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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*)

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ABSTRACT

Sea bass is a 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 salinities: seawater (36 ‰), isotonic water (15 ‰), brackish (4 ‰) and freshwater (0 ‰). 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 among salinities, with the lowest value occurring at seawater salinity (102 pg/ml), and the highest at 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 being observed at the lower salinities in 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 in the fish maintained in freshwater. Taken together, these results revealed that salinity influences melatonin production and modifies the density of binding sites, which would point to a role for this hormone in timing seasonal events in sea bass, including those linked to fish migration between waters of different salinities.

Keywords: melatonin, melatonin binding sites, *Dicentrarchus labrax*, seasonality, salinity

INTRODUCTION

Melatonin is the main product of the pineal organ of vertebrates, including fishes (Ekström and Meissl, 1997). In all species studied to date, melatonin is produced mainly during the dark phase of daily photocycle, with low levels during the light phase (Falcón, 1999). This hormone is secreted into the blood and provides the organism with information regarding the time of day and the season of the year (Reiter, 1993), thus regulating the daily and seasonal rhythms in animals. In addition to this main role, melatonin 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).

Sea bass (*Dicentrarchus labrax*, L.) is a euryhaline fish capable of living in a wide range of salinities, from high salinity environments to freshwater as well as in environments that are subjected to variations in salinity such as estuaries (Chervinsky, 1974). Indeed, sea bass 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 (Lemaire et al., 2000; Varsamos et al., 2001). Therefore, sea bass have to cope with salinity changes in their habitat during their annual cycle, and have consequently developed physiological strategies to adapt to these variations (Claireaux and Lagardère, 1999). In sea bass, seasonal environmental factors that influence melatonin production have been studied, with special attention being paid to the effects of photoperiod and water temperature (García-Allegue et al., 2001), and light intensity and spectrum (Bayarri et al., 2002). Furthermore, water salinity appears to be an important environmental factor influencing food intake and macronutrient selection in sea bass (Rubio et al., 2005). However, to date, the effects of

68 salinity on the melatonin system in this species have not been studied, particularly regarding
69 this environmental factor in sea bass seasonal migrations in the wild.
70 Melatonin actions are mediated through high- and low-affinity receptors. Previous
71 studies have characterized high affinity receptors belonging to the superfamily of G-protein
72 coupled receptors (Vanecek, 1998). Several subtypes of melatonin receptors have been
73 identified, the MT1 and MT2 subtypes described in all vertebrates investigated, and the Mel
74 1c subtype only present in non mammalian vertebrates (Witt-Enderby et al., 2003). A
75 widespread distribution of melatonin receptors in central and peripheral tissues has been
76 described, the higher densities occurring in the central nervous system (Falcón et al., 2007). In
77 fish, melatonin binding sites in the brain and retina may show a daily rhythm in density and/or
78 affinity, depending on the brain area and the species (Iigo et al., 2003; Bayarri et al., 2004b;
79 Park et al., 2007). Moreover, seasonal variations in melatonin binding sites have also been
80 observed, depending on the maturational state, in the masu salmon brain (*Oncorhynchus*
81 *masou*) (Amano et al., 2003). Nevertheless, no study has focused on the possible influence of
82 salinity on melatonin receptors in the fish brain and, thus, modulation of melatonin functions
83 by the changes in water salinity.
84 The aim of this research was to evaluate the influence of water salinity on melatonin
85 concentration in plasma and several tissues (brain, gills, intestine and kidney), and the
86 possible variations in the density of melatonin binding sites in central neural tissues of sea
87 bass exposed to four different salinities, ranging from seawater to freshwater.
88
89 **MATERIALS AND METHODS**
90 **Animals and housing**
91 Sea bass (*Dicentrarchus labrax* L.) were obtained from the Spanish Institute of
92 Oceanography at Mazarrón (Murcia, Spain) and reared at the facilities of the University of
93 Murcia. Fish had an average body weight of 117 ± 37 g. b. w. (mean ± S. D.) and were kept in
94 well aerated 500-l tanks equipped with biological and mechanical filters. Fish were
95 acclimated to lab conditions during the month of May, and the experiments were performed
96 during June. Water temperature was controlled at 23 ºC and the photocycle was set at
97 12L:12D. Light was provided by "daylight" bulbs (Decor A 60W, Osram) placed at 70 cm
98 from the water surface, where light intensity was 300 lx. Fish were fed with a commercial diet
99 for sea bass (Excel 2P, Skretting, Nutreco Holding N.V., Netherlands). During the experiment
100 common water quality criteria were assessed every day by means of commercial kits (Sera,
101 Germany).

Experimental design

The experiments were designed to evaluate the influence of decreasing water salinities on both plasma levels and tissue melatonin content, and melatonin receptors density in central neural tissues in sea bass. For this purpose, four salinities were chosen: 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 has been previously described by Saillant et al. (2003). Fish were reared and the experiments were conducted ethically, following the Spanish legislation on Animal Welfare and Laboratory Practices.

Commercial marine salt (SERA premium sea salt, Germany) was added to freshwater to reach the desired salinity. Salinity changes were made gradually within 2-3 days. When water salinity reached the desired salinity, fish were maintained during one week in these conditions and, after that period, samples for assays were collected. Fish were anaesthetized in clove essence at 50 ppm (Guinama, Valencia, Spain), blood samples were collected by caudal puncture and then fish were sacrificed by decapitation. Tissue samples from brain, intestine, gills and kidneys were collected, frozen immediately in dry ice and stored at -80 ºC until analysis. For each salinity, blood samples for melatonin were taken both at mid-light (ML) and mid-dark (MD) (n=8 for each point), while tissue samples for melatonin analysis were collected only at ML to avoid the influence of the nocturnal rise in circulating melatonin synthesized by the pineal gland. Brains were collected at ML and dissected into optic tectum, telencephalon, hypothalamus and cerebellum, and stored at -80 ºC until assayed for radiobinding. The eye cup was removed at ML and MD and placed under the binocular for removal of the neural retina, which was frozen until assayed.

Melatonin analysis

Samples from brain, kidney, gut and gills were homogenized by sonication in a phosphate buffer saline with 0.01 % thimerosal (Sigma Aldrich Chemicals, St. Louis, USA). Melatonin 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 previous procedure (Kulczykowska and Iuvone, 1998). Melatonin concentration was determined using a commercial radioimmunoassay kit (Melatonin direct RIA, RE 293 01, IBL Hamburg, Germany), and radioactivity was measured using a γ counter (Wallac 1470, Perkin Elmer, MA, USA). Melatonin 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 Chemicals).

Membrane preparation and binding assays

Membranes were prepared as described elsewhere (Bayarri et al., 2004b). Briefly, samples were sonicated in Tris:HCl buffer (50 mM, pH=7.4) and centrifuged, and membranes were resuspended in Tris buffer and stored at -80 ºC until the binding assays were performed. Total protein concentrations in the tissues were measured by Lowry's method (1951), modified to microplates by reducing all the volumes to get 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 ºC for 90 minutes. The reaction was stopped at 4 ºC by adding 750 µl of Tris buffer, and immediately vacuum filtered through 25 mm glass fibre 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 unlabeled melatonin (1 µM) (Sigma Aldrich Chemicals), 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.

Data analysis

Values are expressed as mean ± S.E.M. Statistical analysis was performed using SPSS® software. Data of melatonin in tissues and melatonin binding sites in each brain region were subjected to one-way ANOVA, followed by Duncan's *post hoc* test. Data of plasma melatonin and density of melatonin binding sites were subjected to two-way ANOVA, followed by Duncan's *post hoc* test. Statistical significance threshold was set at $p<0.05$.

RESULTS

All groups showed significant differences between day and night plasma melatonin levels, with higher values during MD (two-way ANOVA, $p<0.05$) (Fig. 1). In addition, nocturnal plasma concentrations of melatonin varied significantly depending on water salinity, the mean values being higher at lower salinities, with FW and BW (151 ± 23 and 123 ± 9 pg/ml, respectively) showing significant differences with SW (102 ± 4 pg/ml) (two-way ANOVA, $p<0.05$). When day and night plasma melatonin values were compared by linear regression, increasing differences between ML and MD plasma melatonin were observed as salinity was reduced from SW to FW (Fig. 2). The statistical analysis of both regression lines revealed that MD melatonin increased significantly as salinity decreased (Spearman correlation, $p<0.05$), while ML melatonin did not change with salinity ($p=0.2$).

Melatonin levels in tissues showed a wide range of variation between different tissues and different salinities (Fig. 3). Significant differences were found in the intestine (ANOVA, $p<0.05$), where melatonin increased threefold in SW compared with other groups. A similar profile could be observed in gills, where melatonin values were significantly higher in animals maintained in seawater, although such differences were less marked than in the intestine. Neither brain nor kidney melatonin levels showed significant differences at the different salinities tested. The highest levels of melatonin were found in the intestine in the SW group (up to 627 ± 89 pg/g tissue) (ANOVA, $p<0.05$).

The radioligand experiments revealed differences in binding capacities in the different brain regions, with optic tectum showing the highest density values, followed by the cerebellum, and the lowest values for telencephalon and hypothalamus (two-way ANOVA, $p<0.05$). When each region of the brain was analyzed for differences between salinities, the optic tectum showed increasing receptor densities with decreasing salinity levels (one-way ANOVA, $p<0.05$) (Fig. 4). Cerebellum, hypothalamus and telencephalon showed similar values in all groups (Fig. 4) (one-way ANOVA, $p>0.05$).

In the retina, the highest receptor density was found in the FW salinity (Fig. 5), showing higher statistically significant densities of melatonin binding sites than SW (12.3 *vs* 8.2 fmol/mg protein at ML in the FW and SW, respectively; and 18.3 *vs* 8.0 fmol/mg protein at MD in the FW and SW, respectively) and IW (12.3 *vs* 9.1 fmol/mg protein at ML in FW and IW; and 18.3 *vs* 8.3 fmol/mg protein at MD in FW and IW, respectively) (two-way ANOVA, $p<0.05$). Although MD values in FW and BW tended to increase when compared with ML values, no significant differences were observed between ML and MD binding sites inside a same salinity (two-way ANOVA, $p=0.11$).

DISCUSSION

Our results revealed that the sea bass melatonin system is influenced not only by light or water temperature, but also by water salinity. In the present study, both circulating melatonin levels and melatonin binding sites in the optic tectum and neural retina of sea bass varied significantly depending on water salinity, showing the highest values at the lowest salinities. In contrast, the melatonin content of gills and intestine was significantly higher in fish exposed to full seawater.

In sea bass, both daily and seasonal melatonin rhythms have been previously reported under different lighting conditions and at different times of the year (Sánchez-Vázquez et al., 1997; García-Allegue et al., 2001). In addition, the influence of light intensity and spectrum

has been studied in this species (Bayarri et al., 2002). However, the influence of salinity, which changes as sea bass migrate seasonally to lagoons, on melatonin production had not been evaluated to date in this species. Salinity is an important environmental factor which affects fish growth (Boeuf and Payan, 2001), food intake and the pattern of macronutrient selection (Rubio et al., 2005). In the wild, sea bass have to cope with salinity changes throughout their life cycle, as they migrate to open sea during autumn-winter and return to coastal lagoons and estuaries during spring (Lemaire et al., 2000; Varsamos et al., 2001).

Previous studies revealed a melatonin seasonal rhythm in this species, with low amplitude during autumn and winter, and high amplitude in spring and summer (García-Allegue et al., 2001). Such seasonal variations of plasma melatonin indicate the time of the year and act as a synchronizer for annual rhythms (Reiter, 1993), as in the case of sea bass for seasonal migrations and reproduction. Curiously enough, lower levels of melatonin were found in higher salinities (coinciding in wild animals 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 influence, together with photoperiod and water temperature, the amplitude of melatonin rhythms observed along seasons. Therefore, not only photoperiod and water temperature, but also salinity changes seem to contribute to the transduction of the seasonal environmental information into melatonin rhythms.

Transitional changes in plasma melatonin levels during long-term adaptation to salinity changes have been reported in salmon (Gern et al., 1984). However, acute changes of salinity may occur during sea bass seasonal migrations, and thus an acute response of melatonin production could be enough to induce physiological changes linked to sea bass annual rhythms.

Apart from plasma, there are no studies on tissue melatonin in fish under different salinities. Herein, the melatonin content remained constant in brain and kidney at the different salinities, but in gills and intestine was higher in fish maintained in seawater. Melatonin has been found in the gastrointestinal tract (GIT) of several fish species (Bubenik and Pang, 1997; Kulczykowska et al., 2006). In addition, the presence of melatonin binding sites in peripheral tissues has been described in three different fish species, gilthead sea bream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and flounder (*Platichthys flesus*) (Kulczykowska et al., 2006). Gastrointestinal melatonin has been suggested to play a paracrine function, as a regulator of feeding rhythms, satiety, intestinal motility and in connection with the osmoregulatory function, 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). However, little is known on melatonin function in gills and, to date, it has only been suggested that might act as an important site of melatonin uptake and excretion in fish (Kulczykowska et al., 2006).

Melatonin binding sites in the brain and retina of sea bass have been previously described by Bayarri and coworkers (2004a, b). However, this is the first study, to our knowledge, that describes variations in melatonin binding sites in different brain areas of fish exposed to different salinities. Some fish show a circadian rhythm of melatonin binding sites and gene expression of melatonin receptors, as occurs in sea bream (Falcón et al., 1996), pike (Gaildrat et al., 1998), goldfish (Iigo et al., 2003) or golden rabbitfish (Park et al., 2007). These findings are similar to studies in mammals, and it has been hypothesized that melatonin down-regulates the expression of melatonin receptors, which would explain the differences in both rhythms (Witt-Enderby et al., 2003). However, recent studies in golden rabbitfish revealed that in this species both melatonin and melatonin receptor rhythms are in phase (Park et al., 2007), showing that melatonin receptor regulation by melatonin itself is a more complex process. Moreover, in the present study, both plasma melatonin concentration and binding site density in the retina and brain tissue increased in parallel with decreasing salinities. The physiological significance of this response is not clear. However, it should be also emphasized that density of melatonin receptors in brain and retina are influenced by melatonin synthesized in the pineal organ and retina. Therefore, in the absence of data on melatonin concentration at the sites of its synthesis, we can only speculate that melatonin up-regulates the expression of its receptors and thus enhances its effect in both tissues. What is more, lack of the data on receptor affinities (Kd) makes any interpretation not conclusive.

In summary, this is the first study describing the influence of salinity on melatonin content in peripheral tissues as kidney, intestine and gills; and the first to report the influence of salinity on the density of melatonin receptors in several brain areas and the neural retina. During the year, sea bass melatonin rhythms decrease their amplitude in autumn-winter, while increase in spring. Curiously, sea bass migrates in winter to seawater, where melatonin is further decreased by the change in salinity, enhancing the seasonal melatonin signalling and describing salinity as a new signal for melatonin synthesis and annual adaptations.

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351
352 **FIGURE CAPTIONS**
353 Figure 1. Plasma melatonin levels at ML (white bars) and MD (dark bars), at the four
354 salinities, FW (freshwater, 0 ‰), BW (brackish water, 4 ‰), IW (isotonic water, 15 ‰), and
355 SW (seawater, 36 ‰). Values are expressed as mean ± S.E.M. (n=4). Data were compared
356 using a two-way ANOVA, followed by Duncan *post hoc* test. Asterisks indicate statistically
357 significant differences between ML and MD values, and different letters indicate statistically
358 significant differences between salinities ($p<0.05$).
359
360 Figure 2. Regression lines for plasma melatonin values at ML (white circles) and MD (black
361 circles) related to salinity (in parts per mile, ppm). Equations for each line are in the right side
362 of the figure. R^2 values are 0.2863 and 0.6602 for ML and MD, respectively.
363
364 Figure 3. Melatonin contents in sea bass brain (3A), intestine (3B), gills (3C) and kidney
365 (3D), at ML at the four salinities (FW, BW, IW and SW). Values in brain, gills and intestine
366 are expressed as picograms of melatonin per gram of tissue; and melatonin contents in kidney
367 are expressed in picograms of melatonin per milligram of protein. Values are expressed as
368 mean ± S.E.M. (n=4). Data from each tissue were subjected to one-way ANOVA, followed
369 by Duncan *post hoc* test. Different letters indicate statistically significant differences
370 ($p<0.05$).
371
372 Figure 4. Density of melatonin binding sites in four regions of sea bass brain, optic tectum,
373 telencephalon, hypothalamus, and cerebellum at ML at the four salinities: FW (white bars),
374 BW (striped bars), IW (grey bars) and SW (black bars). Values are expressed as mean ±
375 S.E.M. (n=7-8). Data from each region were subjected separately to one-way ANOVA,

followed by Duncan *post hoc* test. Asterisk indicate statistically significant differences ($p<0.05$).

Figure 5. Density of melatonin 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 ± S.E.M. (n=7-8). Data were compared using a two-way ANOVA, followed by Duncan *post hoc* test. Different letters indicate statistically significant differences between salinities ($p<0.05$). No significant differences were observed between ML and MD values in the same salinity.

Figure 1

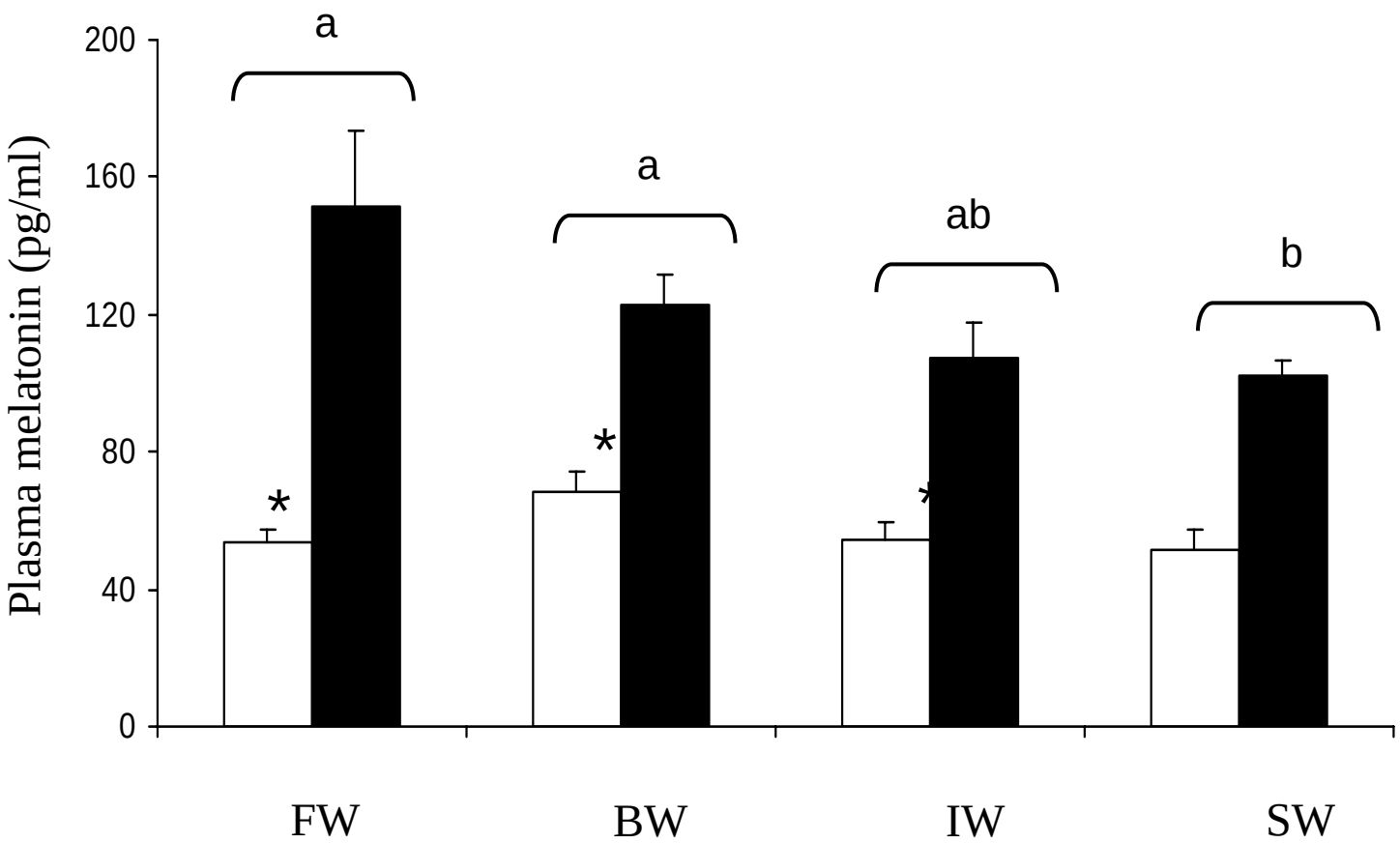
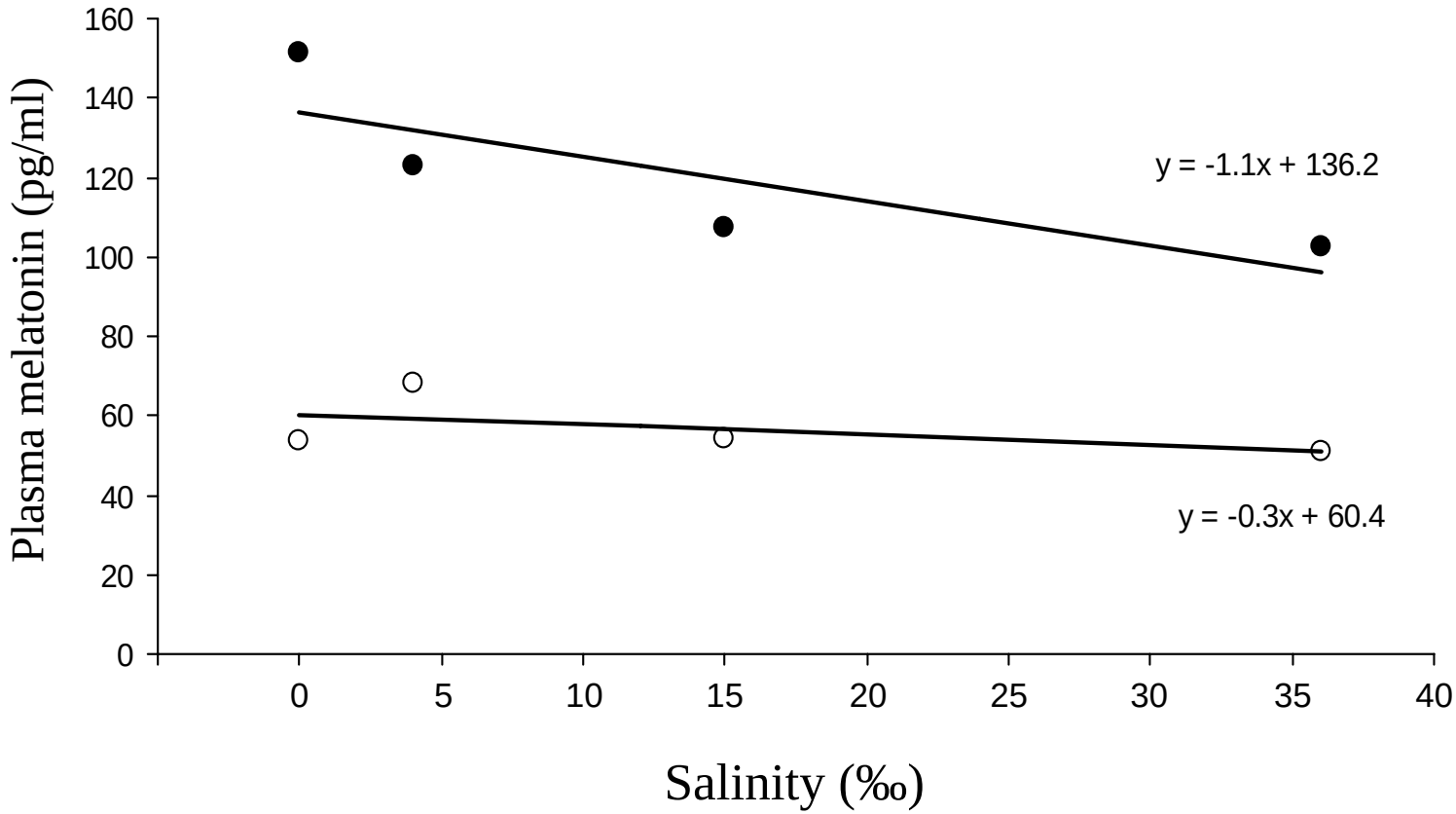


Figure 2


Plasma melatonin (pg/ml)
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Salinity (‰)
y = -1.1x + 136.2
y = -0.3x + 60.4

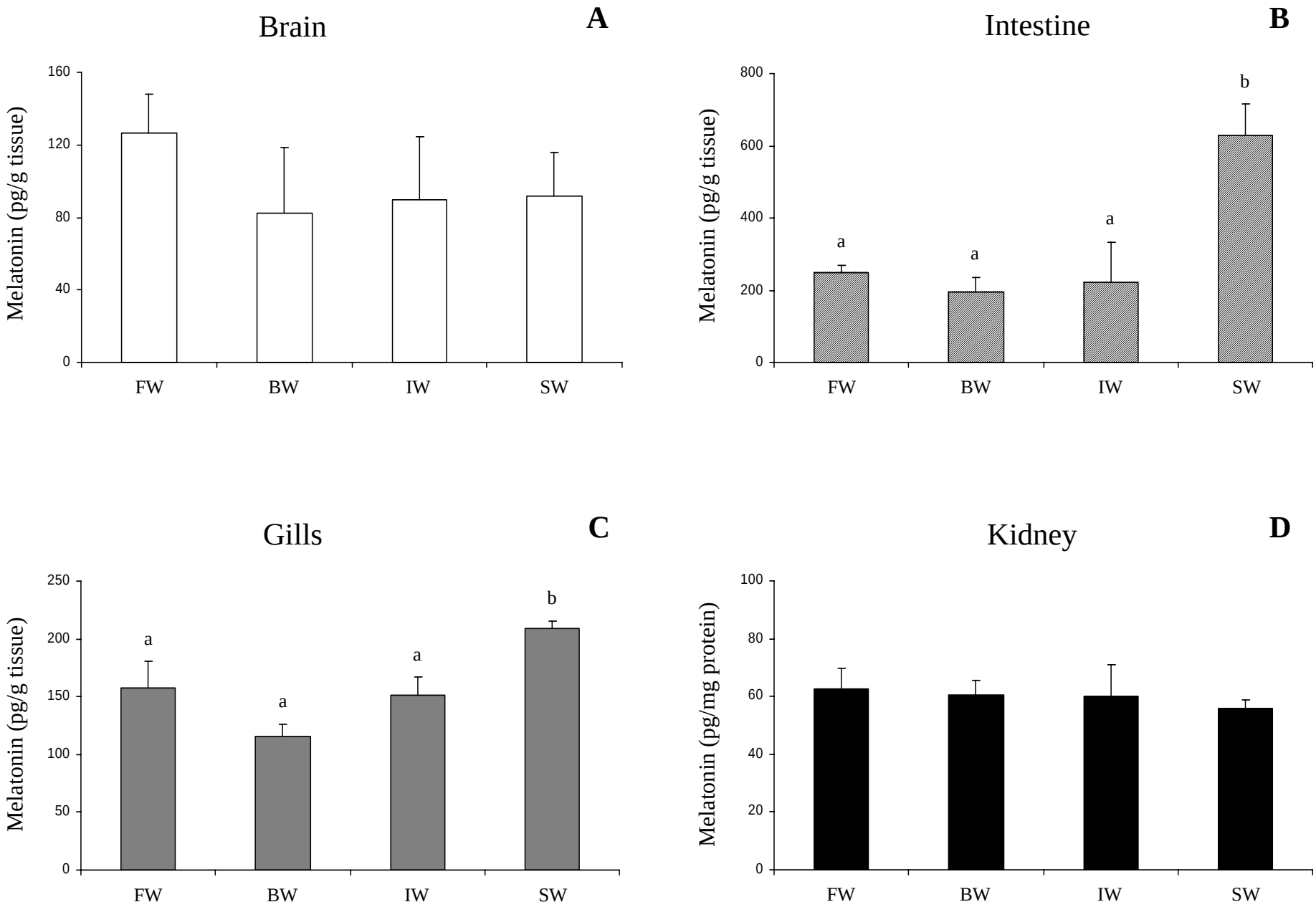

Brain
A
Melatonin (pg/g tissue)
160
120
80
40
0
FW
BW
IW
SW
Intestine
B
Melatonin (pg/g tissue)
800
600
400
200
0
a
a
a
b
FW
BW
IW
SW
Gills
C
Melatonin (pg/g tissue)
250
200
150
100
50
0
a
a
a
b
FW
BW
IW
SW
Kidney
D
Melatonin (pg/mg protein)
100
80
60
40
20
0
FW
BW
IW
SW

Figure 4

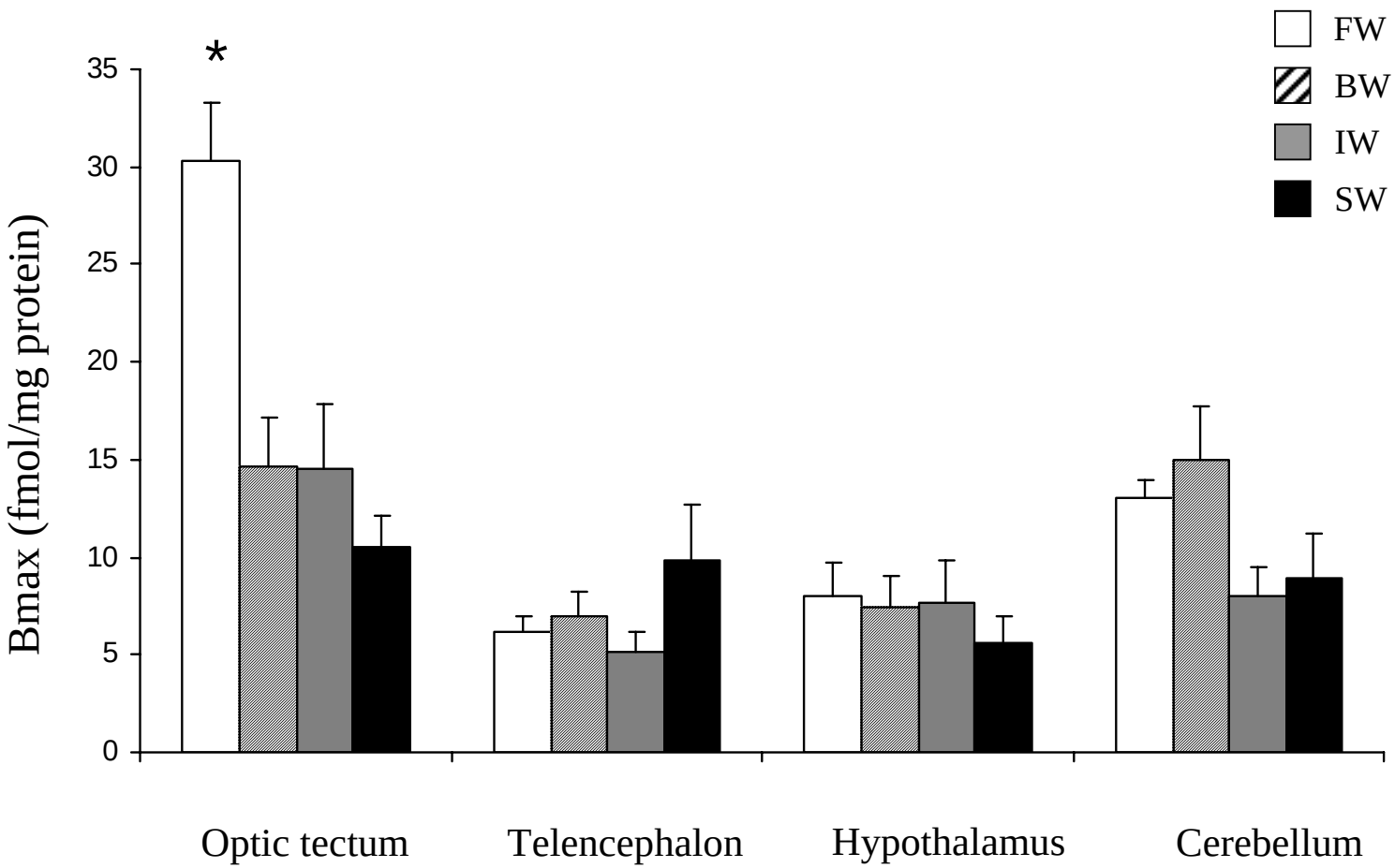

FW
BW
IW
SW
*
Bmax (fmol/mg protein)
35
30
25
20
15
10
5
0
Optic tectum
Telencephalon
Hypothalamus
Cerebellum

Figure 5