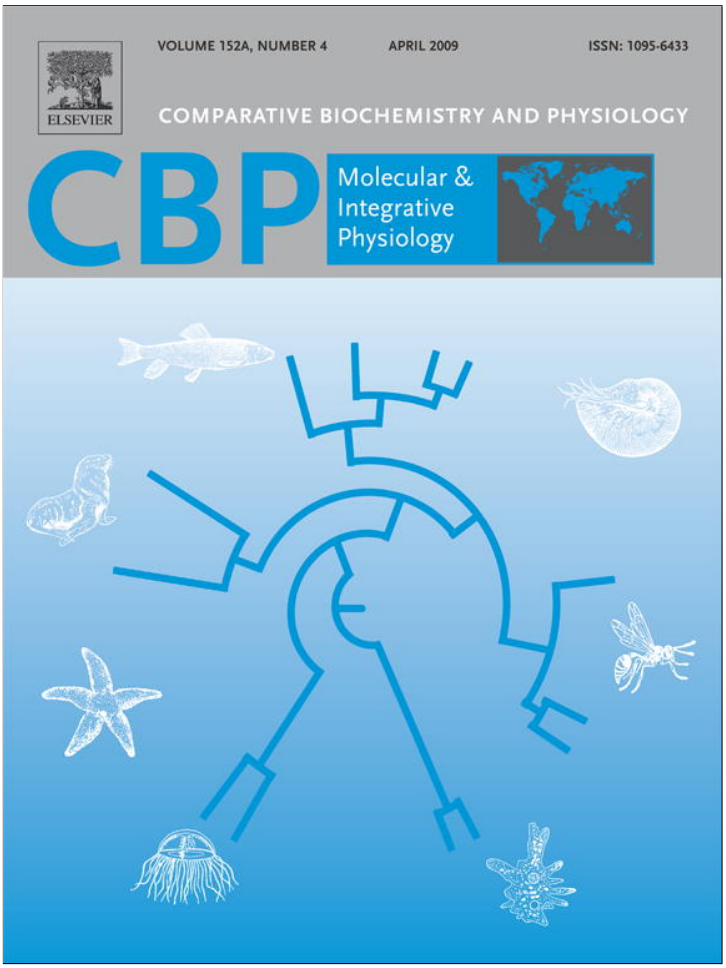

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Effects of water salinity on melatonin levels in plasma and peripheral tissues and on melatonin binding sites in European sea bass (*Dicentrarchus labrax*)

J.F. López-Olmeda [a,*], C. Oliveira [a], H. Kalamarz [b], E. Kulczykowska [b], M.J. Delgado [c], F.J. Sánchez-Vázquez [a]

[a] Department of Physiology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain
[b] Department of Genetics and Marine Biotechnology, Institute of Oceanology of Polish Academy of Sciences, 81-720 Sopot, Poland
[c] Department of Physiology (Animal Physiology II), Complutense University, 28040 Madrid, Spain

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ABSTRACT

Sea bass is an euryhaline fish that lives in a wide range of salinities and migrates seasonally from lagoons to the open sea. However, to date, the influence of water salinity on sea bass melatonin levels has not been reported. Here, we evaluated the differences in plasma and tissue melatonin contents and melatonin binding sites in sea bass under four different salinity levels: seawater (36‰), isotonic water (15‰), brackish water (4‰) and freshwater (0‰). The melatonin content was evaluated in plasma, whole brain, gills, intestine and kidney, while melatonin binding sites were analyzed in different brain regions and in the neural retina. Plasma melatonin levels at mid-dark varied, the lowest value occurring in seawater (102 pg/mL), and the highest in freshwater (151 pg/mL). In gills and intestine, however, the highest melatonin values were found in the seawater group (209 and 627 pg/g tissue, respectively). Melatonin binding sites in the brain also varied with salinity, with the highest density observed at the lower salinities in the optic tectum, cerebellum and hypothalamus (30.3, 13.0, and 8.0 fmol/mg protein, respectively). Melatonin binding sites in the retina showed a similar pattern, with the highest values being observed in freshwater. Taken together, these results reveal that salinity influences melatonin production and modifies the density of binding sites, which suggests that this hormone could play a role in timing seasonal events in sea bass, including those linked to fish migration between waters of different salinities for reproduction and spawning.

1. Introduction

Melatonin (Mel) is the main product synthesized by the pineal organ of vertebrates, including fishes (Ekström and Meissl, 1997). In all species studied to date, Mel peaks mainly during the dark phase of the daily photocycle, while low levels are observed during the light phase (Falcón, 1999). This hormone is secreted into the blood and provides the organism with information on calendar time including time of the day and season throughout the year (Reiter, 1993). Therefore, this conserved signal is likely to regulate the daily and seasonal rhythms in vertebrates. Besides the pineal organ, Mel is produced in other tissues such as the retina and the intestine, where this hormone has been suggested to play a paracrine role (Iigo et al., 1997; Tosini, 2000; Bubenik, 2002). In addition, Mel acts on a wide variety of processes in fish, including food intake and locomotor activity (López-Olmeda et al., 2006), metabolism (Delahunty and Tomlinson, 1984) and the regulation of neuroendocrine factors (Falcón et al., 2007), the dopaminergic system and reproduction (Sébert et al., 2008).

Sea bass (*Dicentrarchus labrax*, L.) are euryhaline fish capable of living in a wide range of salinities, from highly saline to freshwater, as well as in environments that are subjected to variations in salinity, such as estuaries (Chervinsky, 1974), thus they have developed physiological strategies to adapt to such variations (Jensen et al., 1998). Indeed, sea bass usually undergo seasonal migrations that involve changes in salinity: mating and spawning occur in the open sea during autumn and winter, while fish move to tidal lagoons and estuaries in spring to exploit food resources (Lemaire et al., 2000; Varsamos et al., 2001; Pawson et al., 2007). Migration seems to be usual for Mediterranean Sea (Lemaire et al., 2000) and Atlantic (Pawson et al., 2007) European sea bass, and the species can be considered a diadromous, spawning in the sea and growing in freshwater, in a similar way to the European eel (van Ginneken et al., 2005). In contrast, anadromous species such as salmonids, spawn seasonally in freshwater and juveniles migrate to the open sea to continue growing (Klemetsen et al., 2003). Seasonal environmental factors that influence Mel production have been studied in sea bass, with special attention paid to the effects of photoperiod and water temperature (García-Allegue et al., 2001), and light intensity and spectrum (Bayarri et al., 2002). This species shows an inverse rhythm of Mel production in the retina, with high levels during the daytime and low levels at nighttime (Iigo et al., 1997).

Mel actions are mediated through high and low affinity receptors. Previous studies have characterized high affinity receptors belonging

* Corresponding author. Tel.: +34 968 364931; fax: +34 968 363963.
E-mail address: jflopez@um.es (J.F. López-Olmeda).

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to the superfamily of G-protein coupled receptors (Vanecek, 1998). Several subtypes of Mel receptors have been identified, including the MT1 and MT2 subtypes described in all vertebrates investigated to date, and the Mel 1c subtype, which has only been found in non-mammalian vertebrates (Witt-Enderby et al., 2003). The widespread distribution of Mel receptors in central and peripheral tissues has been described, the highest densities occurring in the central nervous system (Falcón et al., 2007). In fish, Mel binding sites in the brain and retina may show a daily rhythm in density and/or affinity, depending on the brain area and the species (Iigo et al., 2003; Bayarri et al., 2004b; Park et al., 2007). Nevertheless, no study has focused on the possible influence of salinity on Mel receptors in the fish nervous system.

The aim of this study was to evaluate the influence of water salinity on Mel concentration in plasma and several tissues (brain, gills, intestine and kidney), and the possible variations in the density of Mel binding sites in the central neural tissues of sea bass exposed to four different salinities, ranging from seawater to freshwater.

2. Materials and methods

2.1. Animals and housing

A total of 64 European seabass (*Dicentrarchus labrax*, Moronidae, Perciformes)were obtained from the Spanish Institute of Oceanography at Mazarrón (Murcia, Spain) and reared at the facilities of the University of Murcia. Fish had an average body mass of 117 ± 37 g (b.w.; mean ± SD) and were kept in well aerated 500-L tanks in a recirculating system equipped with biological and mechanical filters, and an U.V. lamp to sterilize the water before returning to the tanks. A total of 64 fish were used in the experiments. Fish were divided into 4 groups of 16 fish each, thus obtaining a fish density of 4 kg/m^3. Fish were acclimated to lab conditions during May 2005, and the experiments were performed in June 2005. Water temperature was controlled at 23 °C and the photocycle was set at 12 L:12D. Light was provided by "daylight" bulbs (Decor A 60 W, Osram) placed at 70 cm from the water surface, where light intensity was 300 lx. Fish were fed *ad libitum* with a commercial diet for sea bass (Excel D4-2P, Skretting, Nutreco Holding N.V., Netherlands). During the experiment, common water quality criteria were assessed every day by means of commercial kits (Sera, Germany), ensuring that all parameters were at adequate levels for the fish (ammonium, nitrate and nitrite below 0.05 mg/L, and pH between 8.1 and 8.4).

2.2. Experimental design

The experiments were designed to evaluate the influence of decreasing water salinities on both plasma and tissue levels of Mel, and Mel receptor density in the central neural tissues of sea bass. For this purpose, fish were exposed consecutively to four salinities: 36‰ (seawater, SW); 15‰ (isotonic water, IW); 4‰ (brackish water, BW); and 0 ‰ (freshwater, FW). Isotonic salinity for sea bass was set at 15‰, as described by Saillant et al. (2003). Fish were reared and manipulated following the Spanish legislation on Animal Welfare and Laboratory Practices.

Commercial sea salt (SERA marine basic salt, Germany) was added to freshwater, filtered previously throughout an active carbon filter, to reach the desired salinity of 36‰ in the lab facilities where the experiments were performed. Fish were transferred from an open system with running seawater to the laboratory several weeks prior to the experimental phase to allow acclimation. Then, fish were first sampled at 36‰, and again as the salinity was decreased to 15‰, 4‰ and finally 0‰. Salinity changes were made gradually over 2–3 days. When water salinity had reached the desired salinity, fish were maintained for one week in these conditions before samples were collected for analysis. According to Jensen et al. (1998), one week is enough for the full acclimation to different salinities, since this species stabilises plasma osmolality, Na^+ and Cl^- from the fourth day after transferring to a new salinity.

Fish were anaesthetized in clove essence at 50 ppm (Guinama, Valencia, Spain), blood samples were collected by caudal puncture, and fish were sacrificed by decapitation. Tissue samples from brain, intestine, gills and kidneys were collected, frozen immediately in dry ice and stored at −80 °C. For each salinity, blood samples for Mel were taken both at mid-light (ML) and mid-dark (MD) (n=4 for each point), while tissue samples for Mel analysis were collected only at ML to avoid the influence of the nocturnal increase in circulating Mel synthesized by the pineal gland. Brains were collected at ML and dissected into optic tectum, telencephalon, hypothalamus and cerebellum, and stored at −80 °C until assayed for radiobinding (n=8 for each point). The eye cup was removed at ML and MD and placed under the binocular to remove the neural retina, which was frozen until assayed.

2.3. Melatonin analysis

Samples from brain, kidney, intestine and gills were homogenized by sonication in a saline phosphate buffer with 0.01% thimerosal (Sigma Aldrich Chemicals, St. Louis, USA). Mel was extracted from plasma and tissue homogenates using octadecyl C_{18} speedisk columns of 10 µm (J.T. Baker, NJ, USA) and eluted with methanol according to a published procedure (Kulczykowska and Iuvone, 1998). Mel concentration was determined using a commercial radioimmunoassay kit (Melatonin direct RIA, RE 29301, IBL Hamburg, Germany) based on the competition principle between Mel in the sample and added ^{125}I-melatonin. The RIA assay has a sensitivity threshold of 0.9 pg/mL, and intra/inter coefficients of variation of 5.5 and 11.1%, respectively. Radioactivity was measured using a γ counter (Wallac 1470, Perkin Elmer, MA, USA). Mel concentration in tissues was expressed as picograms per gram of tissue (intestine, gill and brain) or as picograms per milligram of protein (kidney). The protein content in kidney was determined using a commercial Total Protein kit (Sigma Aldrich).

2.4. Membrane preparation and binding assays

Membranes were prepared as described by Bayarri et al. (2004b). Briefly, samples were sonicated in Tris–HCl buffer (50 mM, pH=7.4) and centrifuged before being resuspended in Tris buffer and stored at −80 °C until the binding assays were performed. Total protein concentrations in the tissues were measured by Lowry's method (Lowry et al., 1951), modified for use with microplates by reducing all the volumes to obtain a final volume of 300 µL. Binding assays were carried out in triplicate for each sample. Sample membranes (30–40 µg) were incubated with 2-[^{125}I]iodomelatonin as radioligand (GE Healthcare, Spain) at 25 °C for 90 min. The reaction was stopped at 4 °C by adding 750 µL of Tris buffer, and immediately vacuum filtered through 25 mm glass fiber filters (Millipore, APFC, USA) using a Millipore 1225 cell harvester. Filters were washed with 4 mL of Tris–HCl buffer and then radioactivity was quantified using a γ counter (Wallac 1470, Perkin Elmer). Non-specific binding was quantified by adding an excess of unlabelled Mel (1 µM) (Sigma Aldrich), and these values were subtracted from total binding to obtain the specific binding of 2-[^{125}I]iodomelatonin in each sample. The specific binding capacity was expressed as femtomoles per milligram of proteins.

2.5. Data analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using SPSS® software. Data of Mel in tissues were subjected

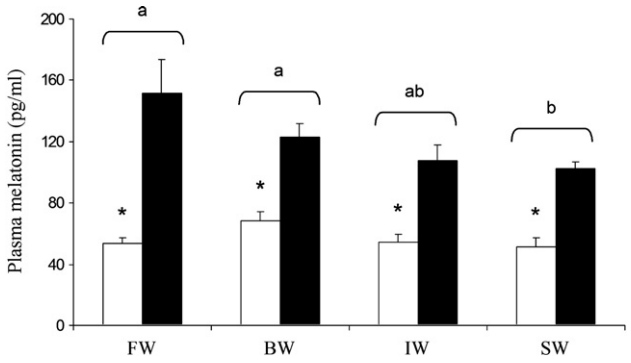

Fig. 1. Plasma Mel levels at ML (white bars) and MD (dark bars), at the four salinities, FW (freshwater, 0‰), BW (brackish water, 4‰), IW (isotonic water, 15‰), and SW (seawater, 36‰). Values are expressed as mean±SEM (n=4). Asterisks indicate statistically significant differences between ML and MD values within the same salinity, and different letters indicate statistically significant differences between salinities for ML and MD (p<0.05).

to one-way ANOVA, followed by Duncan's *post hoc* test, while data of plasma Mel levels and the density of Mel binding sites in both brain regions and the retina were subjected to two-way ANOVA, followed by Duncan's *post hoc* test. The statistical significance threshold was set at p<0.05.

3. Results

All groups showed significant differences between day and night plasma Mel levels, the highest values occurring at MD (Fig. 1). In addition, nocturnal plasma concentrations of Mel varied significantly depending on water salinity, the mean values being higher at lower salinities, FW and BW levels (151±23 and 123±9 pg/mL, respectively) showing significant differences from SW levels (102±4 pg/mL). Differences between day and night plasma Mel levels increased as salinity was reduced from SW to FW (Fig. 1). Statistical analysis of the regression lines for Mel produced during the day or at night, at the four salinities tested, revealed that MD Mel and salinity fitted significantly a linear equation with a negative relation between both factors ($y=-1.1x+136.2$, $R^2=0.66$, $p<0.05$), while ML Mel and salinity did displayed no significant regression ($y=-0.3x+60.4$, $R^2=0.29$, $p=0.2$).

As regards Mel levels in tissues (Fig. 2), significant differences were found in the intestine, where Mel levels were threefold higher in SW than in the other groups. A similar profile could be observed in gills, where Mel values were significantly higher in animals maintained in SW, although such differences were less marked than in the intestine. Neither brain nor kidney Mel levels showed significant differences at the different salinities tested.

The radioligand experiments revealed differences in binding capacities in the different brain regions, with the optic tectum showing the highest density, followed by the cerebellum, and finally the telencephalon and hypothalamus. As regards differences depending on water salinity, the optic tectum showed higher receptor density at FW than the rest of salinities (Fig. 3). The cerebellum, hypothalamus and telencephalon showed similar values in all groups (Fig. 3).

In the retina, the highest receptor density was found in FW (Fig. 4), which showed significantly higher densities of Mel binding sites than SW and IW at both ML and MD. No significant differences were observed between binding sites at ML and MD within the same salinity level (Fig. 4).

4. Discussion

Our results revealed that the sea bass Mel system is influenced not only by light or water temperature, but also by water salinity. In the present study, both circulating Mel levels and Mel binding sites in the

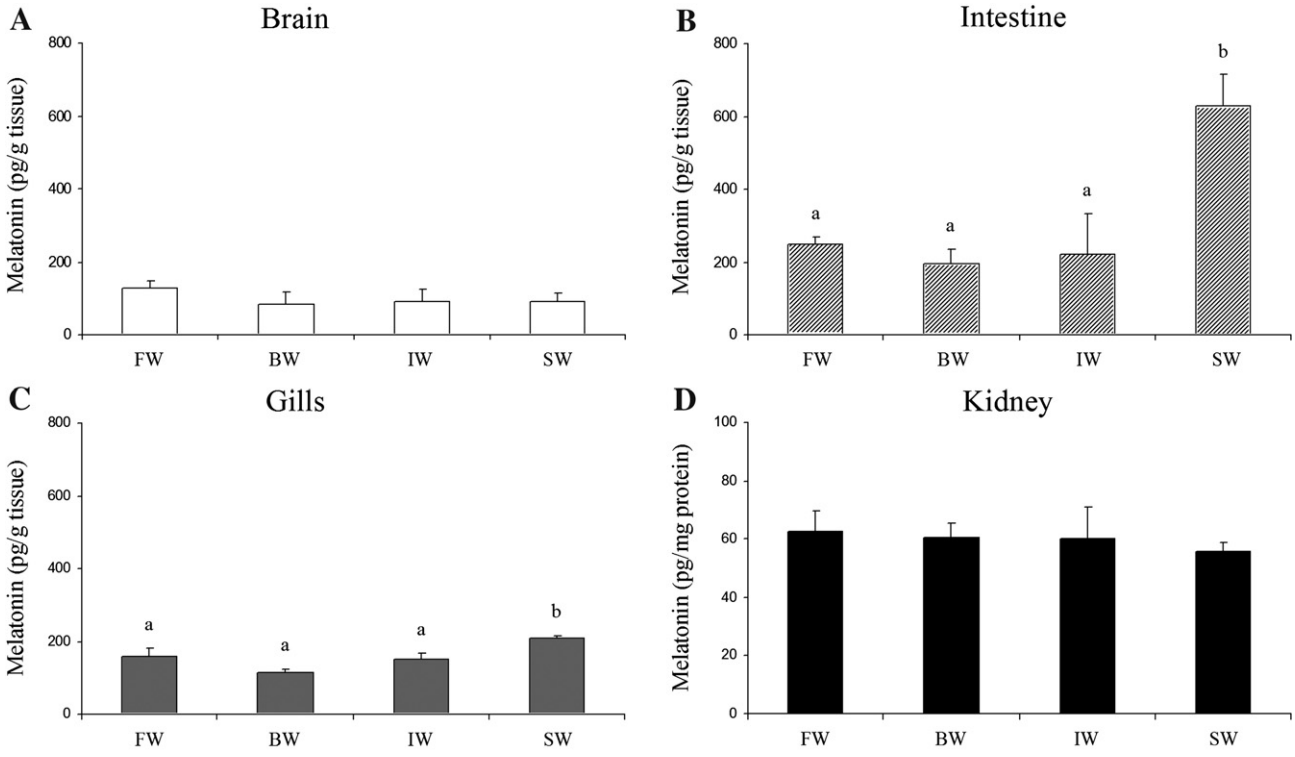

Fig. 2. Mel contents in sea bass brain (3A), intestine (3B), gills (3C) and kidney (3D), at ML at the four salinities (FW, BW, IW and SW). Values are expressed as mean±SEM (n=4). Different letters indicate statistically significant differences (p<0.05).

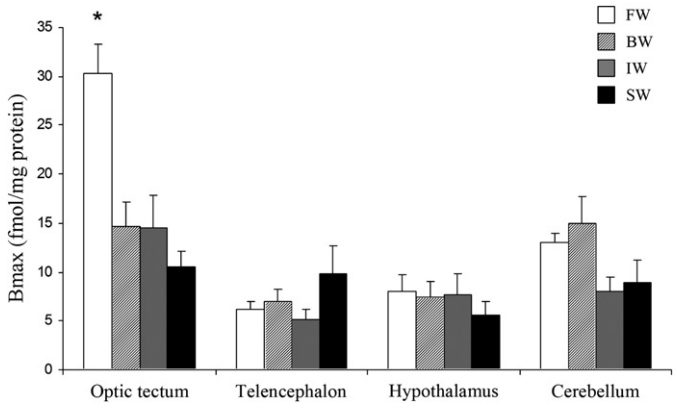

Fig. 3. Density of Mel binding sites in four regions of sea bass brain, optic tectum, telencephalon, hypothalamus, and cerebellum at ML at the four salinities: FW (white bars), BW (striped bars), IW (grey bars) and SW (black bars). Values are expressed as mean ± SEM (n = 7–8). Asterisks indicate statistically significant differences between salinities within the same brain region (Duncan's *post hoc* test).

optic tectum and neural retina of sea bass varied significantly depending on water salinity, the highest values being observed at the lowest salinities. In contrast, the Mel content of gills and intestine was significantly higher in fish exposed to full seawater.

In sea bass, daily and seasonal Mel rhythms have been reported under different lighting conditions and at different times of the year (Sánchez-Vázquez et al., 1997; García-Allegue et al., 2001; Migaud et al. 2007). In addition, the influence of light intensity and spectrum has been studied in this species (Bayarri et al., 2002). Salinity is an important environmental factor which affects fish growth (Boeuf and Payan, 2001) and food intake (Rubio et al., 2005). In gilthead sea bream, an euryhaline fish similar to sea bass, Mel levels varied with salinity in the same way as in the present paper, the lowest values being recorded at the highest salinity (55 ‰) and the highest values at the lowest salinity (5‰) (Kleszczynska et al., 2006). The role of Mel in fish osmoregulation has only been hypothesized, although previous studies have suggested a role for osmoregulatory processes (e.g. ionic efflux and influx, AVT, cortisol and IGF systems) in Mel synthesis in fish (Kulczykowska, 2002).

In the wild, sea bass have to cope with salinity changes throughout their life cycle, as they migrate to the open sea during autumn–winter and return to coastal lagoons and estuaries during spring (Lemaire et al., 2000; Varsamos et al., 2001; Pawson et al., 2007). Previous studies revealed a Mel seasonal rhythm in sea bass, with low amplitude during autumn and winter, and high amplitude in spring and summer (García-Allegue et al., 2001). Such seasonal variations of plasma Mel indicate the time of the year, acting as a synchronizer for annual rhythms (Reiter, 1993), for instance, seasonal migrations and reproduction as in the case of sea bass. In our research, lower levels of Mel were found in higher salinities (which, in wild animals, would coincide with migration to seawater during winter), while higher levels were recorded in lower salinities (coinciding with migration to lagoons during spring). Thus, water salinity might act as a modulator and, together with photoperiod and water temperature, determine the amplitude of Mel rhythms during the different seasons. Furthermore, as the migration of sea bass is related to mating and spawning, it would be interesting to investigate mature fish at different stages of sexual development in further assays.

In contrast with our results in sea bass, Mel levels in coho salmon increased following the transfer from FW to SW (Gern et al., 1984). This inverse response may be related with the fact that the migratory patterns of this two species are opposite, sea bass being a diadromous species that migrates to SW for spawning (Pawson et al., 2007), while coho salmon is an anadromous species that migrates to FW for spawning (Gern et al., 1984).

In peripheral tissues, the Mel content remained constant in brain and kidney at the different salinities, while in gills and intestine it was higher in fish maintained in seawater. Mel has been found in the gastrointestinal tract (GIT) of several fish species (Bubenik and Pang, 1997; Kulczykowska et al., 2006), where it has been suggested to play a paracrine function, as a regulator of feeding rhythms, satiety, intestinal motility and in connection with the osmoregulatory function, such as a regulator of the transmembrane transport of electrolytes and ions (Bubenik, 2002; López-Olmeda et al., 2006). The gills are the main tissue involved in ion transport and osmoregulatory processes in fish (Olson, 2002). It is interesting to note that a similar pattern can be observed in sea bass gills between Mel values found in the present research and Na^+-K^+-ATPase activity previously reported (Jensen et al., 1998). In that paper, Na^+-K^+-ATPase activity displayed a U-shaped regulation, with the highest values at SW. However, little is known on Mel function in gills and, to date, it has only been suggested that gills might act as an important site of Mel uptake and excretion in fish (Kulczykowska et al., 2006).

Mel binding sites in the brain and retina of sea bass have previously been described by Bayarri and coworkers (2004a,b), although, to our knowledge, the effect of different salinities had never been investigated in any fish species. Our results revealed that the density of Mel binding sites peaked in FW, but decreased in brackish and sea water. Recent studies in golden rabbitfish reported that both Mel and Mel receptor rhythms are in phase (Park et al., 2007), showing that Mel receptor regulation by Mel itself is a more complex process. Moreover, in the present study, both plasma Mel concentration and binding site density in the retina and optic tectum increased in parallel with decreasing salinities. However, with the present data, we can only speculate that Mel up-regulates the expression of its receptors and thus enhances its effect in both tissues. Moreover, lack of the data on receptor affinities (Kd) makes any interpretation inconclusive and further research is required.

In summary, our findings revealed that salinity influenced the Mel content in plasma and peripheral tissues (intestine and gills), and the density of Mel receptors in the brain (optic tectum) and the neural retina. During the year, sea bass Mel rhythms decrease their amplitude in autumn-winter but increase in spring-summer, changes that are driven by the seasonal changes in photoperiod and water temperature (García-Allegue et al. 2001). Since European sea bass migrates in winter to seawater, where Mel may be further decreased by the change in salinity, the seasonal Mel signalling may be enhanced. Therefore, salinity may act as a modulator of Mel production.

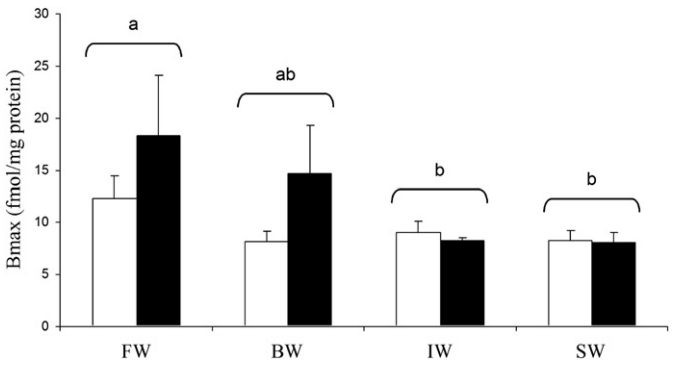

Fig. 4. Density of Mel binding sites in the neural retina at ML (white bars) and MD (dark bars) at the four salinities (FW, BW, IW and SW). Values are expressed as mean ± SEM (n = 7–8). Different letters indicate statistically significant differences between salinities for ML and MD (p < 0.05). No significant differences were observed between ML and MD values in the same salinity.

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