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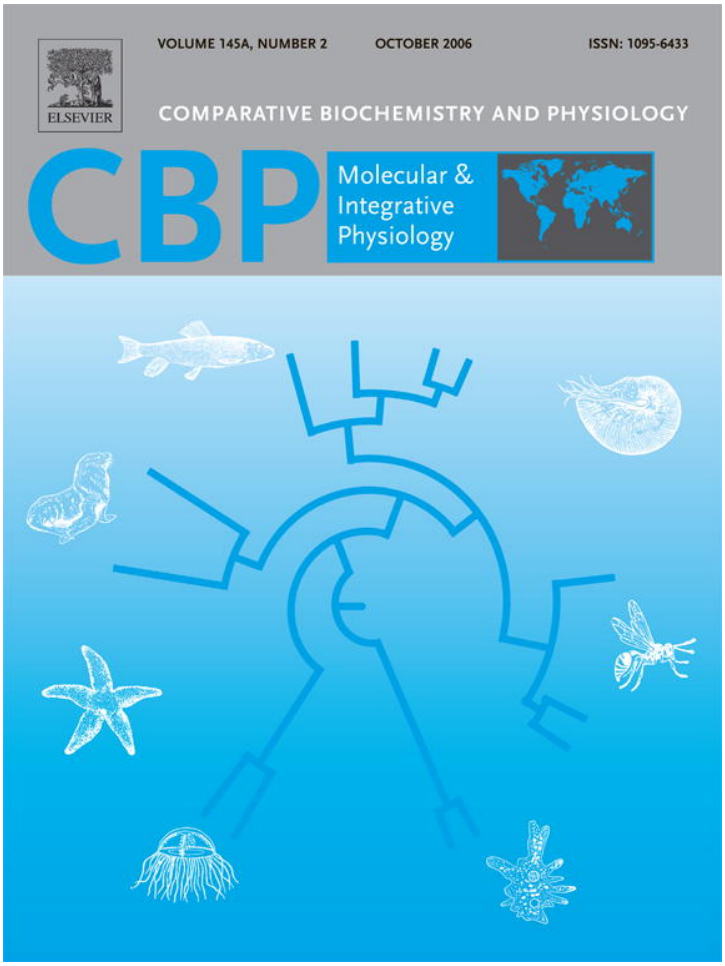

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Arginine vasotocin, isotocin and melatonin responses following acclimation of gilthead sea bream (*Sparus aurata*) to different environmental salinities

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Abstract

Gilthead sea bream (*Sparus aurata*) is a euryhaline species with a capacity to cope with demands in a wide range of salinities and thus is a perfect model-fish to study osmoregulatory responses to salinity-adaptive processes and their hormonal control. Immature sea bream acclimated to different salinities, i.e. SW (38‰), LSW (5‰) and HSW (55‰), were kept at 18 °C under natural photoperiod. Arginine vasotocin (AVT) and isotocin (IT) in plasma and pituitary were determined by HPLC. Plasma melatonin (Mel) was assayed by RIA. Plasma osmolality, ion concentrations (Na^+, K^+, Ca^{2+}, Cl^-) and Na^+,K^+-ATPase activity in gill were measured. A steady increase in plasma AVT, along with increasing water salinity was observed. Pituitary IT concentration in HSW-acclimated fish was significantly higher than that in LSW group. AVT/IT secretory system of sea bream does appear to be involved in the mechanism of long-term acclimation to different salinities. The distinct roles and control mechanisms of both nonapeptides are suggested. Plasma Mel was significantly higher in LSW compared with both HSW and SW groups. Data indicate that the changes in Mel level are linked to osmoregulation. Further studies are required to elucidate a complex role of AVT, IT and Mel in sea bream osmoregulation.
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Keywords: Arginine vasotocin; Gilthead sea bream; Hormones; Isotocin; Melatonin; Neurohypophysis; Osmoregulation; Salinity

1. Introduction

Fish nonapeptides arginine vasotocin (AVT) and isotocin (IT) are produced in the hypothalamic magnocellular and parvocellular neurons of the nucleus preopticus, from where they are transported to the neurohypophysis for storage and release (Bentley, 2002). Several studies have shown that their synthesis and secretion into circulation respond to environmental salinity (Maetz and Lahlou, 1974; Haruta et al., 1991; Hyodo and Urano, 1991; Perrott et al., 1991). The neurohypophysial hormones AVT and IT are presumed to play a role in quick and long-term adaptation of teleost fish to external salinity changes (Kulczykowska, 1997, 2001; Bond et al., 2002; Warne et al., 2005). However, the data on the osmoregulatory role of AVT in fish are often contradictory, while osmoregulatory role of IT remains unclear. Moreover, the physiological role of the hormones in maintenance of water/ions homeostasis does not seem to be uniform among fish species.

Melatonin (Mel; *N*-acetyl-5methoxytryptamine) is primarily synthesized and secreted by the pineal gland in all classes of vertebrate including fish. This hormone is not stored in the pineal, and its plasma concentration reflects the synthesis capacity of the gland (Reiter, 1991). The production and release of Mel display a diurnal rhythm with the higher levels during the darkness (Ekström and Meissl, 1997). Many of the established physiological effects of melatonin are mediated via high-affinity cell membrane receptors (Stankov et al., 1993). The recent finding of the specific 2-[^{125}I] iodomelatonin binding in several fish osmoregulatory organs, i.e. gill, small intestine and kidney (kidney tubules) suggests the possible influence of Mel on water/ion balance in fish (Kulczykowska et al., 2006).

Euryhaline fish manage effective mechanisms of salt and water balance to support life in diverse aquatic environments and several hypophysial and extrahypophysial hormones are

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Table 1
Osmolality and ionic composition of sea bream plasma and tanks' water for three experimental groups

	LSW		SW		HSW	
	Plasma	Water	Plasma	Water	Plasma	Water
Osmolality (mosM)	356±6[a]	130	396±7[b]	1162	415±5[b]	1354
Na^+ (mmol L^{-1})	165±4[a]	55	186±2[b]	468	193±3[b]	721
Cl^- (mmol L^{-1})	142±2[a]	71	150±2[a]	534	156±2[b]	806
Ca^{2+} (mmol L^{-1})	2.1±0.2[a]	1.8	2.4±0.2[a]	11.1	2.7±0.2[b]	14.7
K^+ (mmol L^{-1})	4.5±0.5[a]	1.6	5.0±0.2[a]	10.8	5.2±0.3[a]	13.9

Values are means±S.E.M. (n=8–9).
Different letters indicate significant differences between groups. $P<0.05$; one-way ANOVA followed by SNK test.

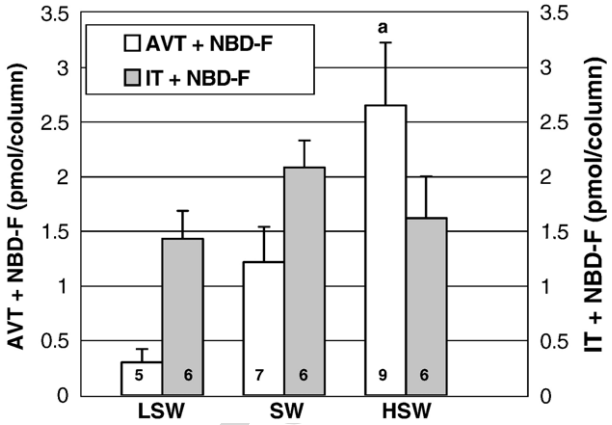

Fig. 1. Plasma AVT and IT in sea bream acclimated to different salinities. Arbitrary units: (AVT/IT+NBD-F complex) pmol per column. Values are means±S.E.M. Number of fish is given in the bars. [a]($P<0.05$; Student's unpaired t-test) vs. LSW.

involved in these processes (Bentley, 2002). Gilthead sea bream (*Sparus aurata*) is a euryhaline species with a capacity to cope with demands in a wide range of salinities and thus is a perfect model-fish to study osmoregulatory response to salinity-adaptive processes (Mancera et al., 1993, 2002; Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrión et al., 2005) and their hormonal control (Mancera et al., 2002; Laiz-Carrión et al., 2003; Guzmán et al., 2004). However, there is not any information about the response of AVT, IT and Mel in *S. aurata* acclimated to different environmental salinities.

In this study, we examined the response of hormones AVT, IT and Mel in *S. aurata* acclimated to salinities of 5‰, 38‰ and 55‰. The purpose of the present work was to gain more information on osmoregulation of this species. The results are discussed in relation to the present knowledge on the osmoregulatory role of AVT, IT and Mel in teleosts.

2. Materials and methods

2.1. Animals and experimental conditions

Immature gilthead sea bream (250–350 g body mass) were provided by Planta de Cultivos Marinos (C.A.S.E.M., Universidad de Cádiz, Puerto Real, Cádiz, Spain). Fish were transferred to the wet laboratories at the Faculty of Marine Sciences (Puerto Real, Cádiz) where they were acclimated for 30 days to full seawater (SW, 38‰: 1162 mosM kg^{-1} H_2O) in 300-L tanks in an open system. After this period, fish were exposed to gradually changing salinity over 2 h until it reached 5‰ (low salinity water, LSW: 130 mosM kg^{-1} H_2O), or 55‰ (high salinity water, HSW: 1354 mosM kg^{-1} H_2O), while the control group was kept at 38‰ (SW) (April–May 2005). The experimental salinities were achieved by either mixing full strength SW with dechlorinated tap water or by mixing full strength SW with natural marine salt (Instant Ocean, Aquarium Systems, Sarrebourg, France).

Fish were kept in recirculating tanks filled with water of different salinities for 2 weeks. During this time common water quality criteria were assessed and no major changes were observed. Average values for those parameters were 5 mg/L for oxygen, 0.3 mg/L for nitrite, 0.4 mg/L for nitrate, 0.4 mg/L for ammonia, and less than 0.1 mg/L for chlorine, calcium and hydrogen sulfide. The water salinity was checked every day and corrected when necessary.

During the experiment fish were maintained at constant temperature of 18 °C under natural photoperiod. Fish were fed once a day with commercial dry pellets at a ration of 1% of body weight (Dibaq-Diprotg SA, Segovia, Spain) and were fasted for 24 h before sampling. No mortality was observed during the experiment.

Table 2
Gill Na^+,K^+-ATPase activity and plasma levels of glucose, lactate, proteins and triglycerides in sea bream acclimated to different salinities for 2 weeks

	LSW	SW	HSW
Na^+,K^+-ATPase activity (μmol ADP mg $protein^{-1}$ h^{-1})	24.5±2.3[b]	14.9±1.4[a]	27.9±1.2[b]
Glucose (mmol L^{-1})	2.35±0.13	2.54±0.08	2.78±0.15
Lactate (mmol L^{-1})	2.86±0.15	2.72±0.19	3.13±0.34
Triglyceride (mmol L^{-1})	1.09±0.08	1.20±0.10	1.09±0.10
Protein (mg mL^{-1})	35.53±2.10	40.14±2.10	41.00±1.40

Values are means±S.E.M. (n=8–9).
Different letters indicate significant differences between groups. $P<0.05$; one-way ANOVA followed by SNK test.

2.2. Sampling

At the end of the experiment fish were anaesthetized in 2-phenoxyethanol (1 mL/L water, Sigma-Aldrich), weighed and sampled. Sampling was started at 9:30 h. The blood was collected from the caudal peduncle into 1-mL ammonia-heparinized syringes. Plasma was separated from cells by centrifugation of whole blood (5 min at 10,000×g), was immediately frozen in liquid nitrogen and stored at −80 °C. A biopsy of gill tissue was placed in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C.

2.3. Analytical methods

AVT and IT in plasma and pituitary were determined by high-performance liquid chromatography (HPLC) with fluorescence detection preceded by solid-phase extraction (SPE). HPLC assay was performed with a Beckman modular system (Beckman

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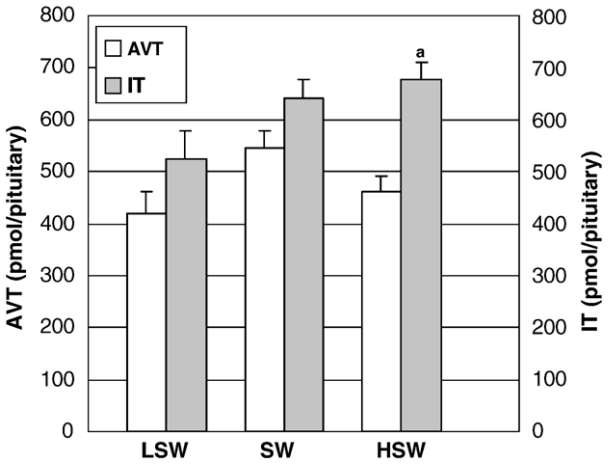

Fig. 2. Pituitary AVT and IT content in sea bream acclimated to different salinities (pmol per pituitary). Values are means±S.E.M. ($n=9$). [a]($P<0.05$; Student's nonpaired t-test) vs. LSW.

Instruments, San Ramon, CA, USA) with spectrofluorometric detector RF-551 (Shimadzu, Columbia, MD, USA). Chromatographic separations were carried out on an Ultrasphere ODS column (250×4.6 mm I.D., 5 μm particle diameter) preceded by a precolumn (45×4.6 mm I.D.) filled with the same material (both from Beckman Instruments, San Ramon, CA, USA). Fluorescence detection was carried out at 530 nm with excitation at 470 nm. SPE procedures were accomplished on Bakerbond spe™ Octadecyl C_{18} Speedisk (20 mg, 1 mL) connected to the Baker SPE 12G column Processor (J.T. Baker, Phillipsburg, NJ, USA). AVT and IT were extracted from 1 mL of plasma and derivatized with NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) to be detected during HPLC-FL analysis. The method has been described in detail by Gozdowska and Kulczykowska (2004) with subsequent modification appended by Gozdowska et al. (2006). However, in this study, plasma AVT and IT are expressed in arbitrary units, *i.e.* (AVT/IT+NBD-F) complex (pmol/column). It represents an amount of derivatized complex of peptide and NBD-F, which is absorbed by HPLC column and detected with spectrofluorometric detector. Peptides are derivatized proportionally to their individual concentrations in plasma, but the high NBD-F background renders the calculation of AVT and IT molar concentrations impossible. This problem was avoided during measurements of pituitary AVT and IT content. Peptides were analysed in every single pituitary and data expressed as pmol of peptide per pituitary according to Gozdowska et al. (2006).

Plasma Mel was assayed using total melatonin RIA kit (IBL, Hamburg), with preceding extraction procedure. Solid phase extraction of melatonin was carried out on Octadecyl C_{18} Speedisk Column, 10 μm (J.T. Baker, USA). Samples were eluted with methanol according to a previous procedure described for melatonin extraction (Kulczykowska and Iuvone, 1998). Before RIA procedure, dried samples were resuspended in Dulbecco's phosphate buffered saline containing 0.01% Thimerosal (Sigma, USA). All samples in duplicate were counted in a Wallac Wizard γ-counter. The detection limit was 2.5 pg/mL plasma. The intra-assay coefficient of variation was 8.0%. The inter-assay variation was not determined, because all samples were measured in the same assay. Plasma Mel concentration was expressed as pg/mL plasma.

Plasma and water osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mosM kg^{-1}. Plasma and water Na^+, K^+ and Ca^{2+} were measured using atomic absorption spectrophotometry (Philips PU7000). Plasma Cl^-, glucose, lactate, and triglyceride levels were measured using commercial kits from Spinreact (Spain) adapted to microplates. Plasma proteins were measured using the bicinchoninic acid method using the BCA protein kit (Pierce, Rockford, IL, USA) adapted for microplates, with serum bovine albumin as standard. The assays were read on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics, Inc. NJ). Gill Na^+,K^+-ATPase activity was determined using the microassay method from McCormick (1993) adapted for *S. aurata* (Mancera et al., 2002).

2.4. Statistics

Values are expressed as means±standard error of the mean (S.E.M.). The statistical differences were analyzed by using one-way ANOVA followed by Student–Newman–Keuls multiple comparison test (SNK), Student's nonpaired t-test and Tukey's test as appropriate. Significance was taken at $P<0.05$.

3. Results

There was an apparent variation in osmolality and ionic composition in sea bream plasma, with increasing values of those parameters concomitantly with water salinity (Table 1). Gill Na^+, K^+-ATPase activity showed significantly higher values in LSW- and HSW-acclimated fish than that in SW-acclimated fish (Table 2). However, plasma metabolite levels from animals acclimated to different salinities did not present significant differences between groups (Table 2).

AVT and IT in plasma of fish acclimated to the three experimental salinities are shown in Fig. 1. A steady increase of AVT, along with the increase in water salinity, has gained significance at the highest salinity of 55‰ ($P<0.05$ vs. LSW). However, no meaningful differences in plasma IT have been observed between salinities. Fig. 2 presents pituitary AVT and IT contents in fish acclimated to three different salinities. There were no differences in pituitary AVT content between groups. However, pituitary IT content in HSW-acclimated fish was significantly higher than that in LSW-acclimated group. Plasma Mel concentration was significantly higher in LSW when compared to HSW and SW groups (Table 3).

Table 3
Plasma melatonin in sea bream acclimated to different salinities

	LSW	SW	HSW
Mel (pg mL^{-1})	52.3±2.5	40.1±0.8[a]	38.3±1.2[a]

Values are means±S.E.M. ($n=9$).
[a] ($P<0.05$; Tukey's post hoc test) vs. LSW.

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4. Discussion

In previous studies, we showed that gilthead sea bream is a euryhaline fish that can be acclimated to extreme salinities, suffering minor changes in plasma osmoregulatory and metabolic parameters (Mancera et al., 1993; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). The values of these parameters measured in the present study were similar to those previously reported for sea bream, and indicated that after 2 weeks in different salinities (LSW, SW and HSW) fish were fully acclimated. Moreover, gill Na^+,K^+-ATPase activity, which increased at the low and high salinities, agreed with previous data (Guzmán et al., 2004; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005).

This is the first study on AVT, IT and Mel responses in the seawater fish long-term acclimated to high and low salinities. Although in this study we can not track the molar concentrations of plasma AVT and IT, we have still an insight into peptides changes after salinity modifications. An increase in plasma AVT, with no response in IT, was observed in sea bream acclimated to higher salinities. AVT response was similar to that presented in long-term acclimated flounder by Bond et al. (2002). On the other hand, the pattern of changes in plasma AVT and IT in sea bream showed a different trend to that reported for rainbow trout (Pierson et al., 1995; Kulczykowska and Stolarski, 1996; Kulczykowska, 1997, 1999) and flounder (Perrott et al., 1991; Balment et al., 1993; Warne et al., 1994) fully acclimated to different salinities. The response of plasma AVT and IT in sea bream, subjected to prolonged osmotic challenge, were rather similar to a transitory reaction observed in rainbow trout (Kulczykowska, 1997) and flounder (Perrott et al., 1991; Balment et al., 1993; Harding et al., 1997) while exposed to acute osmotic challenge.

In HSW-acclimated fish, risen plasma AVT corresponded with high gill Na^+,K^+-ATPase activity. Several hormones (i.e. cortisol, growth hormone, 17β-estradiol, etc.) enhance this activity in teleost, including sea bream, while kept under hyperoosmotic condition (McCormick, 1995; Laiz-Carrión et al., 2003; Guzmán et al., 2004). Moreover, it has been demonstrated that AVT treatment increases significantly gill Na^+,K^+-ATPase activity in sea bream and a role for AVT during hyperosmotic acclimation has been suggested (Sangiao-Alvarellos et al., in press). However, the role of AVT in hypoosmotic adaptation is not clear, because in this study we have observed no link between plasma AVT and high gill Na^+,K^+-ATPase activity in LSW-acclimated sea bream. In addition, in AVT-treated sea bream transferred to LSW no changes in gill Na^+,K^+-ATPase activity has been detected (Sangiao-Alvarellos et al., in press).

In sea bream, the increased plasma AVT corresponded with higher plasma osmolalities and ion concentrations observed in SW and HSW animals. The changes in circulating AVT were probably produced by the changes in plasma osmolality and ion concentrations, as it was shown for rainbow trout and flounder. In rainbow trout transferred from fresh- to brackish-water, a transitory increase of both AVT and IT and a positive correlation between plasma peptides and plasma osmolality were reported (Kulczykowska, 1997). In flounder exposed to hypertonic medium, an elevation in plasma osmolality and ions concentration seemed to be a major factor responsible for the enhanced secretion of AVT into circulation (Warne and Balment, 1995; Bond et al., 2002). However, in flounder and rainbow trout fully acclimated to SW and in trout acclimated to brackish water, higher plasma AVT level was coupled with the lower plasma osmolality (Perrott et al., 1991; Kulczykowska and Stolarski, 1996; Kulczykowska, 1999).

Pituitary AVT content in sea bream acclimated to LSW, SW and HSW did not vary. Thus, the increase of plasma AVT in hyperosmotic environments (SW and HSW) was probably a result of an intensive synthesis of peptide in hypothalamic neurons followed by its immediate release into circulation. On the other hand, in fish kept in extremely high salinity (HSW), pituitary IT content increased significantly. It seems to indicate an activation of neurons producing IT, which does not result in pronounced secretion of the peptide into circulation. After exposure of rainbow trout, medaka and flounder to hyperosmotic media, a significant decrease in pituitary AVT content was observed and a rise of AVT secretion in response to dehydration was suggested (Carlson and Holmes, 1962; Haruta et al., 1991; Perrott et al., 1991). Changes in expression of provasotocin and proisotocin genes in the hypothalamus during acclimation of rainbow trout to hyper- and hypoosmotic environments strongly suggested variations in production of peptides (Hyodo and Urano, 1991), but the direction of changes was opposite to that postulated in sea bream. However, in flounder moved from FW to SW, a rapid increase of proAVT mRNA expression in hypothalamus was followed by elevated secretion of AVT from pituitary, which resulted in a rise of circulating level of the peptide (Warne et al., 2005).

AVT/IT secretory system of sea bream does appear to be involved in the mechanism of long-term acclimation to different salinities. A question arising from this study is the distinct role of AVT and IT in this process. Both, the release and synthesis of AVT and IT, seem to be controlled independently. Discrete roles and control mechanisms of both nonapeptides have been suggested in rainbow trout (Kulczykowska and Stolarski, 1996; Kulczykowska, 1997, 2001). To date, a role of IT in osmoregulation has been seldom addressed. The observation of high pituitary IT content is worth considering. It agrees with data of IT reported for the first time by Pierson et al. (1995) in rainbow trout.

Recently, the role of Mel in fish osmoregulation was strongly suggested (Kulczykowska, 2002; Kulczykowska et al., 2006). In this study, the highest plasma Mel concentration was measured in sea bream acclimated to lowest salinity. On the other hand, plasma Mel in brackish water acclimated rainbow trout was significantly higher than that in freshwater animals (Kulczykowska, 1999). Higher plasma Mel concentration paralleling high Na^+ and Cl^- values was also demonstrated in coho salmon during SW adaptation (Folmar and Dickhoff, 1981) and in common dentex (Pavlidis et al., 1999). Gern et al. (1984) suggested that the increase in plasma Mel during entry of coho salmon into SW is a part of the fish adapting mechanism to osmotic challenge. However, the sea bream, as a typical marine species, may respond to osmotic challenge in different way than freshwater rainbow trout or migratory species, i.e. salmon. It is known that the pineal activity of the cichlid fish *Oreochromis mossambicus* increases in response to

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environmental stresses, i.e. water osmolality, temperature and pH (Relkin, 1989). It might be a case here, while originally seawater fish is acclimated to the extremely low salinity (5‰). We have had no cortisol levels measured in this study, however, in previous experiments in sea bream acclimated to LSW, SW and HSW, similar plasma cortisol levels were shown in all groups (Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). Thus, the data suggest that the higher Mel level observed in LSW-acclimated fish is linked to osmoregulation rather than to stress-related processes. The studies of the presence of 2-[^{125}I]iodomelatonin binding sites in osmoregulatory tissues, i.e. small intestine, kidney tubule and gill, strongly point to new potential targets for Mel action and the influence of Mel on water/ion balance in teleosts, including sea bream (Kulczykowska et al., 2006).

In conclusion, we have undertaken the first studies of simultaneous reaction of neurohypophysial peptides AVT and IT, and indole agent Mel in sea bream acclimated to different environmental salinities. The results confirm that both nonapeptides and Mel are changing in response to external salinity and strongly suggest an osmoregulatory role of these hormones. However, further studies are required to elucidate a complex role for AVT, IT and Mel in sea bream osmoregulation.

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