suggest that Mel can be considered as a modulatory factor in these organs in fish. The small intestine is probably a site of Mel synthesis and/or its active accumulation from the circulation. The results have also pointed to the possibility of Mel uptake and excretion in the non-converted form in the gill and kidney in some fish species. Considering that the binding of an agent to an organ speaks for its action at this site, a physiological role for Mel in osmoregulatory organs in fish is supported and will be explored in future studies.

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