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High density and food deprivation affect arginine vasotocin, isotocin and melatonin in gilthead sea bream (*Sparus auratus*)

Juan Miguel Mancera [a,*], Luis Vargas-Chacoff [a], Angel García-López [c], Agnieszka Kleszczyńska [b], Hanna Kalamarz [b], Gonzalo Martínez-Rodríguez [c], Ewa Kulczykowska [b]

[a] *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain*
[b] *Department of Genetics and Marine Biotechnology, Institute of Oceanology of Polish Academy of Sciences, 81-712 Sopot, Poland*
[c] *Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas, 11510 Puerto Real, Cádiz, Spain*

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Abstract

Arginine vasotocin (AVT) and isotocin (IT) levels in plasma and pituitary, and melatonin (MEL) levels in plasma were determined in gilthead sea bream (*Sparus auratus*) subjected to two different types of stress: i) high density (HD) and ii) food deprivation (NF: non-fed). Fishes were randomly assigned to one of 4 treatments that lasted for 14 days: 1) fed fish under normal low density (ND, 4 kg m^{-3}); 2) non-fed (NF) fish under ND; 3) fed fish under high density (HD, 70 kg m^{-3}); and 4) non-fed fish under HD. Ten fish from each tank were anaesthetized, weighed and plasma and pituitary samples were taken. Plasma and pituitary AVT and IT content were determined by HPLC, while plasma MEL was assayed by RIA. Plasma AVT and IT values were enhanced in all fish kept at high density. The response of AVT was much stronger than that of IT. The highest pituitary AVT and IT levels were shown in NF fish kept at normal density. The significantly higher plasma MEL levels were measured in fed fish kept at HD. These results suggest a role of AVT, IT and MEL in response of sea bream to a common stress factor, high density. Although food deprivation does not influence AVT and IT plasma levels, it seems to affect hypothalamic synthesis of nonapeptides. Further studies are required to elucidate the complex role of AVT, IT and MEL in the sea bream's response to different stress stimuli.

Keywords: Arginine vasotocin; Isotocin; Melatonin; Gilthead sea bream; Stress; High density; Food deprivation

1. Introduction

In teleosts, arginine vasotocin (AVT) and isotocin (IT) are synthetized in the hypothalamic magnocellular and parvocellular neurons of the preoptic nucleus (NPO), stored in the nerve terminal of these neurons at the *pars nervosa* of the hypophysis and released into the blood after appropriate stimuli. AVT and IT may act, either locally in the central nervous system (CNS) as neurotransmitters and/or neuromodulators, or in the peripheral target organs as hormones. In teleosts, a role of AVT in different physiological processes, such as behaviour, metabolism, reproduction, osmoregulation, etc. is well documented (Balment et al., 2006; Kulczykowska, 2007). There is also some evidence of a role of AVT and IT in stress response of teleosts. Studies in goldfish (*Carassius auratus*) show that AVT and IT stimulate cortisol secretion and suggest that both nonapeptides possess corticotropin-releasing factor (CRF) activity (Fryer and Leung, 1982). In *Oncorhynchus mykiss*, CRF and AVT synergize to stimulate adrenocorticotropin (ACTH) release (Baker et al., 1996; Pierson et al., 1996). In *O. mykiss*, acute confinement stress enhances AVT expression in the preoptic nucleus (Gilchriest et al., 2000) and disturbance stress increases plasma AVT, but not IT, in the same species (Kulczykowska, 2001). Recently, a similar activation of AVT expression in NPO following a single acute confinement stress has been observed in *Platichthys flesus* (Balment et al., 2006). IT appears to be involved in the response of female sticklebacks to a specific

* Corresponding author. Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain. Tel.: +34 56 016014; fax: +34 56 016019.
E-mail address: juanmiguel.mancera@uca.es (J.M. Mancera).

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stressful stimuli, i.e. fish stocking density (Kleszczyńska and Kulczykowska, 2006).

The main pineal indole melatonin (*N*-acetyl-5-methoxytryptamine: MEL), is a pleiotropic hormone influencing many physiological processes and behaviours in vertebrates, including fish (Ekström and Meissl, 1997; Kulczykowska, 2002; Dubocovich and Markowska, 2005). An effect of stress on plasma MEL levels seems to be dependent on the type of stress stimuli (Relkin, 1989; Kulczykowska, 2001; Larson et al., 2004). However, the mechanism of MEL regulation in response to stress is not clear. It has been proposed in *O. mykiss*, that MEL synthesis is impaired by elevated AVT in circulation or in vasotocinergic nerve fibers projecting to the pineal organ (Kulczykowska; 2001). On the other hand, in many vertebrate species, MEL appears to be involved in the process of feeding and digesting (Harlow and Weekley, 1986; Sjöblom, 2005). The small intestine is probably an important source of extrapineal and extraretinal MEL, also in fish (Bubenik, 2002; Kulczykowska et al., 2006), and as such, a site of regulation of MEL synthesis.

The aim of the present study was to determine the response of AVT, IT and MEL in *S. auratus* subjected to two different types of stress: i) high density (HD) and ii) food deprivation (NF: non-fed) and explore the potential relationship between hormones. Fish were assigned to one of four treatments that lasted 14 days: 1) fed fish under normal low density (ND, 4 kg m^{-3}), 2) non-fed fish under ND 3) fed fish under high density (HD, 70 kg m^{-3}) and 4) non-fed fish under HD. We selected these factors because sea bream are exposed to both during aquaculture management, and there is evidence that they activate the stress response in *S. auratus* (Arends et al., 1999; Montero et al., 1999; Rotllant et al., 2001; Sangiao-Alvarellos et al., 2005).

2. Materials and methods

2.1. Animals and experimental conditions

Immature *S. auratus* (200–250 g body mass) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cadiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at Faculty of Marine Science (Puerto Real, Cádiz). Animals were acclimated to SW (38 p.p.t. salinity) in 300 L aquaria in an open system for at least 2 weeks. During the experiment (May 2005), fish were maintained under natural photoperiod and constant temperature (18 °C). 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. The described experiment complied with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals.

Fish were randomly assigned to other 300 L tanks containing a plasticized iron wire-net cage with a total volume of 25 L (inner cage diameter 0.60 m) to obtain a fish density of 4 kg m^{-3}. Fish were acclimated to this tank for 7 days. The wire-net cage in the tank was lifted (water depth about 15 cm) to increase the stocking density from 4 to 70 kg m^{-3}. Each tank was randomly assigned to one of 4 treatments (2 replicates/treatment): 1) fed fish under normal low density (ND, 4 kg m^{-3}); 2) non-fed (NF) fish under ND; 3) fed fish under high density (HD, 70 kg m^{-3}); 4) non-fed fish under HD. Food deprived fish did not receive food from the start of the experiment onwards. Food consumption did not differ for fish under ND and HD. After 14 days, 10 fish from each treatment (5 from each tank) were removed by dip-net and samples taken as described below. No mortality was observed. The same experimental model was previously applied in order to successfully activate the stress response in sea bream (Sangiao-Alvarellos et al., 2005).

2.2. Sampling

At the end of the experiment, fish were anaesthetized in 2-phenoxyethanol (8 mM; 1 mL/L water, Sigma-Aldrich), weighed and sampled. Sampling was started at 09:30 h. Blood was collected from the caudal peduncle into 1 mL ammonia-heparinized syringes. Plasma was separated by centrifugation (5 min at 10,000g), immediately frozen in liquid nitrogen and stored at −80 °C. The brain was dissected and collected pituitaries were frozen and kept at −80°C.

2.3. Analytical methods

AVT and IT in plasma and pituitary were determined by high-performance liquid chromatography (HPLC-FL) with fluorescence detection preceded by solid-phase extraction (SPE). HPLC assay was performed with a Beckman modular system (Beckman 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). 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, which is absorbed by the HPLC column and detected by spectrofluorometry. Peptides are derivatized proportionally to their individual concentrations in plasma, but the NBD background does not allow the calculation of AVT and IT molar concentrations. AVT and IT contents were measured in every pituitary and data were expressed as pmol of peptide per pituitary according to Gozdowska et al. (2006) and Kleszczyńska et al. (2006).

Table 1
Effect of density and feeding for 14 days on selected plasma parameters in *S. auratus*

Parameter	Density	Feeding condition	
		Fed	Non-fed
Cortisol (ng mL^{-1})	Normal	4.99±1.31	12.8±1.91*
	High	28.5±2.88#	39.9±2.67*#
Glucose levels (μmol mL^{-1})	Normal	3.73±0.23	4.34±0.30
	High	5.21±0.32#	6.21±0.35#
Lactate levels (μmol mL^{-1})	Normal	3.54±0.28	2.69±0.22*
	High	2.66±0.23#	3.11±0.11
Protein levels (mg mL^{-1})	Normal	43.20±1.01	40.76±0.72
	High	37.38±0.93#	34.09±1.33*#
Triglyceride levels (μmol mL^{-1})	Normal	1.37±0.152	0.94±0.04*
	High	0.99±0.07#	0.88±0.07*#

Normal density was 4 kg m^{-3}, high density was 70 kg m^{-3}.
Data are means±S.E.M., $n=10$ fish per group. *, Significantly different ($P<0.05$) from fed group at the same density. #, Significantly different ($P<0.05$) from normal density at the same feeding condition.

Plasma MEL of extracted samples was assayed in duplicate using total melatonin RIA kit (IBL, Hamburg, Germany). 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 were assayed in a Wallac Wizard γ-counter. The detection limit was 2.5 pg mL^{-1} of 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.

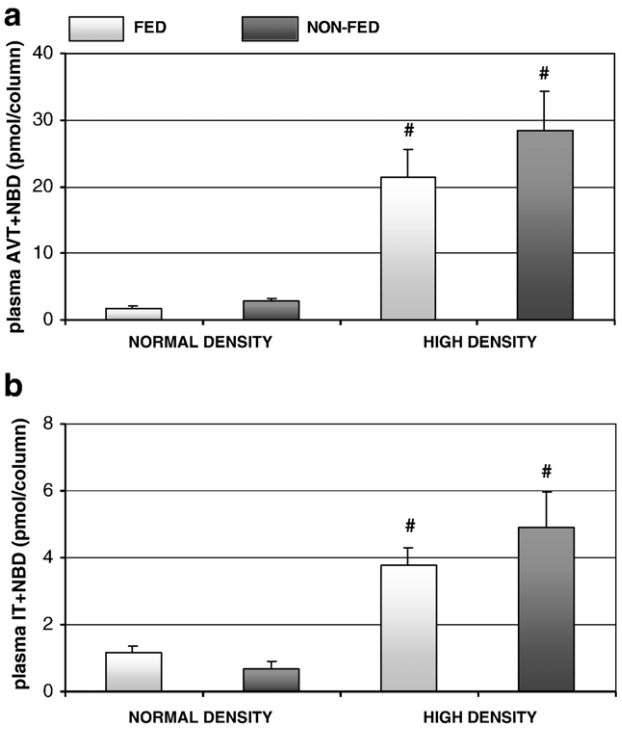

Fig. 1. Plasma AVT (a) and IT (b) concentrations in *S. auratus* under different density and feeding conditions. Arbitrary units: [AVT/IT+ NBD-F]-complex (pmol/column). Values are means±S.E.M., $n=8$ fish per group. Post-hoc comparisons were made using Tukey's test. #, Significantly different ($P<0.05$) from normal density at the same feeding condition.

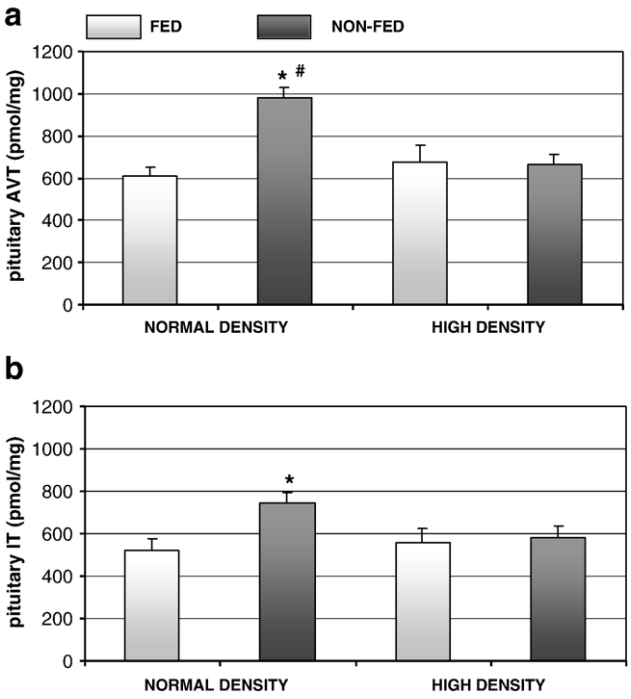

Fig. 2. Pituitary AVT (a) and IT (b) concentrations in *S. auratus* under different density and feeding conditions. Values are mean±S.E.M., $n=7–8$ fish per group. Post-hoc comparisons were made using the Spjotvoll/ Stolin test. *, Significantly different ($P<0.05$) from fed group at the same density. #, Significantly different ($P<0.05$) from normal density at the same feeding condition.

Plasma cortisol levels were quantified by enzyme-linked immunosorbent assay (ELISA) adapting the method described by Rodríguez et al. (2000) for testosterone. Cortisol was extracted from 5 μL plasma in 1.5 mL methanol. Cortisol standard, mouse anti-rabbit IgG monoclonal antibody, and specific anti-steroid express antibody and enzymatic tracer (steroid acetylcholinesterase conjugate) were purchased from Cayman Chemical Company (Michigan, USA). Microtiter plates (MaxiSorp™) were purchased from Nunc (Roskilde, Denmark). Standards and extracted plasma samples were run in duplicate. The lower limit of detection (90% of binding, ED90) was 0.37 ng mL^{-1} plasma. The inter-assay coefficient of variation at 50% of binding was 4.6% ($n=3$), while the mean intra-assay coefficient of variation (calculated from the samples duplicates) was 3.1%. The mean percentage of recovery was 95% ($n=4$). Main cross-reactivity (>1%; given by the supplier) for anti-cortisol express antibody was detected with prednisolone (22%), cortexolone (6.1%), cortisone (2.0%), and corticosterone (1.3%).

Plasma, 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 the standard. The assays were read on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments,

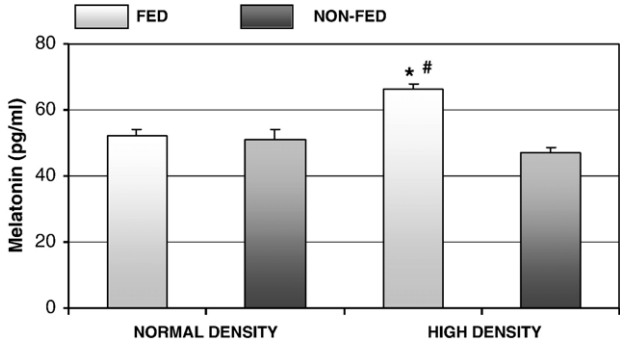

Fig. 3. Plasma MEL concentrations in *S. auratus* under different density and feeding conditions. Values are means ± S.E.M., $n = 7–8$ fish per group. Post-hoc comparisons were made using the Spjotvoll/Stolin test. *, Significantly different ($P < 0.05$) from non-fed group at the same density. #, Significantly different ($P < 0.05$) from normal density at the same feeding condition.

Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics, Inc. NJ, USA).

2.4. Statistics

The differences among groups were tested using two-way ANOVA with density (normal and high) and feeding conditions (fed and food deprived) as main factors. Post-hoc comparisons were made by the Tukey's test and the Spjotvoll/Stolin test. Results were considered significantly different when $P < 0.05$.

3. Results

Values of plasma parameters are shown in Table 1. Under HD conditions, plasma cortisol levels increased significantly in both fed and food deprived fish, but an effect was more pronounced in NF group. Plasma glucose increased significantly in all fish kept at HD. Plasma lactate decreased in fed fish under HD and food deprived fish under ND. Proteins and triglycerides levels decreased in response to density and feeding conditions and showed the lowest values in food deprived fish kept at HD.

Plasma AVT and IT values were enhanced in all fish kept at high density. The response of AVT was much stronger than that of IT (Fig. 1). The highest pituitary AVT and IT levels were shown in food deprived fish kept at normal density (Fig. 2).

The significantly higher plasma MEL levels were measured in fed fish kept at HD (Fig. 3).

4. Discussion

High density (HD) and food deprivation (NF) are two kinds of stressors frequently used to study stress system in teleosts, including *S. auratus* (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Sangiao-Alvarellos et al., 2005). This is the first study of AVT, IT and MEL responses in *S. auratus* subjected to one (HD or NF) or two combined stressors (HD plus NF). Our results on plasma cortisol, glucose, lactate and protein concentrations agreed with those reported previously for *S. auratus* exposed to similar experimental conditions (Sangiao-Alvarellos et al., 2005). Data indicated that the experimental model used here was effective in stimulating the stress response in this species (Arends et al., 1999; Power et al., 2000; Montero et al., 1999; Rotllant et al., 2001; Sangiao-Alvarellos et al., 2005). As it has been previously demonstrated, the sensitivity of *S. auratus* to the stress induced by HD conditions is intensified by food deprivation (Sangiao-Alvarellos et al., 2005).

AVT plays a clear osmoregulatory role in fish, with evident effects on osmoregulatory organs. It has been shown that the synthesis of AVT in hypothalamus and its secretion into circulation from the neurohypophysis are sensitive to an important environmental factor: salinity. There are three main osmoregulatory organs in fish: gill, GIT and kidney, which are considered as potential goals for neurohypophysial nonapetides action (Warne, 2002; Warne et al., 2002; Kulczykowska, 2007). In addition, a possible role of AVT in stress response in teleosts has been suggested (Gilchriest et al., 2000; Kulczykowska, 2001; Balment et al., 2006). According to this hypothesis, higher hypothalamic AVT expression and plasma AVT concentration could be expected in stressed fish. Our results showed clearly a significant increase in plasma AVT in all fish subjected to high density; food deprivation seems to intensify this effect slightly. In sea bass, the specific AVT binding sites in adrenocorticotropin (ACTH)-producing cells of the pituitary have been observed (Moons et al., 1989). Furthermore, in goldfish and trout, AVT is known to synergize with CRF to enhance ACTH release, which, in turn, increases plasma cortisol levels (Fryer and Leung, 1982; Baker et al., 1996; Pierson et al., 1996). Thus high AVT level observed in *S. auratus* subjected to HD in this study could also result in enhanced cortisol level which was indeed the highest in fed fish and food deprived fish under HD. In animals under stress, mobilization of metabolic substrates is necessary to cope with higher energy demand. AVT receptors have been found in hepatocytes of *O. mykiss* (Guibbolini et al., 2000) and AVT treatment is associated with increased glycogenolytic potential in liver (Janssens and Lowrey, 1987; Moon and Mommsen, 1990). Thus, the higher plasma AVT level observed in *S. auratus* under HD, could support cortisol to trigger a typical reaction to stress, which results in increased plasma glucose level.

To our knowledge, the evidence for a potential role of IT in the stress response of fish is scarce. It has been demonstrated that osmotic or acute disturbance stress did not affect plasma IT level in *O. mykiss* (Kulczykowska, 2001). On the other hand, IT appears to be involved in the response of female sticklebacks to fish stocking density (Kleszczyńska and Kulczykowska, 2006). Our results indicate that in *S. auratus*, plasma IT level increases significantly in response to HD, while food deprivation has no effect. We can only speculate here that IT responds to very specific stimuli, for instance, high density. Future studies involving new experimental approaches (IT injection, different stress stimuli) and/or analytical studies (localization of receptors, expression of hormone and receptors, etc.) are needed to clarify IT's potential role in stress response.

Pituitary AVT and IT contents were the highest in food deprived *S. auratus* at normal density. It may suggest an activation of synthesis of both peptides in hypothalamic neurons followed

by their storage in pituitary in response to food deprivation. This is not a case in fed fish, in which pituitary and plasma hormone levels are significantly lower. In fish subjected to HD, pituitary hormone levels are comparable to those observed in fed fish under normal density, but their plasma levels are significantly higher. It may suggest an intense release of hormones from pituitary to circulation together with their enhanced synthesis in hypothalamic neurons. Thus a stress produced by high density condition seems to be a strong stimulus for release of nonapetides from pituitary to circulation, but food deprivation seems to affect hypothalamic synthesis of the hormones. The results show a parallel response of AVT and IT to both stressors, i.e. food deprivation and high density, and point to a joint mechanism of regulation. On the other hand, discrete roles and control mechanisms of both nonapeptides have been suggested previously, in *S. auratus* during adaptation to different salinities (Kleszczyńska et al., 2006) and in rainbow trout exposed to osmotic and disturbance stress (Kulczykowska 1997, 2001). Further studies on the expression of provasotocin and proisotocin genes in hypothalamus are needed to elucidate the cause of this apparent difference in neurohypophysial peptides' regulation and response to various stressors in fish.

Melatonin is known as a pleiotropic agent involved in different physiological processes in fish, i.e. rhythmic adaptation, reproduction, osmoregulation, etc. (Ekström and Meissl, 1997; Kulczykowska, 2002; Kulczykowska et al., 2006). The influence of stress on plasma MEL levels seems to be dependent on the type of stress stimuli. Thus, in tilapia, *O. mossambicus*, chronically subjected to extreme osmolality, pH and temperature, increased plasma MEL levels were observed (Relkin, 1989). Also chronic social stress enhanced MEL levels in *O. mykiss* (Larson et al., 2004). In *S. auratus*, kept at HD for two weeks, plasma MEL was also enhanced, similarly to that observed in other fish species exposed to chronic stressors. On the other hand, a decreased plasma MEL concentration was observed in *O. mykiss*, while exposed to acute disturbance stress (Kulczykowska, 2001). The mechanism of MEL response to acute stressors, linked to an impairment of MEL synthesis by elevated AVT in circulation or in vasotocinergic nerve fibers projecting to pineal organ, obviously did not apply to the chronically stressed sea bream. Interestingly, an increase of plasma MEL observed in *S. auratus* kept at HD was suppressed by food deprivation. In this study, MEL was measured during the day, when its synthesis in the pineal organ and retina was probably constrained and plasma concentration was low. However, there is an evidence that, in sea bass (*Dicentrarchus labrax*) and rainbow trout, the eye produces more MEL in the light than in the dark (Sanchez-Vazquez et al., 1997; Besseau et al., 2006). Thus some of the daytime MEL could also be from the eyes. On the other hand, in day light, the main extrapineal and extraretinal source of MEL in vertebrates is the small intestine (Bubenik and Pang, 1997; Van't Hof and Gwinner, 1999). The high levels of intestinal MEL, together with high affinity MEL binding sites, were reported in the small intestine of three fish species, including *S. auratus* by Kulczykowska et al. (2006). MEL appears to regulate food intake, digestion and motility in the intestinal tract in mammals (Harlow and Weekley, 1986; Bubenik, 2002; Kvetnoy et al., 2002). In human and rat, melatonin is known as a potent stimulant of mucosal bicarbonate secretion in duodenal lumen (Sjöblom, 2005). However, in fish, the role and regulation of MEL production in gastrointestinal tract (GIT) are not known yet. In this study, plasma MEL was high in fed fish kept at HD, suggesting that the hormone synthesis in the GIT is stimulated by both: the presence of food and the chronic HD stress. However, at the present state of our knowledge, we can only speculate, that GIT MEL plays a role in the sea bream response to HD and that the mechanism of local stimulation of MEL synthesis in the intestine depends on the presence of food.

In conclusion, our results suggest a role of AVT, IT and MEL in response of sea bream to a common stress factor, high density. Although food deprivation does not influence AVT and IT plasma levels, it seems to affect hypothalamic synthesis of both. Parallel response of AVT and IT to both stressors points to a joint mechanism of nonapeptides' regulation. Probably, MEL synthesis in GIT depends on a presence of food at site of the hormone synthesis. Further studies are required to elucidate the complex role of AVT, IT and MEL in the sea bream stress response.

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