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Oral administration of melatonin counteracts several of the effects of chronic stress in rainbow trout

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ABSTRACT

To assess a possible antistress role of melatonin in fish, we orally administered melatonin to rainbow trout for 10 d and then kept the fish under normal or high stocking density conditions during the last 4 d. Food intake; biochemical parameters in plasma (cortisol, glucose, and lactate concentrations); liver (glucose and glycogen concentrations, and glycogen synthase activity); enzyme activities of amylase, lipase, and protease in foregut and midgut; and content of the hypothalamic neurotransmitters dopamine and serotonin, as well as their oxidized metabolites, 3,4-dihydroxyphenylacetic acid and 5-hydroxy-3-indoleacetic acid, were evaluated under those conditions. High stocking density conditions alone induced changes indicative of stress conditions in plasma cortisol concentrations, liver glycogenolytic potential, the activities of some digestive enzymes, and the 3,4-dihydroxyphenylacetic acid-to-dopamine and 5-hydroxy-3-indoleacetic acid-to-serotonin ratios in the hypothalamus. Melatonin treatment in nonstressed fish induced an increase in liver glycogenolytic potential, increased the activity of some digestive enzymes, and enhanced serotoninergic and dopaminergic metabolism in hypothalamus. The presence of melatonin in stressed fish resulted in a significant interaction with cortisol concentrations in plasma, glycogen content, and glycogen synthase activity in liver and dopaminergic and serotoninergic metabolism in the hypothalamus. In general, the presence of melatonin mitigated several of the effects induced by stress, supporting an antistress role for melatonin in rainbow trout.

1. Introduction

Stress effects are usual causes of undesirable damages in modern intensive fish cultures. High circulating concentrations of catecholamines and cortisol are involved in the first line of the integrated response to stress in fish [1]. Hormonal changes induced by stress trigger immediate tissue alterations to restore the effect of the stressor and to satisfy the increased energy demand. Thus, although catabolic actions in metabolic processes are considered the main secondary responses to stress, the effects at organism level such as alterations in food intake (FI), growth, and reproduction are considered as tertiary responses to stress [1,2].

Besides the complexity of neuroendocrine mechanisms of stress, a characteristic behavioral response to intensive stress in fish is a reduction in appetite [2]. Several central and peripheral peptides and hormones have been investigated in relation to their effects to induce or suppress fish appetite (reviewed in Volkoff et al [3]). Among them, a particular focus has been placed on corticotropin-releasing hormone, which initiates the activation of the hypothalamus-pituitary-interrenal (HPI) axis and seems to have a predominant role in the feeding inhibition under stress conditions [4]. Several other peptides, and also brain monoamine neurotransmitters such as serotonin (5HT) and dopamine (DA), which are important in triggering the initial steps of stress, are candidates to mediate some of the physiological and behavioral stress effects on FI [5,6].

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The hormone melatonin is mainly synthesized in the pineal organ and plays a crucial role in the regulation of seasonal and circadian rhythms of physiology and behavior in all vertebrates, including fish [7]. Melatonin is also likely to be synthesized in other fish organs such as the gastrointestinal tract (GIT) where it could be involved in the regulation of gut motility and other digestive- and metabolism-related processes [8–10]. As a multifunctional molecule, melatonin is believed to affect several neural and endocrine mechanisms, mediating relevant functions, such as feeding, osmoregulation, reproduction, and stress response [7]. An antistress function of melatonin at both central and peripheral levels has been proposed in vertebrates, including mammals [11,12] and birds [13]. In fish, there is some evidence about a suppressor effect of melatonin treatment on the HPI axis, such us reduction in glucocorticoid secretion and locomotor activity [14,15]. Other precursors of melatonin synthesis, such as 5HT and the amino acid L-tryptophan also have been reported to induce mitigating effects on stress responsiveness in fish [16].

The presumptive role of melatonin in FI of fish regulation is also far from fully understood. For instance, studies in goldfish (*Carassius auratus*) found that melatonin induced an anorexigenic effect when injected peripherally but not after central intravenous treatment [17]. An inhibitory effect of oral melatonin treatment on feeding was also described in European sea bass, *Dicentrarchus labrax* [18], and tench, *Tinca tinca* [19], suggesting that some melatonin effects on FI might not be centrally but peripherally mediated. However, a central action of melatonin is also possible because treatments with the hormone alter gene expression of some brain neuropeptides involved in feeding regulation in zebrafish, *Danio rerio* [20], and rainbow trout, *Oncorhynchus mykiss* [21]. Moreover, the brain monoaminergic systems that are believed to participate in the regulation of feeding behavior in fish [22,23] are also altered by melatonin treatments, which in addition mitigated some effects of stress [15].

Integrative studies in fish about the possible mitigating effect of melatonin on stress response are still lacking. The aim of this study was to explore a putative antistress role of melatonin in the rainbow trout by evaluating changes in some neuroendocrine and metabolic parameters related to the stress response. Food intake and the activity of some digestive enzymes were also measured. Melatonin was administered as a dietary supplementation to avoid any negative effect of fish handling and to better define changes in the assessment of the stress response.

2. Material and methods

2.1. Fish

The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and of the Spanish Government (RD 1201/2005) for the use of animals in research. Female rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Noia, Spain). Fish were maintained for 1 mo in 100-L tanks under laboratory conditions and a fixed 12:12 light/dark photoperiod (lights on at 8 AM; light intensity of 300 lux) in dechlorinated tap water at 15°C. Fish mass was 145 ± 3 g. Commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed) were used to fed fish once daily (10 AM) to satiety.

2.2. Experimental protocol

2.2.1. Preliminary experiment: Time course of plasma melatonin concentrations after oral administration

Food pellets were submerged in a solution with melatonin (Sigma, Indianapolis, IN, USA) at 2 different concentrations (0.04 and 0.2 g/kg food) and then dried at 37°C for 24 h. After acclimation, fish were separated into 2 experimental groups and fed with pellets supplemented with the 2 doses of melatonin. After anesthesia with MS-222 (50 mg/L) buffered to pH 7.4 with sodium bicarbonate, blood samples were taken before feeding and at 0.5, 1, 2, 4, and 6 h after feeding. Plasma was obtained after centrifuging blood samples for 10 min at 9,000 × *g* and stored at −80°C for melatonin analysis.

2.2.2. Effects of oral administration of melatonin on stress response

After acclimation, fish were distributed into 6 tanks and fed once daily (10 AM) for 10 d with commercial pellets alone (control) or supplemented with a low (0.04 g/kg food) or a high (0.2 g/kg food) concentration of melatonin (2 tanks per treatment). The last 4 d of the experiment, half of the tanks (1 tank per treatment) were kept at 10 kg fish mass/m^3 and denoted as nonstressed groups. In the remaining tanks (1 tank per treatment) a quantity of water was removed until reaching stressful high stocking density conditions (70 kg fish mass/m^3) and denoted as stressed groups. Therefore, 6 experimental treatments were used: a) nonstressed fish fed with commercial pellets, b) nonstressed fish fed with pellets supplemented with a low concentration of melatonin (0.04 g/kg food), c) nonstressed fish fed with pellets supplemented with a high concentration of melatonin (0.2 g/kg food), d) stressed fish fed with commercial pellets, e) stressed fish fed with pellets supplemented with a low concentration of melatonin (0.04 g/kg food), and f) stressed fish fed with pellets supplemented with a high concentration of melatonin (0.2 g/kg food).

On the last day, 4 h after feeding, fish were anesthetized with MS-222 (50 mg/L) buffered to pH 7.4 with sodium bicarbonate. Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation and divided into 2 aliquots. One aliquot was immediately frozen on liquid nitrogen for the assessment of plasma cortisol concentrations, whereas the other aliquot, for the assessment of plasma metabolites, was deproteinized immediately (using 0.6 *M* perchloric acid) and neutralized (using 1 mol/L potassium bicarbonate) before freezing on liquid nitrogen and storage at −80°C until further assay. Fish were sacrificed rapidly by decapitation, and the liver was removed, snap-frozen in liquid nitrogen, and stored at −80°C until assayed. Foregut and hindgut were removed, cleaned from

surrounding vessels and fat, opened, cleaned, snap-frozen in liquid nitrogen, and stored at −80°C until assayed. The brain was removed and placed on a chilled Petri dish, and the hypothalamus was obtained, snap-frozen in liquid nitrogen, and stored at −80°C until assayed.

Only the animals with food content in the GIT were evaluated.

2.3. Assessment of food intake

Food intake was registered throughout the experiment and also during the 10 previous days to determine the basal concentrations of this variable in each experimental tank. To assess FI, food was supplied in batches of 5 g until satiation; the overall process took approximately 10 min until fish did not eat any more. Uneaten food was removed from the tank, dried, and weighed to subtract this weight value to the amount of food offered (the loss of water during dry procedure was also taking in account). Values of FI registered after treatment are referred to those of basal values.

2.4. Concentrations of melatonin, cortisol, and metabolites in plasma and digestive enzyme activities

Plasma melatonin concentrations were assessed according to Muñoz et al [24] with modifications. Briefly, a 100-μL aliquot of plasma was mixed (1:1; vol/vol) with 0.1 *M* acetic acetate buffer (pH 4.6), and 1 mL of chloroform for 1 min, centrifuged (6000 × *g*, 10 min), and the aqueous phase was aspirated. The organic layer was separated, and 200 μL of 0.1 *M* NaOH was added. After stirring and centrifuging again, the aqueous phase was aspirated, and the organic layer was dried out under a Speed-vac Concentrator plus (Eppendorf, Hamburg, Germany). The residue was dissolved in 100 μL of mobile phase and filtered through a 0.5-μm filter. An aliquot (20 or 50 μL) of the filtrate was injected into the HPLC system. The chromatographic system consisted of a Gilson (Middleton, WI, USA) 321 solvent delivery pump equipped with a 50-μL Rheodyne (IDEX-HSS, Oak Harbor, WA, USA) injection valve, a 5-μm analytical column (Phenomenex Inc, Torrance, CA, USA; Kinetex C18, 50-mm length × 4.6-mm diameter) and a Jasco FP-1520 fluorescence detector (JASCO Corp, Tokyo, Japan) set at 280/345 nm excitation/emission wavelengths. The mobile phase consisted of a solution of 85 m*M* acetic acetate, 0.1 m*M* Na_2-EDTA, and acetonitrile (14% of final volume) with the pH adjusted to 4.7. All analyses were performed at room temperature at a flow rate of 1.0 mL/min. In tissues, melatonin content was assessed similarly to plasma by using the supernatant obtained after homogenization and centrifugation of 50 to 200 mg of each tissue (liver, foregut, and midgut) diluted in a volume of 0.1 molar acetic acetate buffer.

Plasma cortisol concentrations were analyzed by an ELISA kit (product 500360; Cayman Chemical Company, Ann Arbor, MI, USA) according to the instructions of the manufacturer. No sample extraction was required, and detection limit was approximately 35 pg/mL. Plasma glucose and lactate concentrations were determined enzymatically with commercial kits (Biomérieux, Madrid, Spain, and Spinreact, Girona, Spain, respectively) adapted to a microplate format. Tissues used for the assessment of metabolite concentrations were homogenized immediately by ultrasonic disruption with 7.5 volumes of ice-cooled 0.6 *M* perchloric acid and neutralized (using 1 *M* potassium bicarbonate). The homogenate was centrifuged, and the resulting supernatant was immediately assayed. Tissue glycogen concentrations were assessed with the method of Keppler and Decker [25]. Glucose obtained after glycogen breakdown (after subtracting free glucose concentrations) was determined with a commercial kit (Biomérieux).

Tissue pieces used to assess enzyme activities were homogenized by ultrasonic disruption with 10 volumes of ice-cold phosphorylation-dephosphorylation stopping buffer that contained 50 m*M* imidazole-HCl (pH 7.6), 15 m*M* 2-mercaptoethanol, 100 m*M* KF, 5 m*M* EDTA, 5 m*M* EGTA, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged, and the supernatant was used immediately for glycogen synthase (GSase) activity assay as previously described [26]. Gut tissue was homogenized as described earlier but with 4.5 volumes of ice-cold buffer of 100 m*M* Tris-HCl (pH 8.0). Amylase and lipase activities were determined with commercial kits (Spinreact) modified with microplates, whereas protease activity was assessed according to the casein method, using 1% casein as substrate; 1 unit of enzyme activity was defined as 1 mg of tyrosine released per minute and mg of protein. Enzyme activities were determined with a microplate reader INFINITE 200 PRO (Tecan, Grödig, Austria) and microplates. Enzymatic analyses were all performed at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities. Enzyme activities were normalized by milligram of protein. Protein was assayed in triplicate in homogenates with the use of microplates according to the bicinchoninic acid method, with bovine serum albumin (Sigma) as standard.

2.5. Assessment of monoamines and its metabolites

The contents of DA, 3,4-dihydroxyphenylacetic acid (DOPAC; a major DA metabolite), 5HT, and 5-hydroxyindole-3-acetic-acid (5HIAA; a major 5HT metabolite) in hypothalamus were analyzed by HPLC with electrochemical detection as previously described [27]. Tissues were weighed and homogenized by ultrasonic disruption in 0.5 mL of mobile phase used in the chromatography. Homogenates were centrifuged (16,000 × *g*, 10 min), and supernatants were diluted with mobile phase before the analysis. The HPLC system was equipped with a Jasco PU-2080 Plus pump connected to a Jasco AS-2057 autosampler, a 5-μm analytical column (Phenomenex Inc; Nucleosil C18, 150-mm length × 4.6-mm diameter) and an ESA Coulochem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials set at +40 mV (first electrode) and +340 mV (second electrode). The mobile phase was composed of 63.9 m*M* NaH_2PO_4, 0.1 m*M* Na_2EDTA, 0.80 m*M* sodium 1-octanesulfonate, and 15.3% (vol/vol) methanol; its pH was adjusted to 2.95 with orthophosphoric acid and was filtered (0.20-μm filter; Millipore, Bedford, MA USA) and degasified at vacuum before use. Analytical run time was 15 min at an isocratic flow rate of 1.0 mL/min at room temperature. Acquisition and integration of

chromatograms were performed with the ChromNAV version 1.12 software (Jasco Corp, Tokyo, Japan).

2.6. Statistical analysis

Comparisons among groups in the experiment were performed with 2-way ANOVA with doses of melatonin treatment (0, 0.04, and 0.2 g/kg) and stress (not stressed and stressed) as main factors. Post hoc comparisons were performed with Student-Newman-Keuls tests, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Time-course of plasma melatonin concentrations after oral administration of melatonin

A postprandial increase of plasma melatonin content was noticed 0.5 h after treatment with food containing both low and high doses of melatonin (Fig. 1). Concentrations of melatonin were high only for 60 min after treatment with the low dose, whereas with the higher dose they remained elevated up to 4 h then decreasing to values similar to those at the beginning (after 6 h of treatment).

3.2. Food intake

Food intake is shown in Figure 2. In controls, FI decreased by >50% under stress conditions (high stocking density), and this effect was abolished in the groups fed with dietary melatonin supplementation. Moreover, melatonin treatment per se induced a slight (nonsignificant) anorectic effect in nonstressed fish.

3.3. Plasma variables

Variables assessed in plasma are shown in Figure 3. Melatonin concentrations increased in groups fed with the high dose of melatonin after 4 h, but the increase was not significant in the nonstressed group. At the melatonin dose of 0.04 g/kg, the concentration of melatonin in plasma was unchanged compared with controls (Fig. 3A). Cortisol concentrations increased in control fish subjected to stress conditions, whereas no differences were observed in the groups that ate melatonin compared with nonstressed fish. A slight (nonsignificant) increase in cortisol concentration was noted in the melatonin-treated group in the absence of stress (Fig. 3B). No changes were found in plasma glucose concentrations related to the supplement of melatonin in nonstressed fish (Fig. 3C), whereas in the stressed group values were lower than their respective unstressed groups (this difference was significant at low and high melatonin doses). The concentration of lactate in plasma did not show any significant differences (Fig. 3D).

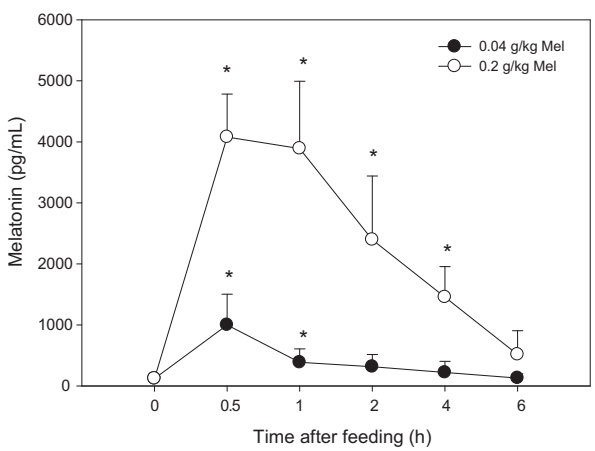

Fig. 1. Time-course of plasma melatonin concentration in rainbow trout fed with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin. Values represent the mean ± SEM of 3 fish. * $P < 0.05$ vs basal value (time 0). Mel, melatonin.

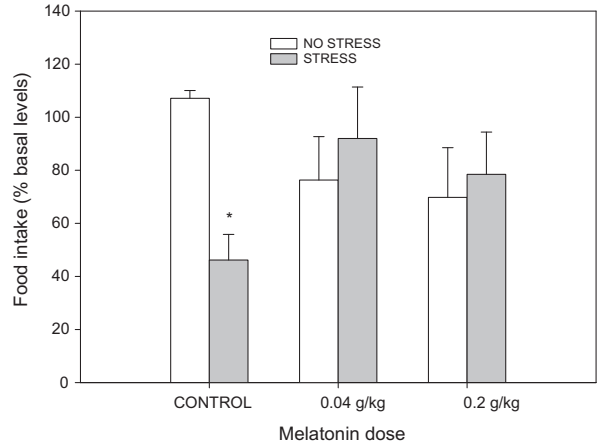

Fig. 2. Food intake of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin and kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) during the last 4 d. Values represent the mean ± SEM of food intake values of each group. Food intake values measured after treatment were referred to those of basal values. * $P < 0.05$ vs the unstressed fish group at the same melatonin treatment.

3.4. Liver variables

Liver variables are shown in Figure 4. Melatonin content in this tissue significantly increased at low and high doses of melatonin when fish were not stressed. Under stress conditions, only the high dose of melatonin induced a significant increase in melatonin concentration compared with the control group and its respective nonstressed group (Fig. 4A). Glucose concentration in liver decreased in stressed fish without melatonin and remained unaltered in the other groups (Fig. 4B). Glycogen content (Fig. 4C) decreased in stressed fish compared with unstressed fish; the content decreased in melatonin-treated fish under nonstressed conditions, whereas in stressed fish an increase was noted. A similar pattern was observed in GSase activity (Fig. 4D), which was lower in stressed fish without melatonin, whereas the decrease noted in activity induced by melatonin treatment in nonstressed fish disappeared in the stressed fish.

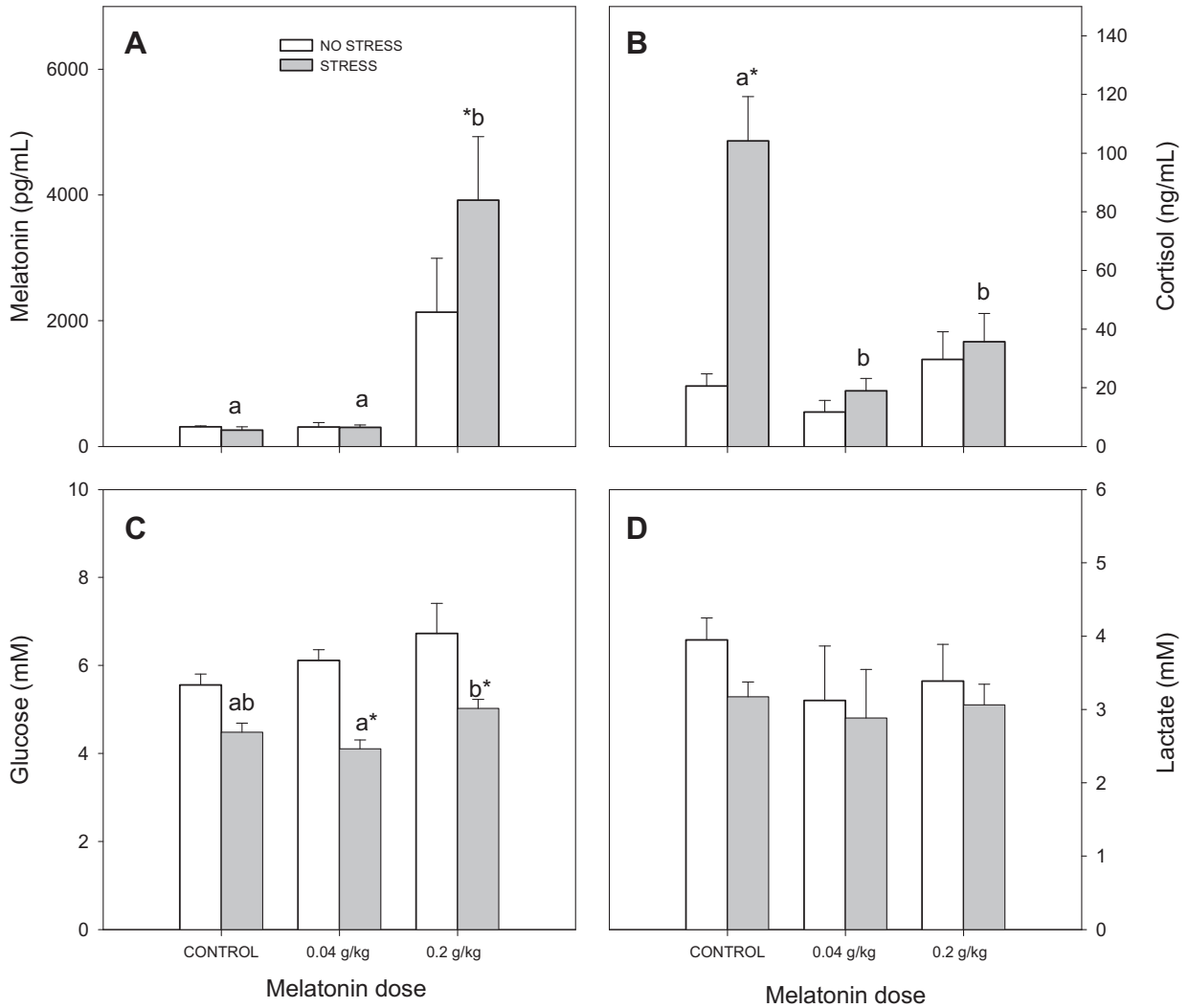

Fig. 3. Melatonin (A), cortisol (B), glucose (C), and lactate (D) concentrations in plasma of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) for 4 d. Values represent the mean ± SEM of 7 to 8 fish per group. * $P < 0.05$ vs the unstressed group within the same melatonin treatment. Different letters indicate significant ($P < 0.05$) differences among melatonin treatments within fish groups at the same stocking density (stress and no stress).

3.5. Foregut and midgut enzyme activities

Melatonin concentrations in foregut of either unstressed or stressed trout increased significantly with the high dose of melatonin (Fig. 5A). In contrast, in midgut the highest dose of melatonin induced a significant increase in tissue melatonin concentrations only when stress was present (Fig. 6A).

Changes in the activity of digestive enzymes are shown (Fig. 5B–D and Fig. 6B–D). Amylase activity decreased in stressed fish compared with the nonstressed group, although it was increased with the treatment of melatonin in both GIT regions (Figs. 5B and 6B). Lipase activity in foregut (Fig. 5C) displayed higher values in fish under crowding stress than in their respective unstressed groups, and these changes were significant only for the 0.2-g/kg dose of melatonin, whereas in midgut no significant changes were found (Fig. 6C). The activity of alkaline protease in foregut displayed differences between the control and the nonstressed fish treated with the high dose of melatonin (Fig. 5D). In midgut the activity of alkaline protease decreased with stress, but this difference disappeared with melatonin treatment (Fig. 6D).

3.6. Hypothalamic neurotransmitters

Melatonin treatment induced changes in the contents of monoamines (5HT and DA) and their main oxidative metabolites (5HIAA and DOPAC) in trout hypothalamus (Fig. 7). Monoamine contents increased in nonstressed and stressed fish treated with melatonin (Fig. 7A, D). Stress induced significant increases in the concentrations of the metabolites DOPAC and 5HIAA (Fig. 7B, E) that were suppressed when fish were treated with melatonin. The content of DOPAC increased in nonstressed fish treated with the lower dose of melatonin. These changes resulted in

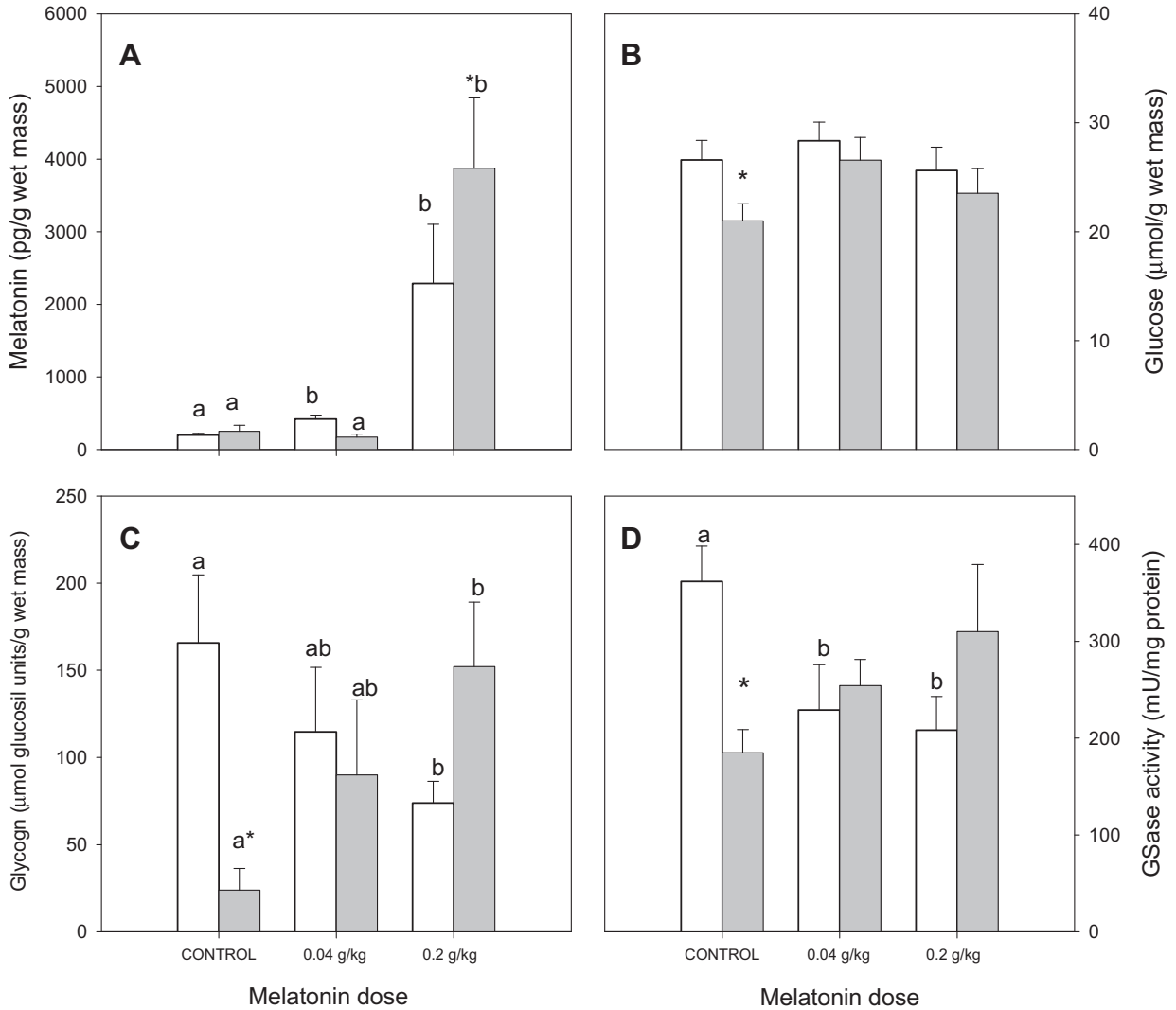

Fig. 4. Melatonin (A), glucose (B), and glycogen (C) concentrations and activity of GSase (D) in liver of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) for 4 d. Values represent the mean ± SEM of 7 to 8 fish per group. * $P < 0.05$ vs the unstressed group within the same melatonin treatment. Different letters indicate significant ($P < 0.05$) differences among melatonin treatments within fish groups at the same stocking density (stress and no stress). GSase, glycogen synthetase.

significant increases in the ratios of metabolite to monoamine (DOPAC/DA and 5HIAA/5HT) under stress conditions, and this effect was abolished in the presence of melatonin (Fig. 7C, F).

4. Discussion

High stock density is one of the most common stress situations in fish culture that lead to alterations in different parameters related to animal welfare, including hormonal and metabolic status and FI. Most of these changes are secondary to the activation of the HPI axis through enhanced corticotropin-releasing hormone production [4] which promotes cortisol release from interrenal cells. The increase in plasma cortisol concentrations observed in the rainbow trout kept under high stocking density confirmed the activation of the HPI axis and validates the experimental protocol (to induce a prolonged stress response). In addition, the observed reduction in FI under stress conditions is in agreement with similar studies in this species [28,29].

Another characteristic response to stress is the increase in glucose and lactate concentrations in plasma to satisfy the increased energy expenditure. However, in our experiment the stress response was not reflected by increases in those parameters, and even a decrease was noted for plasma glucose concentrations in the stressed group. This was probably because of prolonged stress of 4 d, resulting in a sustained consumption of energy resources (limiting glucose availability in blood). The decreased hepatic contents of glycogen and glucose also agree with data in the same species under stress conditions [28,29] and are

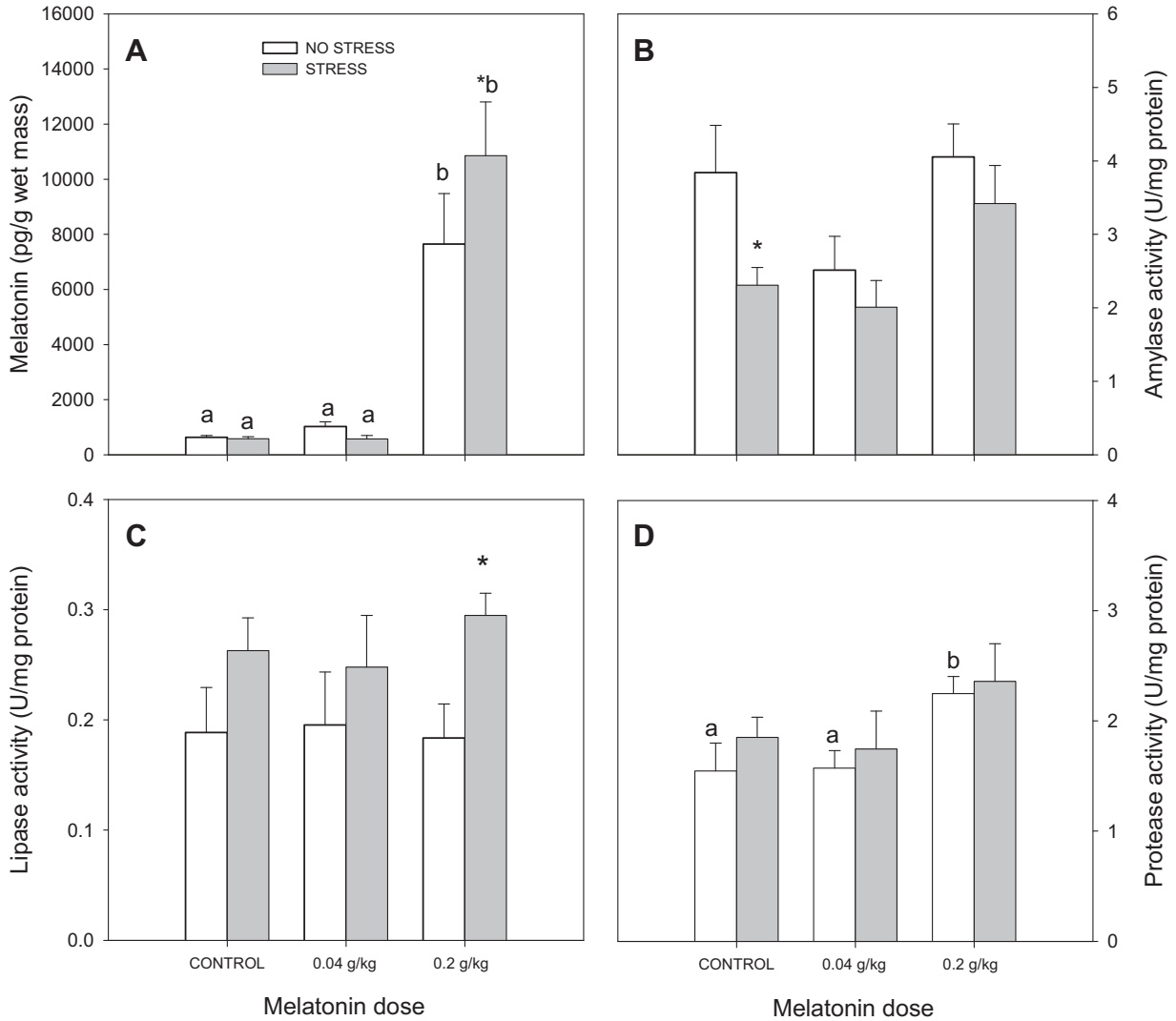

Fig. 5. Concentration of melatonin (A) and activities of amylase (B), lipase (C), and protease (D) in foregut of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) for 4 d. Values represent the mean ± SEM of 7 to 8 fish per group. * $P < 0.05$ vs the unstressed group within the same melatonin treatment. Different letters indicate significant ($P < 0.05$) differences among melatonin treatments within fish groups at the same stocking density (stress and no stress).

thought to result from activation of glycogenolysis and gluconeogenesis in response to increased plasma catecholamines and cortisol [1]. The decreased GSase activity found in liver of stressed fish is also in agreement with changes observed in glycogen content.

It is known that behavioral and physiological stress responses in fish are linked by common control mechanisms in the brain [5]. Several brain monoamine neurotransmitters, in particular 5HT and DA, have been suggested to respond to handling and predator exposure by increasing concentrations of their major metabolites (5HIAA and DOPAC, for serotonin and dopamine, respectively) [30,31] with similar consequences in the DOPAC-to-DA and 5HIAA-to-5HT ratios, which are considered as indicators of the activity/release rate of both neurotransmitters [27,30,32]. Thus, fish under high stocking density conditions showed an enhanced hypothalamic serotoninergic and dopaminergic function as indicated by the high metabolite-to-monoamine ratios. These changes occurred in parallel with the activation of the HPI axis and can be interpreted as that both 5HTergic and DAergic neurons have a role in triggering the initial steps of the neural activation under stress [6]. Moreover, both 5HT and DA are known to participate in the central control of feeding behavior and are likely to be involved in the anorectic action of CRF [22,23]. Taken together, these data suggest that decreased FI observed in stressed trout may be mediated, at least in part, by an increased hypothalamic dopaminergic and serotoninergic activity.

Studies in mammals have allowed us to know that GIT is responsive and sensitive to a wide range of stressors [33,34]. In addition, a number of studies in fish support a role of stress on digestive morphology and function. Thus,

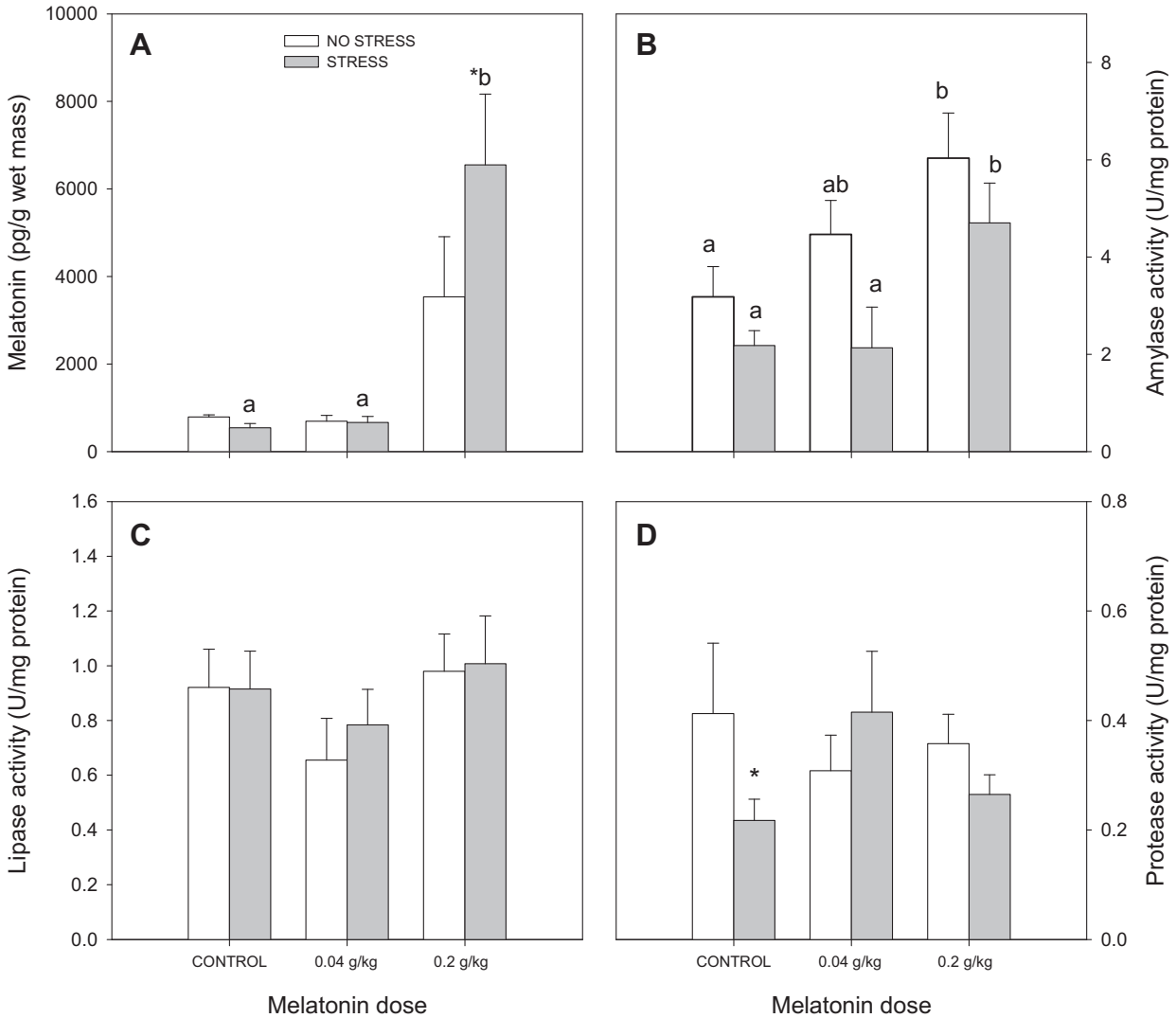

Fig. 6. Concentration of melatonin (A), and activities of amylase (B), lipase (C), and protease (D) in midgut of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) for 4 d. Values represent the mean ± SEM of 7 to 8 fish per group. * $P < 0.05$ vs the unstressed group within the same melatonin treatment. Different letters indicate significant ($P < 0.05$) differences among melatonin treatments within fish groups at the same stocking density (stress and no stress).

several ultrastructural and functional damages were found in the intestine of rainbow trout after acute stress, affecting enterocyte membrane properties and permeability among others [35]. The observed damages may lead to enhanced uptake of potentially noxious materials, that is, increased bacterial translocation, and inflammatory response with consequences on digestive activity and nutritional state. Therefore, the assessment of activities of digestive enzymes has been suggested as an indicator of digestive condition of fish kept under stress [36]. However, in our study few changes in enzyme activities were noted in stressed trout. These changes suggested a decreased potential for digestive capacity of carbohydrates (amylase activity in foregut) and proteins (alkaline protease in midgut) under stress, which could be related to local oxidative stress processes or could indirectly result from endocrine disturbance [36]. The possibility that elevated cortisol concentrations mediate the changes induced by stress in the activity of digestive enzymes would deserve further studies.

The supplementation of the diet with melatonin was apparently an appropriate way of administration for this hormone because plasma values of this hormone increased after oral treatment in all tissues studied. This type of treatment avoids invasive procedures, that is, intraperitoneal or central intravenous injections, which are stressful for the animals. Moreover, the concentrations of melatonin observed in plasma of trout receiving melatonin-supplemented food were among 2-fold (lower dose) and 10-fold (higher dose) higher than those measured normally in vivo at night (approximately 300–400 pg/mL) [37]. The higher values of plasma melatonin obtained with both oral

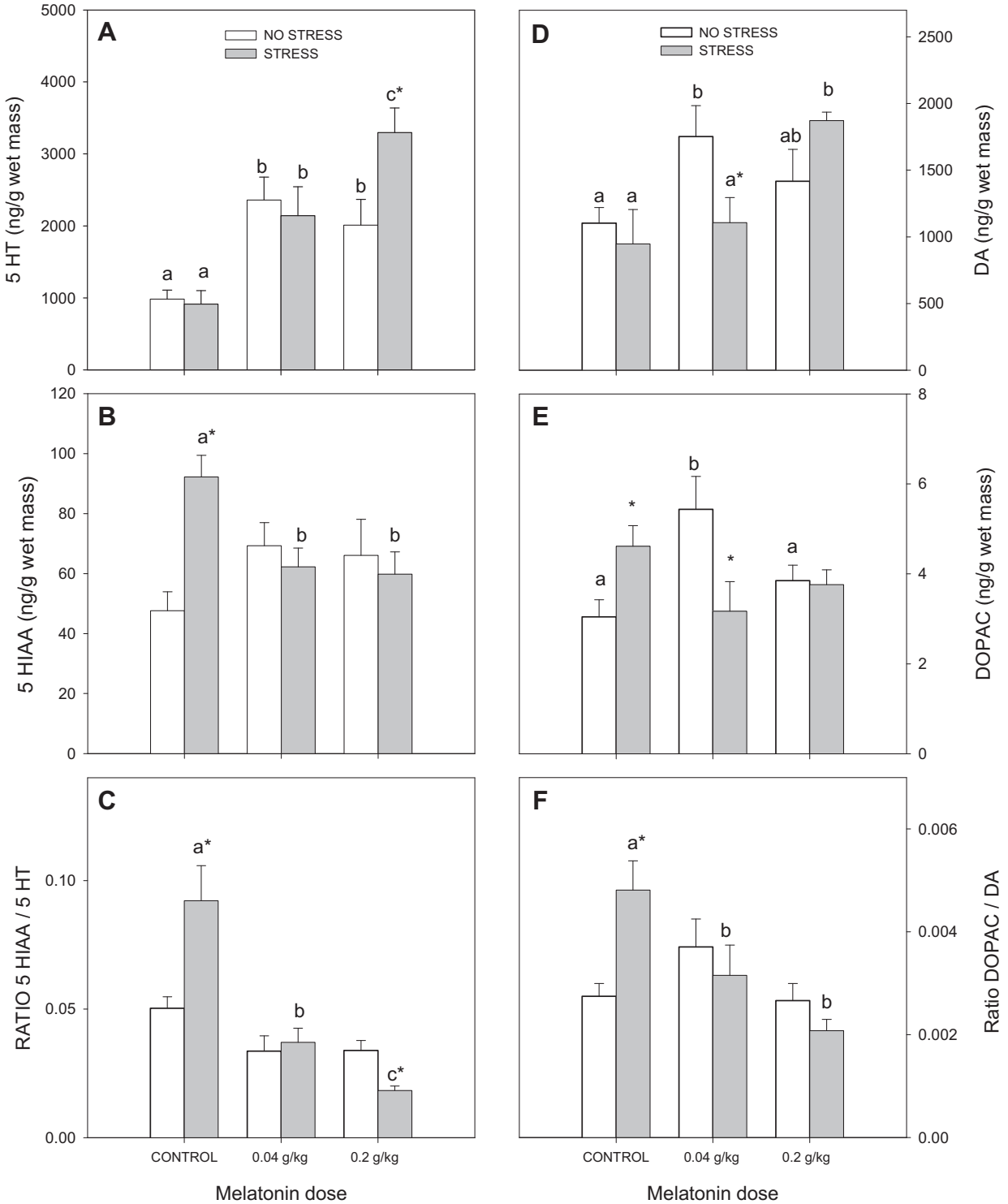

Fig. 7. Serotonin (A), 5HIAA (B), DA (D), and DOPAC (E) contents and 5HIAA-to-5HT (C) and DOPAC-to-DA (F) ratios in the hypothalamus of rainbow trout fed with commercial food (control) or with food supplemented with a low (0.04 g/kg) or a high (0.2 g/kg) dose of melatonin kept at normal stocking density (NO STRESS; 10 kg/m^3) or high stocking density (STRESS; 70 kg/m^3) for 4 d. Values represent the mean ± SEM of 7 to 8 fish per group. * $P < 0.05$ vs the unstressed group within the same melatonin treatment. Different letters indicate significant ($P < 0.05$) differences among melatonin treatments within fish groups at the same stocking density (stress and no stress). DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5HIAA, 5-hydroxy-3-indoleacetic acid; 5HT, serotonin.

doses were noted as soon as 0.5 h after food administration and remained elevated for 4 h after feeding with the higher dose. With the low dose of melatonin plasma concentrations of the hormone decreased rapidly after feeding (1 h), which is in agreement with the rapid clearance rate of melatonin in plasma [38].

Melatonin has been reported to have anorexigenic properties in some fish species, such as goldfish [17], tench [19], and European sea bass [18]. Our results in trout partially agree with a possible modulatory role of melatonin because nonstressed fish receiving oral melatonin tended to decrease FI. Moreover, this effect seems to be independent of changes in plasma cortisol, and some metabolic resources (plasma glucose and lactate concentration) that were not modified by treatments, which is also in agreement with previous studies in trout receiving intraperitoneal melatonin [10]. In contrast, fish fed with melatonin-supplemented food exhibited an increased content of 5HT, DA, and DOPAC in the hypothalamus, suggesting that alterations in these neurotransmitters might have a role in the inhibition of FI associated with melatonin treatment.

In liver, a clear glycogenolytic effect of melatonin (decreased glycogen concentrations and decreased GSase activity) was found, which is in agreement with decreased liver glycogen content previously reported in goldfish [39] and trout [40] treated with melatonin. Meanwhile, at the intestine few effects of melatonin were observed in the activity of studied digestive enzymes in unstressed fish, and only with the higher dose of melatonin used was an increase noted in the activity of alkaline protease in foregut and the amylase activity in midgut. Interestingly, the effects of melatonin alone (in general an up-regulation of the activity of digestive enzymes) were the converse than those elicited by stress.

Changes induced by high stocking density were largely modified by melatonin treatment for several parameters assessed in plasma, hypothalamus, and liver and in a few of those assessed in foregut or midgut. Food intake was higher in stressed fish receiving food supplemented with melatonin than in fish receiving control food. For plasma cortisol values, a clear suppressive action was noted in stressed trout that received oral melatonin, suggesting that increased melatonin concentrations in blood reduced the activation of the HPI axis under a prolonged stress condition. This effect is in agreement with results of a recent study showing that melatonin treatment (dissolved in the water of the tanks) mitigates stress-related neuroendocrine and metabolic effects in Senegalese sole exposed to high stocking density and low-water replacement [15]. Oral melatonin treatment also reduced the handling-induced increase in cortisol concentrations in European sea bass [41], which also agrees with the present study. An antistress effect of melatonin also has been suggested in other vertebrates such as birds [13] and mammals, including rodents [11] and primates [12], for which melatonin inhibits the activation of pituitary adrenal axis or directly attenuates ACTH responses in the adrenal gland. In addition, our results also show that melatonin effects in both FI and plasma cortisol concentrations were different, depending on whether the HPI axis was activated, thus suggesting that either peripheral or central melatonin actions are modulated by stress.

In addition to melatonin, an antistress effect has been reported for L-tryptophan in trout because elevated dietary intake of this amino acid decreased either basal or stress-induced elevated plasma cortisol values [16]. Because L-tryptophan is the precursor of neuronal 5HT, some of the antistress effects of L-tryptophan were suggested to involve changes in brain 5HTergic activity. In our study, the most relevant data in relation with the effect of melatonin treatment on hypothalamic 5HTergic activity in stressed trout was a reduction in 5HIAA content and the 5HIAA-to-5HT ratio, then suggesting that melatonin treatment operates in a sense contrary to stress at this level. In addition, the DOPAC-to-DA ratio (which was enhanced under stress) was reduced by melatonin treatment. These results agree with previous data reported in Senegalese sole showing that melatonin is able to modulate the stress-associated changes in brain monoaminergic neurotransmitters [15]. Because melatonin treatment also partially recovered FI in stressed trout, a role for brain neurotransmitters (in particular 5HT) can be suggested to interplay in the potential action of melatonin on neural circuits that integrate the inhibitory stress signals on FI.

To conclude, crowding stress associated with high stocking density induced changes in parameters related to stress response in rainbow trout (decreased FI, increased plasma cortisol concentrations, enhanced liver glycogenolytic potential, decreased activity of some digestive enzymes, and increased DOPAC-to-DA and 5HIAA-to-5HT ratios in the hypothalamus) that agree in general with those previously reported in the same species. Melatonin treatment in nonstressed fish increased liver glycogenolytic potential and the activity of some digestive enzymes. In stressed trout, oral melatonin treatment mitigated most of the effects induced by stress in the parameters evaluated, supporting that this hormone has an antistress role in rainbow trout. Overall, our results suggest a different role of melatonin in unstressed and stressed fish that could be explained by complex interactions with central or peripheral mechanisms or both mechanisms related to the activation of the HPI axis. Future studies specifically addressed to evaluate separately the interaction of melatonin with brain-pituitary processes involved in the stress response and also the effect on interrenal glucocorticoid secretion under basal and stress-related hormonal activation would be useful for understanding the role of melatonin to mitigate stress effects in cultured fish.

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