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Effect of L-tryptophan and melatonin supplementation on the serotonin gastrointestinal content and digestive enzymatic activity for *Salmo salar* and *Oncorhynchus kisutch*

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ABSTRACT

Several studies describe gastrointestinal tract (GIT) melatonin (MEL) synthesis from 5-HT, which itself derives from the essential amino acid L-tryptophan (L-trp) in the intestine. Supplementing L-trp and MEL through diet has shown social-response effects and suppresses neuroendocrine stress in teleosts. In this study, the effects of a MEL and L-trp-supplemented diet on the endocrine intestinal function and enzymatic response activity of two salmonid species were examined. To assess the possible effect of L-trp and MEL on intestinal serotonin content and digestive enzyme activity, three L-trp-supplemented diets and three MEL-supplemented diets were orally administered to a group of *Salmo salar* and *Oncorhynchus kisutch* for seven days under normal density conditions. Plasma biochemistry (cortisol, L-trp, MEL) as well as enzyme activity (amylase, lipase, and total protease) and serotonin content were measured in the pyloric caeca, midgut, and hindgut. Plasma L-trp levels were found to be directly related to L-trp supplemented diet levels. Similarly, MEL supplementation increased plasma MEL levels, and the presence of MEL in both salmon species resulted in a significant interaction with cortisol concentrations in plasma, and the highest concentrations of L-trp caused an increased GIT content for 5-HT in *S. salar*. No differences were seen in the GIT content for 5-HT for the L-trp supplemented diets in *O. kisutch*. An inhibitory effect was found on digestive enzymes in the supplemented diets of both salmonid species. In general, the presence of MEL in the diet reduced cortisol levels; diets supplemented with L-trp and MEL had either a stimulatory or inhibitory effect on digestive enzyme activity, which seemed to be indirect and tissue dependent.

1. Introduction

Serotonin (5-HT) and melatonin (MEL) are neuroendocrine transmitters with a wide array of biological activities in organisms (Míguez et al., 1995; Muñoz-Pérez et al., 2016). Several studies agree that MEL synthesis occurs from 5-HT in the gastrointestinal tract (GIT), and this latter is derived from the essential amino acid L-tryptophan (L-trp) in mammal, bird, and fish intestines (Muñoz-Pérez et al., 2016). In addition, this amino acid is indispensable in all fish species (National Research Council (NRC), 2011). Previous studies have reported behavioural effects of dietary Trp supplementation and MEL, which are related to neuroendocrine responses, such as decreased aggression and stress attenuation, in several teleost species as Tilapia, Atlantic Cod and Rohu (Herrera et al., 2017; Kumar et al., 2014; Martins et al., 2013), and particularly salmonids as *Salmo salar* (Atlantic salmon) and *Oncorhynchus mykiss* (rainbow trout) (Basic et al., 2013; Höglund et al., 2007; Lepage et al., 2002; Winberg et al., 2001; Wolkers et al., 2012). The ability of MEL to reduce the stress effects in fish has been investigated in different teleost species, including *O. mykiss*, *Carassius auratus* (goldfish), and *Solea senegalensis* (Senegalese sole) (Azpeleta et al., 2010; Conde-Sieira et al., 2014; Herrero et al., 2006; López-Patiño et al., 2013). A few studies have also attributed L-trp and MEL supplemented diets to a stimulating effect on the digestive enzyme activity in fish and mammals (Jaworek et al., 2004; Thakur et al., 2006). Such supplemental increases in L-trp should lead to increased serotonin (5-HT) and MEL levels in the GIT. Oral supplementation with L-trp and MEL lead to an increased serotonin (5-HT) and MEL levels in the GIT.

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5-HT is a very important neurotransmitter in the digestive processes. It is synthesized in the GIT, specifically in the mucosa enterocromafin cells (EC), which contain high 5-HT levels (Gershon and Tack, 2007). High 5-HT concentrations were found in the *O. mykiss* GIT wall, mostly related to myenteric plexus neurons (Anderson and Campbell, 1988; Caamaño-Tubío et al., 2007; Muñoz-Pérez et al., 2016). This neurotransmitter is synthesized from the neutral essential amino acid L-trp, which is brought into the cell through an active transport mechanism for neutral amino acids. In the cell, tryptophan hydroxylation, catalysed by tryptophan hydroxylase (TpOH), synthesizes hydroxytryptophan, which is then decarboxylated by decarboxylase, forming 5-HT (Senatori et al., 2003). MEL is synthesized from 5-HT by *N*-acetylation followed by methylation (Muñoz et al., 2009). MEL receptors has been described in pancreatic tissue of mammals (Jaworek et al., 2004), so that it could participate in the regulation of GIT enzymatic activity, even in fish.

Alkaline digestion in fish takes place in the intestine, using hydrolytic enzymes (lipase, amylase and alkaline proteases). The influence of diet composition, amount of food, salinity, and feeding habits on digestive enzyme activity has been studied (Montoya et al., 2010; Vargas-Chacoff et al., 2015). Other studies have compared the enzyme activity of species with different eating habits, showing great variability (Furné et al., 2005; Hidalgo et al., 1999). *S. salar*, for example, have pancreatic enzyme activity that decreases aborally along the intestinal tract (Chikwati et al., 2012; Chikwati et al., 2013; Krogdahl et al., 2015).

In vertebrates was described exocrine pancreatic secretion is controlled by autonomic neural reflexes triggered by gastrointestinal hormones (Konturek et al., 2003). Cholecystokinin (CCK) and secretin are the main pancreatic secretagogues. Exogenous MEL or L-trp causes a dose-dependent stimulation of pancreatic amylase secretion in mice (Jaworek et al., 2004). There are a few published studies on the effects of L-trp on intestinal enzymes. For example, an *in vitro* study in rats showed the activation of amylase, lipase, and trypsin (Svatos, 1994), and another study in *Cyprinus carpio* var. *Jian* (Jian carp) described the positive or negative effects of L-trp over intestinal enzymes (Tang et al., 2013).

Knowledge of digestive physiology is essential in fish aquaculture, and our current understanding of those processes is still limited (Volkoff et al., 2005). This study will examine the effects of supplementing fish diets in two economically important aquaculture fish species to improve aquaculture practice and gain a better understanding of the impacts to digestive physiology. The specific objective is to quantify and compare the gastrointestinal 5-HT content and digestive enzyme activity in the pyloric caeca, midgut, and hindgut, of smoltified *Salmo salar* (Atlantic salmon) and *Oncorhynchus kisutch* (Coho salmon) specimens in a pre-stress condition, after having supplementing their diet with different concentrations of L-trp and MEL and comparing this with control diet group.

2. Material and methods

2.1. Animals

A group of immature Atlantic salmon (100 ± 15 g body weight [Mean and SD], n = 84) and Coho salmon (150 ± 30 g body weight [Mean and SD], n = 84) in a post-smolt stage were obtained from Salmones Frío Sur fish farm (Hornopirén, Chile) and Salmones Austral fish farm (Rupanco lake, Chile), respectively. They were acclimated to seawater for 30 days in 1000 l tanks under laboratory conditions consisting of a 12:12 light:dark photoperiod, 12 ± 1 °C water temperature, 11 pp. salinity, and continuously renovated and aerated water. During the acclimation period, fish were fed daily at 11:00 h with commercial dry pellets for salmonids (Ewos, size 100, proximate food analysis was 45.5% crude protein, 20.5% lipids, 9.5% carbohydrates, 11% ashes, 11% water, and 2.5% fibre) at 1% of their body mass daily. All the experimental procedures and animal manipulation were designed according to the ethical handling of live animals' standards from the Chilean National Commission of Scientific and Technological Research (CONICYT), the Universidad de Los Lagos, and the Universidad Austral de Chile.

2.2. Experimental design

2.2.1. Supplemented diets experiment

Six different experimental MEL- and L-trp supplemented diets were prepared. Food pellets were submerged in a MEL solution (Sigma, Indianapolis, IN, USA) at three different concentrations, 0.002%, 0.01% and 0.05%, and dried at 37 °C for 24 h, following Conde-Sieira et al. (2014). Other food pellets were prepared with three different concentrations of L-trp, 0.5%, 1.5% and 2.5%, following Herrero et al. (2006). The feeds were identical in energy value and differed only in L-trp and MEL concentrations; the control feed contained 0.04% of L-trp and no detectable concentrations of MEL.

The fish were distributed into 14 tanks (20 fish per tank), kept at 10 kg/m^3, and fed once a day (10 am) for 10 days with commercial pellets (control) or supplemented with different concentrations of MEL (0.002%, 0.01%, or 0.05%) or L-trp (0.5%, 1.5%, or 2.5%). Tanks were randomly assigned to the MEL or L-trp-supplemented groups, dietary treatments were assessed in duplicates (six tanks per group).

2.3. Sampling procedure

On the final day (day 10), 240 min after feeding, fish were netted and deeply anesthetized by immersion in MS-222 (50 mg/kg), buffered to pH 7.4 with sodium bicarbonate. Fish were weighed and blood was collected from the caudal vein into 1 ml heparinized syringes (25,000 units of ammonium heparin, 3 ml saline solution, 0.6% NaCl). Plasma was separated from cells by whole blood centrifugation (5 min, 2000 × *g*, 12 °C), snap frozen in liquid N_2, and stored at − 80 °C until analysis for cortisol, MEL, and L-trp. Fish were then euthanized by spinal section, before removing samples of pyloric caeca, midgut, and hindgut. A portion of each sample (50–70 mg) was separated and frozen; one for MEL analysis and the other for 5-HT quantification, measured using HPLC techniques (Gesto et al., 2006; Muñoz-Pérez et al., 2016). For the evaluation of digestive enzyme activity, 100–120 mg samples of pyloric caeca, midgut, and hindgut were taken for colorimetrical quantification of amylase, lipase, and alkaline proteases (Vargas-Chacoff et al., 2015). Only animals with food content in the GIT were evaluated.

2.4. Analytical procedures

2.4.1. Serotonin

Prior to evaluating serotonin levels, tissues were homogenized by ultrasonic disruption in 0.5 ml of PCA (0.3 mM) and centrifuged (16,000 × *g*, 10 min). An HPLC system with electrochemical detection (HPLC-EC) was used for 5-HT quantification, following Gesto et al. (31). The HPLC system consisted of a Dionex ISO-3100 isocratic pump, a 5 μm analytical column, and an ESA Coulochem III electrochemical detector. The detection system included a dual analytical cell with oxidation potentials adjusted to + 40 mV (first electrode) and + 340 mV (second electrode). The mobile phase consisted of 63.9 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 1.63 mM Sodium 1-Octanesulfonate, and 14.9% methanol (v/v); pH was adjusted to 2.79 with phosphoric acid, filtered, and degassed before use. All measurements were performed at a flow of 0.8 ml/min. A Dionex (Sunnyvale, CA, USA) Chromeleon, version 6.8, chromatography data management system was used for system control and data collection. Quantification of the sample peaks was estimated in relation to the peak areas of their respective standards.

2.4.2. Digestive enzymatic activity

For digestive enzyme extracts, pyloric caeca, midgut, and hindgut

tissues were finely minced in an ice-cooled Petri dish, following Vargas-Chacoff et al. (2015). Tissue homogenization was performed using an Ika Ultra-turrax HG-15A with a buffer solution of 50 mM Tris-HCl pH 7.5 (Alarcón et al., 1998). The homogenates were subsequently centrifuged at 16,000*g* for 30 min at 4 °C, and the supernatant was recovered and stored at − 80 °C until further use. Determinations of the soluble protein in the enzyme extract were tested in triplicate, using the Pierce BCA Protein Assay Kit #23225, using bovine serum albumin as a standard protein. Total alkaline proteolytic activity was determined using 5% casein as substrate, following Psochiou et al. (2007). The α-amylase-like activity was quantified using 1% soluble starch as a substrate (Worthington and Worthington, 2011). Finally, bile salt-dependent lipase activity was quantified using 1 ml of a substrate consisting of 4-nitrophenyl caproate (4-NPC, 100 mM ethanol to give a final concentration of 0.35 mM in the reaction mixture), 6 mM sodium taurocholate (Na-TC), 0.1 M NaCl, and 0.5 M Tris-HCl at pH 7.4, following Gjellesvik et al. (1992). All enzyme assays were performed with a MultiscanGo Microplate Reader (Thermo Scientific), using ScanIT, version 3.2.

2.4.3. Plasma measures

MEL was measured using the Muñoz et al. (2009) protocol in 200 µl of plasma. The L-trp in plasma was measured following the Cervantes et al. (2009) protocol, using a HPLC-FD system and separating the amino acid with octadecylsilane column (5 µm particle size), using mobile phase methanol:water (20:80) at pH 4.0 and a flow of 1 ml/min.

Plasma cortisol concentrations were analysed using an ELISA kit (product 500360; Cayman Chemical Company, Ann Arbor, MI, USA) following manufacturer instructions.

2.5. Statistics

Assumptions of normality and homogeneity for the variances were tested. Logarithmic transformations of the data were performed as necessary to satisfy conditions for the parametric analysis of variance. A one-way ANOVA was performed for each physiological variable. A post-hoc Tukey-test was used to identify significantly different supplemented diet in each type of intestinal tissue. Differences in the experiment were considered significant at a level of $p < 0.05$.

3. Results

3.1. Plasma measures

MEL and L-trp plasma levels increased according to the dose (Table 1).

In the case of *S. salar* L-trp plasma levels, the diet supplemented with 0.5% L-trp showed L-trp levels nearly 8 times higher than the control group. The diet with 1.5% showed a further increase of 2.2, and the diet with 2.5% showed an additional increase of nearly 1.6 (Table 1). In *O. kisutch* plasma L-trp we found at L-trp 1.5 and 2.5% significantly increments higher than control. Cortisol plasma levels in our pre-stress individuals were significantly less than control for L-trp treatments of 1.5% and 2.5% in *S. salar* and 1.5% for *O. kisutch*.

Table 2
Plasma cortisol levels expressed as mean ± SEM for *S. salar* and *O. kisutch*. Data are represented as mean ± SEM from 8 to 10 animals for each diet. An asterisk (*) indicates significant differences between the control and MEL or L-trp diet-supplemented groups (one-way ANOVA, post-hoc Tukey-test, $p < 0.05$).

Diet	Plasma cortisol (ng/ml) *S. salar*	Plasma cortisol (ng/ml) *O. kisutch*
Control	10.12 ± 1.38	30.05 ± 4.5
L-trp 0.5%	11.41 ± 2.7	26.04 ± 7.1
L-trp 1.5%	5.33 ± 1.1*	17.81 ± 1.4*
L-trp 2.5%	5.52 ± 0.9*	21.99 ± 2.7
MEL 0.002%	11.18 ± 1.7	14.04 ± 3.1*
MEL 0.01%	8.60 ± 1.6	16.70 ± 2.4*
MEL 0.05%	3.97 ± 0.6*	15.54 ± 2.1*

For the plasma MEL levels, 0.002% supplemental diet showed an increase of 14 fold over the control in *S. salar* and 8 fold over control in *O. kisutch*. The diet supplemented with 0.01% showed a further 4.2 fold increase, and the diet supplemented with 0.05% MEL showed an additional 96 fold increase (Table 1). In the case of MEL-supplemented diets, cortisol levels were significantly reduced at 0.05% for *S. salar* and at 0.002%, 0.01%, and 0.05% for *O. kisutch.* (Table 2) in the pre-stress fish.

3.2. Quantification of 5-HT content in the GIT of Salmo salar and Oncorhynchus kisutch with L-trp and MEL supplemented diets

The gastrointestinal tract (GIT) 5-HT content was increased for *S. salar,* with higher doses of L-trp supplemented diets (Fig. 1A), but there were no differences in 5-HT content in any GIT tissue among the L-trp supplemented diets (Fig. 1C).

The gastrointestinal tract (GIT) 5-HT content of *S. salar* with MEL supplemented diets increased with the higher MEL doses (Fig. 1B). For *O. kisutch*, there were significant differences in 5-HT content in the pyloric caeca and hindgut at higher doses of MEL (Fig. 1D).

3.3. Quantification of digestive enzymatic activity in Salmo salar with L-trp and MEL-supplemented diets

Lipase activity in *S. salar* with L-trp supplemented diets showed no differences between L-trp doses (Fig. 2A). The alkaline protease activity with L-trp supplemented diets (Fig. 2C) shows an increase in the pyloric caeca and hindgut for higher L-trp doses. Finally, the amylase activity with L-trp supplemented diets showed an increase in the pyloric caeca and a decrease in midgut for all L-trp doses (Fig. 2E). MEL supplemented diets among *S. salar* showed no lipase activity differences in the pyloric caeca and hindgut, but did show a decrease in the midgut (Fig. 2B).

MEL supplemented diets showed an increase in alkaline protease activity in pyloric caeca and hindgut, but a decrease in midgut activity (Fig. 2D). Finally, there was a decrease in amylase activity for MEL

Table 1
Plasma melatonin and tryptophan measures expressed as mean ± SEM for *S. salar* and *O. kisutch*. Data are represented as mean ± SEM from 8 to 10 animals for each diet. An asterisk (*) indicates significant differences between control and MEL or L-trp diet-supplemented groups (one-way ANOVA, post-hoc Tukey-test, $p < 0.05$).

Diet	Plasma MEL (pg/ml) *S. salar*	Plasma trp (µg/ml) *S. salar*	Plasma MEL (pg/ml) *O. kisutch*	Plasma trp (µg/ml) *O. kisutch*
Control	51.46 ± 1.2	3.58 ± 0.2	85.71 ± 3.1	4.55 ± 0.2
L-trp 0.5%	61.12 ± 2.7	28.37 ± 2.2*	93.52 ± 6.3	15.69 ± 2.2
L-trp 1.5%	68.46 ± 3.2	64.29 ± 6.3*	97.21 ± 5.4	65.1 ± 6.3*
L-trp 2.5%	51.46 ± 1.2	101. 20 ± 9.1*	105.32 ± 2.4	91.18 ± 9.1*
MEL 0.002%	721.16 ± 4.7*	4.52 ± 0.8	697.36 ± 3.1*	4.11 ± 0.3
MEL 0.01%	2479.80 ± 163.9*	3.24 ± 0.4	2553.50 ± 171.9*	5.73 ± 0.4
MEL 0.05%	5993.00 ± 260.1*	4.17 ± 0.7	5834.10 ± 180.6*	3.38 ± 0.3

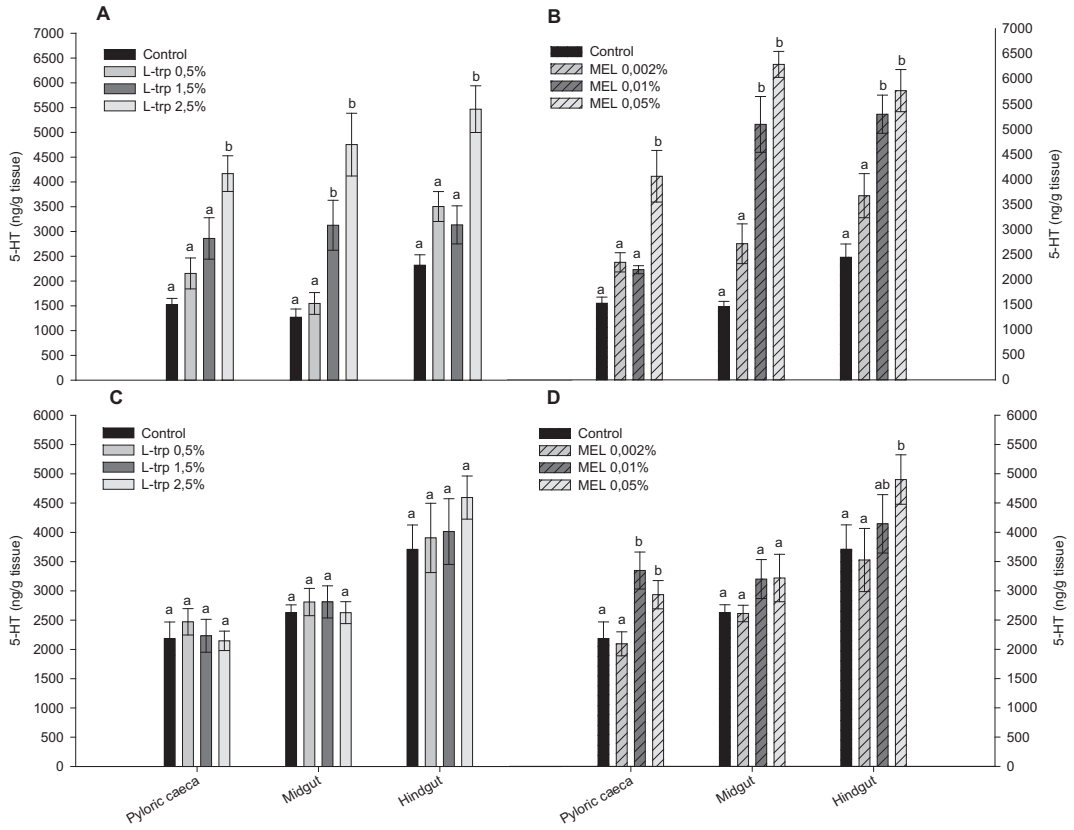

Fig. 1. 5-HT content by dietary supplementation of *S. salar* with different L-trp (A) or MEL (B) concentrations and 5-HT content by dietary supplementation of *O. kisutch* with different L-trp (C) or MEL (D) concentrations. Data are represented as mean ± SEM. 5-HT is expressed in ng/g of tissue in each TGI region, 8 to 10 animals for each diet. Different letters indicate statistically significant differences (one-way ANOVA, post-hoc Tukey-test, $p < 0.05$) compared with controls for the same tissue.

supplemented diets in the midgut and hindgut (Fig. 2F).

3.4. Quantification of digestive enzymatic activity in Oncorhynchus kisutch with L-trp and MEL supplemented diets

Among *O. kisutch*, there were no significant differences in lipase activity between L-trp supplemented diets in all GIT regions (Fig. 3A). The alkaline protease activity for L-trp supplemented diets showed no significant differences in the pyloric caeca and midgut among any of the L-trp supplemented diets, but there was a significant decrease in the hindgut for the L-trp 0.5% dose (Fig. 3C). The amylase activity for L-trp supplemented diets showed a decrease in the pyloric caeca for the L-trp 0.5% dose and a decrease in all L-trp doses in the midgut (Fig. 3E).

For *O. kisutch* GIT samples taken from MEL supplemented diets, there was a lipase activity increase in the midgut, but an activity decrease in pyloric caeca; there was no significant change in hindgut activity (Fig. 3B). There was a decrease in alkaline protease activity in the midgut for the 0.01% MEL dose, and an increase in activity in the hindgut for all MEL-supplemented diets (Fig. 3D). Among all MEL supplemented diets, there was a decrease of amylase activity in the pyloric caeca and midgut (Fig. 3F).

4. Discussion

4.1. L-trp

High doses of L-trp reduced plasma cortisol levels in *S. salar*, in our pre-stress fish, and also in *O. kisutch*. Several articles in fish exposed to stressors have described the effects of tryptophan supplementation diet, having effects on plasma cortisol levels, for example tryptophan reduces cortisol levels in fish exposed to high temperature (Kumar et al., 2014), exposure to air (Herrera et al., 2017), chasing (Martins et al., 2013) and high stocking density conditions (Conde-Sieira et al., 2014). In agreement with a previous study's results with *S. salar* (Basic et al., 2013), demonstrating a long-term (10 days) effects of elevated dietary L-trp on the neuroendocrine response suppressed cortisol levels in our pre-stress fish.

The diets supplemented with the highest L-trp concentrations caused increases in intestinal 5-HT content in *S. salar* tissues, but there were no such differences in the 5-HT intestinal content for the L-trp supplemented diets in *O. kisutch*. This difference indicates that there are variations according to species and the enzyme activity that synthesizes 5-HT from L-trp. This is the first report, to our knowledge, about the effects of L-trp supplemented diets on 5-HT intestinal content in fish, the next step is study the origin of these serotonin.

In fish, very little is known about the metabolic regulation of intestinal 5-HT, although studies in sea bass demonstrated a progressive increase of plasma 5-HT after the intake of diets supplemented with L-Trp, being attributed this effect to a greater release of 5-HT from the intestine (Herrero et al., 2006). In mammals, however, it is known that both oral ingestion of tryptophan and its absence in the diet alter serotonergic activity (Hansen and Witte, 2008), in agreement with what was shown in our study. In pigs, supplemented diet increased intestinal villi height, crypt depth, and trp concentration differentials in different parts of the intestine (Koopmans et al., 2005).

4.2. MEL

The effectiveness of MEL diet supplementation indicates that this is an appropriate way to administer this hormone, because plasma values increased after oral treatment, which aligns with the results found in *Oncorhynchus mykiss* (Conde-Sieira et al., 2014). This type of treatment

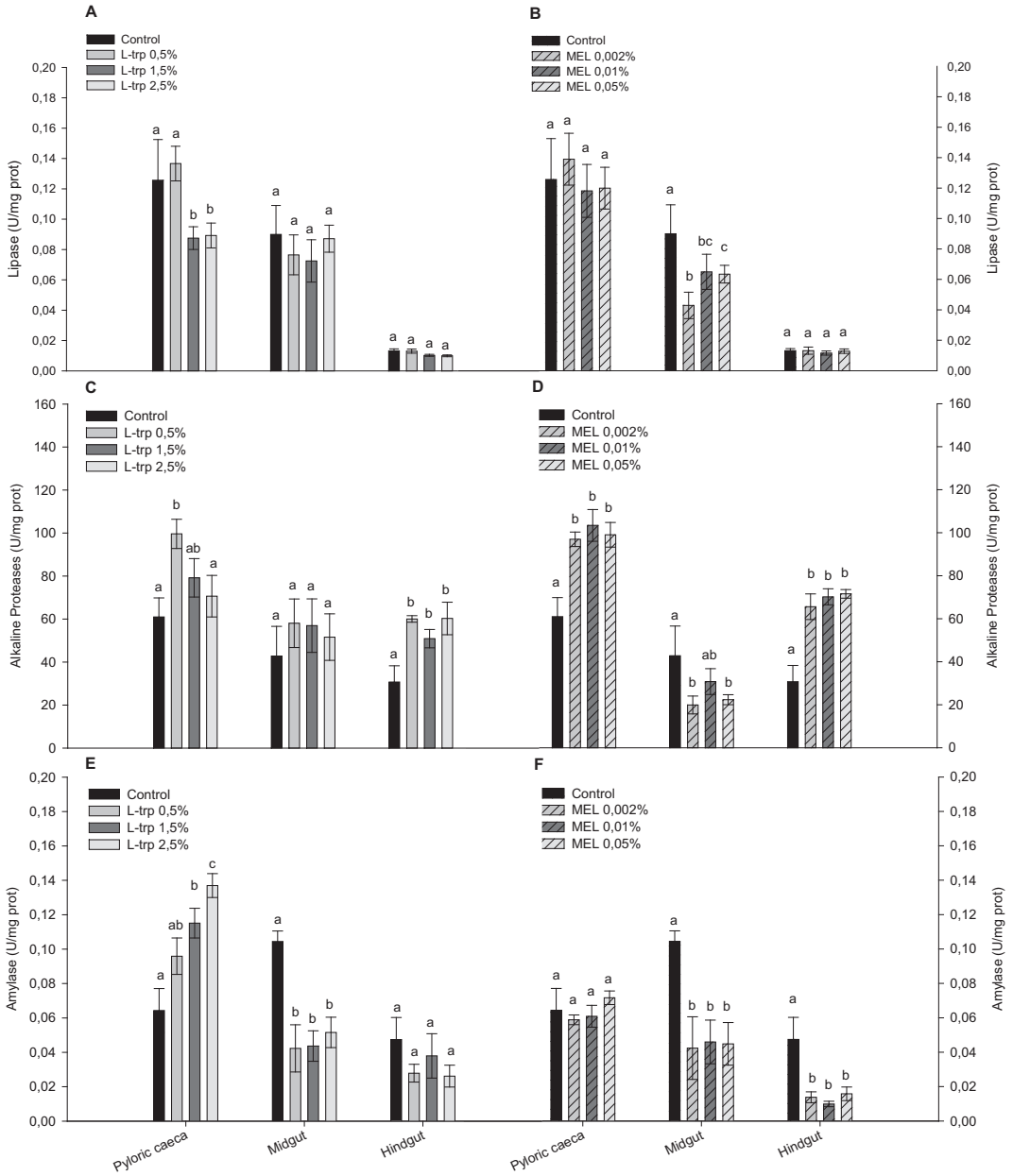

Fig. 2. Digestive enzymatic activity (lipase, alkaline proteases, and amylase) of *S. salar* fed with diets supplemented with different doses of L-trp (A, C and E) and MEL (B, D and F). Data are represented as mean ± SEM of enzyme activity expressed in U/mg in each tissue of 8 to 10 animals for each diet. Different letters indicate statistically significant differences (one-way ANOVA, post-hoc Tukey-test, $p < 0.05$) between diets according to tissue.

avoids invasive procedures, such as intraperitoneal or central intravenous injections, which are stressful to the animals (Gesto et al., 2016). Moreover, the MEL concentrations observed in the plasma of *O. mykiss* receiving MEL-supplemented food were between 2-fold (lower dose) and 10-fold (higher dose) higher than those measured normally *in vivo* at night (approximately 300–400 pg/ml) (Ceinos et al., 2008). The higher plasma MEL values obtained for both oral doses were noted as soon as 0.5 h after food administration, and remained elevated for 4 h after feeding with the higher dose. Plasma hormone concentrations for the low MEL-dose decreased rapidly after feeding (1 h), which aligns with the rapid MEL clearance rate in plasma (Hernández-Rauda et al., 2000).

The MEL supplemented diet in both species significantly reduced basal plasma cortisol levels in comparison to the control diet (Table 2). In other teleost species submitted to stress melatonin reduce cortisol levels (Conde-Sieira et al., 2014; López-Patiño et al., 2013) In European sea bass melatonin reduce basal cortisol levels at 15 min post-ingest (Herrero et al., 2006). *O. kisutch* had reduced cortisol levels in all MEL-supplemented diets, as did *S. salar*, but only at higher doses. MEL can inhibit ACTH-mediated cortisol production in mammals' adrenal gland in (Torres-Farfan et al., 2003; Rao et al., 2001), and could also have direct central actions within the hypothalamus (Xu et al., 1995).

An increase was also observed in the 5-HT content in most analysed tissues from *S. salar* and *O. kisutch*. Since there is no described metabolic pathway for forming 5-HT from MEL, these results can be attributed to an indirect effect mediated by exogenously administered dietary MEL, which is believed to inhibit reuptake of 5-HT level endings of adrenergic nerve fibres, increasing extracellular availability (Míguez et al., 1995). Matheus et al. (2010), describes a MEL inhibitory effect on the serotonin transporter (SERT), reducing the 5-HT uptake after its release, resulting in increased 5-HT extracellular availability. This could explain why MEL-supplemented diets yielded higher 5-HT levels than in with L-trp supplemented diets (see below).

Our results for both species suggest that there is an increase in the

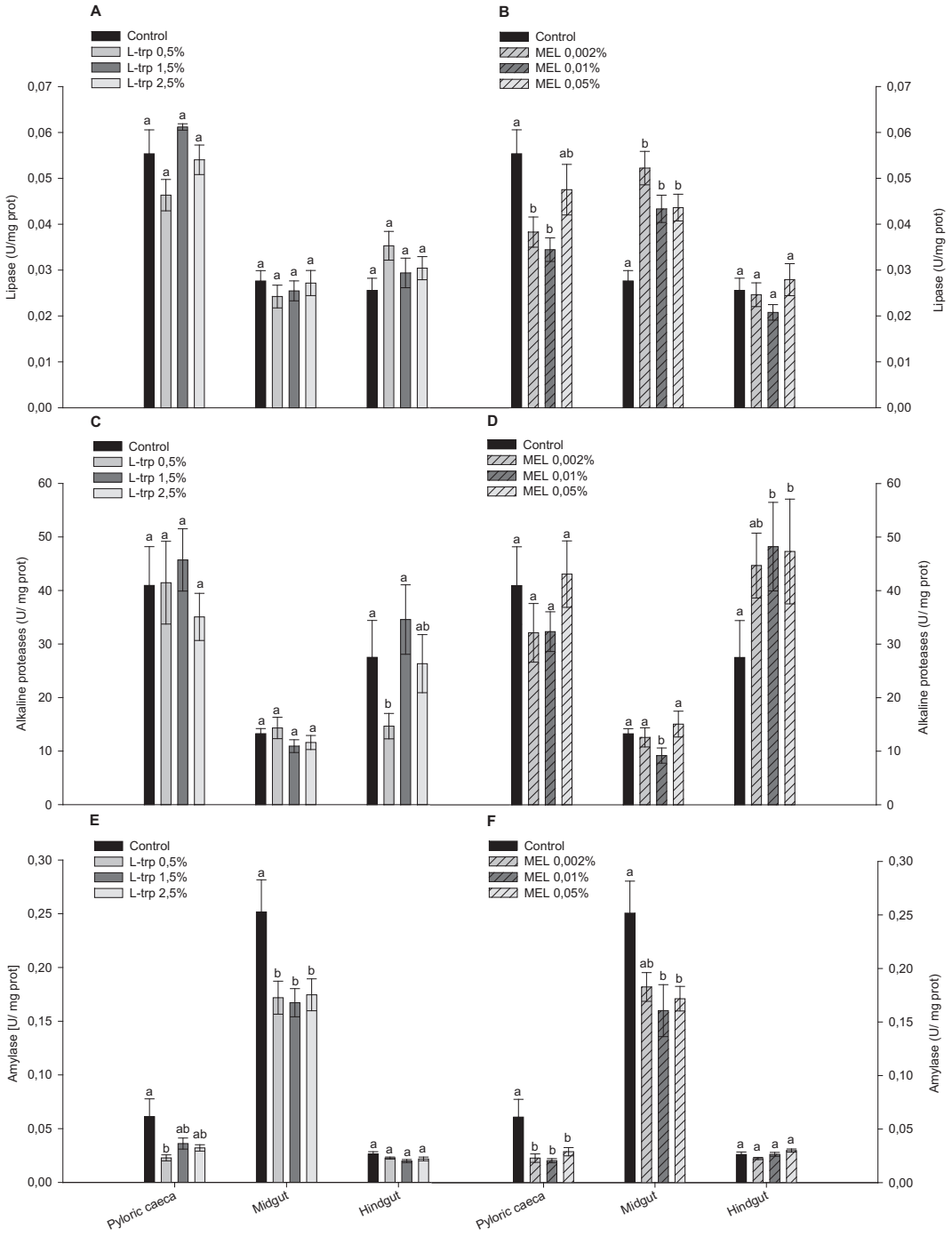

Fig. 3. Digestive enzymatic activity (lipase, alkaline proteases, and amylase) of *O. kisutch* fed with diets supplemented with different doses of L-trp (A,C and E) and MEL (B, D and F). Data are represented as mean ± SEM of enzyme activity expressed in U/mg in each tissue of 8 to 10 animals for each diet. Different letters indicate statistically significant differences (one-way ANOVA, post-hoc Tukey-test, $p < 0.05$) between diets according to tissue.

neuroendocrine activity toward the distal GIT segments, indicating each segment's functional and structural differences in the digestive process. The higher distal portion content maybe due to an important modulatory function during nutrient absorption. MEL decreased longitudinally in trout guts, being highest in the muscular foregut wall and decreasing through the pyloric caeca and more distal gut regions (Muñoz-Pérez et al., 2016). Studies in mammals also reported different MEL levels in anterior and posterior GIT segments, although non-homogeneous patterns have been reported in cows and pigs (Bubenik et al., 1999). Early immunohistochemical studies in mammals (Bubenik, 1980) that reported higher MEL levels attempted to assign MEL production to the enterochromaffin (EC) cells, which contain very high levels of 5-HT (Gershon and Tack, 2007). Specifically, the intestinal tissues (including pyloric caeca and gut segments) displayed the highest content of 5-HT.

4.3. Digestive enzyme activity

The digestive enzyme activity data in this study present a similar pattern of activity decline along the GIT intestine as has been previously described in *S. salar* (Chikwati et al., 2013; Hartviksen et al., 2014 and Krogdahl et al., 2015) and *O. kisutch*. Some studies have compared

different enzyme activities between species with different dietary habits, demonstrating variability (Furné et al., 2005; Hidalgo et al., 1999). Other studies have described GIT enzyme dynamics in a single fish species over the day and how it relates to feeding patterns, leading to adjustments in behaviour and physiology, causing the phenomenon known as food anticipatory activity (FAA) (Montoya et al., 2010; Vera et al., 2007). The variations in digestive enzyme activity in *S. salar* are related to its optimum pH (Krogdahl et al., 2015); an important factor in diets supplemented with L-trp and MEL is the effect of the latter on intestinal pH regulation, as it stimulates bicarbonate secretion (Sjöblom and Flemström, 2003), which could impact digestive enzyme activity.

L-trp has been shown to activate amylase, lipase, and trypsin *in vitro* (Svatos, 1994). This amino acid increases amylase activity in rats, and this effect is associated with its protein structure, because the enzyme contains tryptophan units (Kushak et al., 2002). The effect of tryptophan on digestive enzyme activity may be related to cholecystokinin (CCK), which is known to be one of the regulators of pancreatic enzyme secretion in fish (Aldman et al., 1992) and that may be regulating the enzyme activity in both salmon.

An inhibitory effect, which has been described in guinea pigs (*Cavia porcellus*), of the 5-HT on exocrine pancreatic secretion (coupling with 5-HT_3 type receptors), leads to increase intraductal pressure and consequent reduction of pancreatic secretion by acting as a negative feedback mechanism against such discharge (Suzuki et al., 2001). The inhibitory effect on digestive enzymes associated with L-trp and MEL supplemented diets is therefore unsurprising, because these diets increase the 5-HT intestinal content, and there is a possible inhibitory effect on pancreatic secretory activity mediated by 5-HT_3 receptors. In a study related to stress response in *O. mykiss*, a MEL supplemented diet produced some changes in enzyme activity (Conde-Sieira et al., 2014). Compared to our results, the controls levels of 5-HT and MEL are lower than those of *O. mykiss*, and this difference probably suggests an increase in the endocrine response to disturbance, produced by supplemented diets.

5. Conclusion

Treatment with MEL-supplemented diets over 10 days resulted in suppressed basal cortisol levels in Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynchus kisutch*). L-trp reduced cortisol plasma levels in Atlantic salmon. Diets supplemented with MEL and L-trp have either a stimulatory or inhibitory effect on digestive enzyme activity. Such effects seem to be indirect and dependent on tissue. The underlying mechanisms driving these MEL and L-trp effects require further study and analysis.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

All experimental procedures complied with the guidelines regarding the use of laboratory animals of the Chilean National Commission of Scientific and Technological Research (CONICYT), the Universidad de Los Lagos, and the Universidad Austral de Chile.

References

Alarcón, F.J., Díaz, M., Moyano, F.J., Abellán, E., 1998. Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol. Biochem. 19, 257–267.
Aldman, G., Grove, D., Holmgren, S., 1992. Duodenal acidification and intra-arterial injection of CCK8 increase gallbladder motility in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 86, 20–25.
Anderson, C., Campbell, G., 1988. Immunohistochemical study of 5-HT-containing neurons in the teleost intestine: relation to the presence of enterochromaffin cells. Cell Tissue Res. 254, 553–559.
Azpeleta, C., Martínez-Álvarez, R.M., Delgado, M.J., Isorna, E., De Pedro, N., 2010. Melatonin reduces locomotor activity and circulating control in goldfish. Horm. Behav. 57, 323–329.
Basic, D., Krogdahl, A., Schjolden, J., Winberg, S., Vindas, M.A., Hillestad, M., Mayer, I., Skjerve, E., Höglund, E., 2013. Short- and long-term effects of dietary L-tryptophan supplementation on the neuroendocrine stress response in seawater-reared Atlantic salmon (*Salmo salar*). Aquaculture 388-391, 8–13.
Bubenik, G.A., 1980. Localization of melatonin in the digestive tract of the rat. Effect of maturation, diurnal variation, melatonin treatment and pinealectomy. Horm. Res. 12, 313–323.
Bubenik, G.A., Hacker, R.R., Brown, G.M., Baros, L., 1999. Melatonin concentrations in the luminal fluid, mucosa and muscularis of the bovine and porcine gastrointestinal tract. J. Pineal Res. 26, 56–63.
Caamaño-Tubío, R.I., Pérez, J., Ferreiro, S., Aldegunde, M., 2007. Peripheral serotonin dynamics in the rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol Part C 145, 245–255.
Ceinos, R.M., Polakof, S., Rodríguez, A., Soengas, J.L., Míguez, J.M., 2008. Food deprivation and refeeding effects on pineal indoles metabolism and melatonin synthesis in the rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 156, 410–417.
Cervantes, C.R.C., Barbosa, R.D., Faro, L.R., Adan, L.V., Gago-Martínez, A., Pallares, M.A., 2009. Differential changes of neuroactive amino acids in simples obtained from discrete rat brain regions alter systemic administration of saxitoxin. Neurochem. Int. 54, 308–313.
Chikwati, E.M., Venold, F.F., Penn, M.H., Rohloff, J., Refstie, S., Guttvik, A., Hillestad, M., Krogdahl, Å., 2012. Interaction of soyasaponins with plant ingredients in diets for Atlantic salmon, *Salmo salar* L. Br. J. Nutr. 107, 1570–1590.
Chikwati, E., Sahlmann, C., Penn, M.H., Krogdahl, A., Bakke, A.M., 2013. Alterations in digestive physiology during early stages of diet-induced enteritis in Atlantic salmon, *Salmo salar* L. Aquaculture 402–403, 28–37.
Conde-Sieira, M., Muñoz, J.L.P., López-Patiño, M.A., Gesto, M., Soengas, J.L., Míguez, J.M., 2014. Oral administration of melatonin counteracts several of the effects of chronic stress in rainbow trout. Domest. Anim. Endocrinol. 46, 26–36.
Furné, M., Hidalgo, M.C., López, A., García-Gallego, M., Morales, A.E., Domezain, A., Domezainé, J., Sanz, A., 2005. Digestive enzyme activities in Adriatic sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*). A comparative study. Aquaculture 250, 391–398.
Gershon, M.D., Tack, J., 2007. The serotonin signalling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 132, 397–414.
Gesto, M., Tintos, A., Soengas, J.L., Míguez, J.M., 2006. Effects of acute and prolonged naphthalene exposure on brain monoaminergic neurotransmitters in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C 144, 173–183.
Gesto, M., Álvarez-Otero, R., Conde-Sieira, M., Otero-Rodiño, C., Usandizaga, S., Soengas, J.L., López-Patiño, M.A., 2016. A simple melatonin treatment protocol attenuates the response to acute stress in the sole (*Solea senegalensis*). Aquaculture 452, 272–282.
Gjellesvik, D.R., Lombardo, D., Walther, B.T., 1992. Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): purification and properties. Biochim. Biophys. Acta 1124, 123–134.
Hansen, M.B., Witte, A.B., 2008. The role of serotonin in intestinal luminal sensing and secretion. Acta Physiol. 193 (4), 311–323.
Hartviksen, M., Bakke, A.M., Vecino, J.G., Ringo, E., Krogdahl, A., 2014. Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (*Salmo salar* L.): digestive and metabolic investigations. Fish Physiol. Biochem. 40, 1621–1637.
Hernández-Rauda, R., Míguez, J.M., Ruibal, C., Aldegunde, M., 2000. Effects of melatonin on dopamine metabolism in the hypothalamus and the pituitary of the rainbow trout, (*Oncorhynchus mykiss*). J. Exp. Zool. 287, 440–444.
Herrera, M., Herves, M.A., Giráldez, I., Skar, K., Mogren, H., Mortensen, A., Puvanendran, V., 2017. Effects of amino acid supplementations on metabolic and physiological parameters in Atlantic cod (*Gadus morhua*) under stress. Fish Physiol. Biochem. 43, 591–602.
Herrero, M.J., Martínez, F.J., Míguez, J.M., Madrid, J.A., 2006. Response of plasma and gastrointestinal melatonin, plasma cortisol and activity rhythms of European sea bass (*Dicentrarchus labrax*) to dietary supplementation with tryptophan and melatonin. J. Comp. Physiol. B 177, 319–326.
Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267–283.
Höglund, E., Sørensen, C., Bakke, M.J., Nilsson, G.E., Øverli, Ø., 2007. Attenuation of stress-induced anorexia in brown trout (*Salmo trutta*) by pre-treatment with dietary L-tryptophan. Br. J. Nutr. 97, 786–789.
Jaworek, J., Nawrot, K., Konturek, S.J., Leja-Szpak, A., Thor, P., Pawlik, W.W., 2004. Melatonin and its precursor, L-tryptophan: influence on pancreatic amylase secretion *in vivo* and *in vitro*. J. Pineal Res. 36, 155–164.
Konturek, S.J., Pepera, K., Zabielski, K., Konturek, P.C., Pawlik, A., Szlachcic, A., Hahn,

E.G., 2003. Brain-gut axis in pancreatic secretion and appetite control. J. Physiol. Pharmacol. 54 (3), 293–317.
Koopmans, S.J., Ruis, M., Dekker, R., Diepen, H., Korte, M., Zdzislaw, Mroz, 2005. Surplus dietary tryptophan reduces plasma cortisol and noradrenaline concentrations and enhances recovery after social stress in pigs. Physiol. Behav. 85, 469–478.
Krogdahl, Â., Sundby, A., Holm, H., 2015. Characteristics of digestive processes in Atlantic salmon (*Salmo salar*). Enzyme pH optima, chyme pH, and enzyme activities. Aquaculture 449, 27–36.
Kumar, P., Saurabh, S., Pal, A.K., Sahu, N.P., Arasu, A.R.T., 2014. Stress mitigating and growth enhancing effect of dietary tryptophan in Rohu (*Labeo rohita*, Hamilton, 1822) fingerlings. Fish Physiol. Biochem. 40, 1325–1338.
Kushak, R.I., Drapeau, C., Winter, H.S., 2002. Pancreatic and intestinal enzyme activities in rats in response to balanced and unbalanced plant diets. Plant Food Hum. Nutr. 57, 245–255.
Lepage, O., Tottmar, O., Winberg, S., 2002. Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 205, 3679–3687.
López-Patiño, M.A., Conde-Sieira, M., Gesto, M., Librán-Pérez, M., Soengas, J.L., Míguez, J.M., 2013. Melatonin partially minimizes the adverse stress effects in *Senegalese sole* (*Solea senegalensis*). Aquaculture 388–391, 165–172.
Martins, C.I.M., Silva, P., Costas, B., Larsen, B.K., Gonçalo, A., Santos, G.A., Conceição, L.E.C., Dias, J., Øverli, Ø., Höglund, E., Schrama, J.W., 2013. The effect of tryptophan supplemented diets on brain serotonergic activity and plasma cortisol under undisturbed and stressed conditions in grouped-housed Nile tilapia *Oreochromis niloticus*. Aquaculture 400–401 (2013), 129–134(http://www.sciencedirect.com/science/article/pii/S0044848613001002. Keywords: Stress; Welfare; Monoamines; Flesh quality; Serotonin; Cortisol).
Matheus, N., Mendoza, C., Iceta, R., Mesonero, J., Alcalde, A., 2010. Melatonin inhibits serotonin transporter activity in intestinal epithelial cells. J. Pineal Res. 48, 332–339.
Míguez, J.M., Simonneaux, V., Pévet, P., 1995. Evidence for a regulatory role of melatonin on serotonin release and uptake in the pineal gland. J. Neuroendocrinol. 7, 949–956.
Montoya, A., López-Olmeda, J.F., Yúfera, M., Sánchez-Muros, M.J., Sánchez-Vásquez, F.J., 2010. Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (*Sparus aurata*). Aquaculture 306, 315–321.
Muñoz, J.L.P., Ceinos, R.M., Soengas, J.L., Míguez, J.M., 2009. A simple and sensitive method for determination of melatonin in plasma, bile and intestinal tissues by high performance liquid chromatography with fluorescence detection. J. Chromatogr. B 877, 2173–2177.
Muñoz-Pérez, J.L., López-Patiño, M.A., Álvarez-Otero, R., Gesto, M., Soengas, J.L., Míguez, J.M., 2016. Characterization of melatonin synthesis in the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*): distribution, relation with serotonin, daily rhythms and photoperiod regulation. J. Comp. Physiol. B 186 (4), 471–484.
National Research Council (NRC), 2011. Nutrient Requirements of Fish and Shrimp. National Academy Press, Washington DC (376 pp.).
Psochiou, E., Mamuris, Z., Panagiotaki, P., Kouretas, D., Moutou, K.A., 2007. The response of digestive proteases to abrupt salinity decrease in the euryhaline sparid (*Sparus aurata*) L. Comp. Biochem. Physiol. B 147, 156–163.
Rao, N.V.A., Raza, B., Prasad, J.K., Razi, S.S., Gottardo, L., Ahmad, M.F., Nussdorfer, G.G., 2001. Melatonin decreases glucocorticoid blood concentration in the rat and palm squirrel, acting directly on the adrenal gland. Biomed. Res. 22 (2), 115–117.
Senatori, O., Pierucci, F., Parvez, S.H., Scopelliti, R., Nicotra, A., 2003. Monoamine oxidase in teleosts. Biog. Amines 17, 199–213.
Sjöblom, M., Flemström, G., 2003. Melatonin in the duodenal lumen is a potent stimulant of mucosal bicarbonate secretion. J. Pineal Res. 34, 288–293.
Suzuki, A., Naruse, A., Kitagawa, M., Ishiguro, H., Yoshikawa, T., Ko, S., Yamamoto, A., Hamada, H., Hayakawa, T., 2001. 5-Hydroxtryptamine strongly inhibits fluid secretion in guinea pig pancreatic duct cells. J. Clin. Invest. 108, 749–756.
Svatos, A., 1994. The effect of amino acids, sugars and fats on activation of pancreatic enzymes. Cas. Lek. Cesk. 133, 140–143.
Tang, L., Feng, L., Sun, C.Y., Chen, G.F., Jiang, W.D., Hu, K., Liu, Y., Jiang, J., Li, S., Kuang, S., Zhou, X., 2013. Effect of tryptophan on growth, intestinal enzyme activities and TOR gene expression in juvenile Jian carp (*Cyprinus carpi var.* Jian): studies *in vivo* and *in vitro*. Aquaculture 412, 23–33.
Thakur, R., Tyagi, J.S., Mohan, J., Sastry, K.V.H., 2006. Effect of pinealectomy and melatonin administration on jejunal mucosal enzymes and performance of young broiler chickens. J. Appl. Anim. Res. 30, 137–140.
Torres-Farfan, C., Richter, H.G., Rojas-García, P., Vergara, M., Forcelledo, M.L., Valladares, L.E., Torrealba, F., Valenzuela, G.J., Serón-Ferré, M., 2003. Mt1 melatonin receptor in the primate adrenal gland: inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. J. Clin. Endocrinol. Metab. 88 (1), 450–458.
Vargas-Chacoff, L., Saavedra, E., Oyarzún, R., Martínez-Montaño, E., Pontigo, J.P., Yáñez, A., Ruiz-Jarabo, I., Mancera, J.M., Ortiz, E., Bertrán, C., 2015. Effects on the metabolism, growth, digestive capacity and osmoregulation of juvenile of Sub-Antarctic Notothenioid fish (*Eleginops maclovinus*) acclimated at different salinities. Fish Physiol. Biochem. 41, 1369–1381.
Vera, L.M., De Pedro, N., Gómez-Milán, E., Delgado, M.J., Sánchez-Muros, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., 2007. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiol. Behav. 90 (528-524).
Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142, 3–19.
Winberg, S., Øverli, Ø., Lepage, O., 2001. Suppression of aggression in rainbow trout (*Oncorhynchus mykiss*) by dietary L-tryptophan. J. Exp. Biol. 204, 3867–3876.
Wolkers, C.P.B., Serra, M., Hoshiba, M.A., Urbinati, E.C., 2012. Dietary L-tryptophan alters aggression in juvenile matrinxã *Brycon amazonicus*. Fish Physiol. Biochem. 38, 819–827.
Worthington, K., Worthington, V., 2011. Worthington Enzyme Manual. Worthington Biochemical Corporation. http://www.worthingtonbiochem.com/AA/assay.html.
Xu, F., Li, J.C., Ma, K.C., Wang, M., 1995. Effects of melatonin on hypothalamic gamma-aminobutyric acid, aspartic acid, glutamic acid, beta-endorphin and serotonin levels in male mice. Biol. Signals 4, 225–231.