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Serotonergic outcome, stress and sexual steroid hormones, and growth in a South American cichlid fish fed with an L-tryptophan enriched diet

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ABSTRACT

Reared animals for edible or ornamental purposes are frequently exposed to high aggression and stressful situations. These factors generally arise from conspecifics in densely breeding conditions. In vertebrates, serotonin (5-HT) has been postulated as a key neuromodulator and neurotransmitter involved in aggression and stress. The essential amino acid L-tryptophan (trp) is crucial for the synthesis of 5-HT, and so, leaves a gateway for indirectly augmenting brain 5-HT levels by means of a trp-enriched diet. The cichlid fish *Cichlasoma dimerus*, locally known as *chanchita*, is an autochthonous, potentially ornamental species and a fruitful laboratory model which behavior and reproduction has been studied over the last 15 years. It presents complex social hierarchies, and great asymmetries between subordinate and dominant animals in respect to aggression, stress, and reproductive chance. The first aim of this work was to perform a morphological description of *chanchita's* brain serotonergic system, in both males and females. Then, we evaluated the effects of a trp-supplemented diet, given during 4 weeks, on brain serotonergic activity, stress and sexual steroid hormones, and growth in isolated specimens. Results showed that *chanchita's* brain serotonergic system is composed of several populations of neurons located in three main areas: pretectum, hypothalamus and raphe, with no clear differences between males and females at a morphological level. Animals fed with trp-enriched diets exhibited higher forebrain serotonergic activity and a significant reduction in their relative cortisol levels, with no effects on sexual steroid plasma levels or growth parameters. Thus, this study points to food trp enrichment as a "neurodietary" method for elevating brain serotonergic activity and decreasing stress, without affecting growth or sex steroid hormone levels.

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1. Introduction

Since the monoamine serotonin (5-HT) was isolated and characterized by Rapport et al. (1948), increasing evidence has been pointing out its role as a major neurotransmitter in the brain (Murphy et al., 1998). The serotonergic system is widely present in the animal kingdom (Hay-Schmidt, 2000), modulating multiple behavioral, neuropsychological, and physiological processes (Berger et al., 2009; Deemyad et al., 2013; Meneses and Liy-Salmeron, 2012). In teleost fish, the brain serotonergic system

is composed of at least two major populations of neurons located in the hypothalamus and raphe (Lillesaar, 2011; Lorenzi and Grober, 2012; Margolis-Kazan et al., 1985; Rodriguez-Gomez et al., 2000), whereas an additional pretectal cluster is present in some fish species (Kaslin and Panula, 2001; Loveland et al., 2014). This organization differs from that of mammals, in which serotonergic cell bodies are almost exclusively found in the raphe (Hornung, 2003).

The essential amino acid L-tryptophan (trp) is the substrate of the rate-limiting step for 5-HT biosynthesis, a reaction catalyzed by the enzyme tryptophan hydroxylase (Hamon et al., 1981). This enzyme seems to be only half-saturated with trp (Young and Gauthier, 1981) and, as a consequence, enriched trp diets can

enhance the rate of 5-HT synthesis (Young, 2013). On the other hand, acute trp depletion has been broadly employed as a safe and effective physiological method for lowering the synthesis rate of human brain 5-HT (Zepf, 2013).

Dietary trp supplementation has succeeded in "calming down" edible and commercial farmed animals, by lessening aggression and stress generated in highly social and/or densely reared species. For example, augmenting trp diet content has proven to reduce aggression in fish (Höglund et al., 2005; Wolkers et al., 2012), pigs (Poletto et al., 2014), chickens (Shea et al., 1991), and dogs (DeNapoli et al., 2000). In accordance with the 5-HT deficit hypothesis initially posed in mammals (Krakowski, 2003), these effects of trp-enriched diets are most likely and at least partially mediated by 5-HT. In teleosts, as well as in other vertebrates, high 5-HT activity has been related to low levels of aggression (Clotfelter et al., 2007; Teles et al., 2013; Zubizarreta et al., 2012) and/or lower social rank (Dahlbom et al., 2012; Larson and Summers, 2001; Lepage et al., 2005). In addition, stress mitigation with trp-enriched diets has been broadly reported in fish (Basic et al., 2013; Hoseini et al., 2012; Kumar et al., 2014; Lepage et al., 2002; Martins et al., 2013) and other animals (Koopmans et al., 2005), including humans (Capello and Markus, 2014). 5-HT is known to be intricately related to the hypothalamic–pituitary-adrenal axis (Dinan, 1996; Fuller, 1992; Winberg et al., 1997a), regulating stress coping behaviors (Puglisi-Allegra and Andolina, 2015). The most accepted notion is that elevated serotonergic activity counteracts acute stress-induced elevation of plasma cortisol (the main bioactive corticosteroid in fish; Ellis et al., 2012) (Gesto et al., 2013; Martins et al., 2013), although 5-HT can also excite the aforementioned axis (Höglund et al., 2002).

In 2012, approximately 400 thousand tons of cichlid fish were captured or reared in South American inland waters for edible and ornamental purposes, with an increasing number over the past years (FAO, 2009–2012). In this context, trp-enriched diets would become a resourceful "neurodietary" method for aquaculture management, particularly in highly aggressive species that render stress-derived consequences, such as cichlids (e.g., Earley et al., 2006; Mileva et al., 2009; Parikh et al., 2006). However, the addition of trp in the diet must not adversely affect reproduction or growth. Although not entirely understood, it is generally accepted that 5-HT plays a key role in the modulation of reproductive and sexual behavior (Gonzalez et al., 1994; Mcintosh and Barfield, 1984; Snoeren et al., 2014; Zhang et al., 2013), probably acting through the regulation of hypophyseal and steroid hormone levels, and receptors (Ito et al., 2014; Prasad et al., 2015; Senthilkumaran et al., 2001; Somoza et al., 1988; Vitale and Chiocchio, 1993). To our knowledge, there are no previous reports in teleosts on the effects of trp dietary supplements on reproductive physiological parameters, such as reproductive-related steroid plasma levels. 5-HT is also clearly involved in food intake: high 5-HT reduces feeding, with concomitant weight loss (Reviewed in Lam et al., 2010). However, Shen et al. (2012) reported an improvement on growth with trp supplementation in pigs.

Cichlasoma dimerus, locally known as "*chanchita*", is a South American cichlid fish that has emerged as a fruitful laboratory model for neuroendocrinological and behavioral studies (reviewed in Pandolfi et al., 2009 and Ramallo et al., 2014). In the aquarium, the largest male usually raises as the territorial and reproductively active member of the group, and great asymmetries occur between territorial and non-territorial *chanchitas* with respect to aggression, stress, sexual steroid levels, and gonadal state of maturation (Alonso et al., 2012; Morandini et al., 2014; Ramallo et al., 2015).

In order to elucidate the location of 5-HT-producing cells within *chanchita's* brain, our first goal was to characterize the brain serotonergic system in both females and males by means of immunohistochememechemical assays. Secondly, we aimed to evaluate the effects of a trp-enriched diet on brain serotonergic activity, cortisol and sex steroid plasma levels, and growth.

2. Methods

2.1. Animals and housing

Adult *chanchita* were caught in Esteros del Riachuelo (27°25′S, 58°15′W), Corrientes, Argentina. Upon arrival to the laboratory, animals were maintained in community tanks (one fish/15 L; 6–8 fish per tank) provided with plants and stones, under conditions mimicking their natural habitat (25–27 °C, 14:10 light:dark cycle) (Almirón et al., 2008) for at least one month before the experiments. These were conducted in accordance with international standards on animal welfare, as well as being compliant with institutional and national regulations (Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Comité Nacional de Ética en la Ciencia y la Tecnología).

2.2. Immunohistochemical characterization of brain serotonergic system

2.2.1. General procedure

Adult females (body mass: 27.2 ± 5.1 g; length: 10.4 ± 0.9 cm; $n = 5$) and males (body mass: 31.2 ± 5.4 g; length: 10.8 ± 1.1 cm; $n = 5$) were anesthetized with 0.1% benzocaine and euthanized by decapitation. Brains were rapidly excised and fixed by immersion in 4% paraformaldehyde in 0.1 M saline phosphate buffer (PBS, pH 7.4) for 18–24 h at 4 °C. Then, samples were rinsed in PBS, dehydrated through an ascending series of alcohols, clarified with xylene, and embedded in Paraplast. Finally, brains were serially sectioned at 10 μm in the transverse plane, mounted on gelatin-coated slides and stretched overnight at 37 °C.

Samples sectioned at 10 μm were deparaffinized in xylene, rehydrated through a graded ethanol series to PBS and treated with 3 % hydrogen peroxide. Then, sections were immersed in a blocking solution containing 5% of non-fat dry milk for 35 min. After washing them in PBS, samples were incubated overnight at room temperature in a moist chamber with a rabbit polyclonal primary anti-serotonin antibody (1:1000; catalog No. 20080, Immunostar, Hudson, WI, USA). This antiserum was successfully used to identify serotonergic cells in the cichlid fish *Astatotilapia burtoni* (Loveland et al., 2014). To avoid false positives, primary antibody was substituted with PBS. Neither fibers nor cells were labeled with the latter omission. Sections were then rinsed in PBS and incubated for 1 h with a biotinylated anti-rabbit IgG (Sigma®) diluted 1:600. Amplification of the signal was achieved by incubation with peroxidase-conjugated streptavidin (Dako) diluted 1:700 for 1 h at dark. Finally, slides were washed in PBS and visualized with 0.1% 3,3′-diaminobenzidine in TRIS buffer (pH 7.6) and 0.06% H_2O_2. Sections were lightly counterstained with haematoxylin, cover-slipped, examined with a Microphot FX (Nikon) microscope, and digitally photographed (Coolpix 4500, Nikon).

2.2.2. Morphometric comparisons

We computed the average soma area from digitalized images of 5-HT-ir cells using the software Image Pro Plus (Media Cybernetics), which was previously calibrated with a stage micrometer. All somata areas were measured as the cross-sectional area ($μm^2$) by tracing the cell's profile with a digitizing pen, taking into account only those cells whose margins were clearly distinguishable. Ten randomly chosen pretectal and raphe 5-HT-ir somata were measured for each animal. Because hypothalamic cells were densely arranged, entire hypothalamic nuclei areas (followed along

all the sections where they appeared), rather than individual cell boundaries, were employed for comparing females and males within these populations.

2.3. Trp-enriched diet

2.3.1. Experimental setup and feeding

Adult *chanchitas* from group tanks were randomly chosen as experimental subjects. Across the study, these animals were entirely isolated in 21 L aquaria, maintaining previous housing conditions (25–27 °C, 14:10 light:dark cycle, artificial plants, and stones). During the first 7 days (acclimation period), individuals were fed with commercial fish pellets (Peishe Koi Tradicional, Shulet®), whereas in the following 28 days, animals either continued with a CTL diet (normal commercial fish pellets) or received an 8 times L-trp-enriched supplement (TRP) one. In all cases, animals were fed daily with doses corresponding to approximately 1.5% of individual body weight.

Supplemented diets were prepared through the sprinkle method (Wolkers et al., 2012). Briefly, commercial fish pellets were sprinkled with a mixture of 10% hydrochloric acid and 96% ethanol, in equal parts, and 2.1 g of trp/100 g of food. Supplemented pellets were then dried at 37 °C. To avoid palatable effects, CTL food was also sprinkled with the solution of hydrochloric acid and ethanol, omitting the addition of trp.

To compare between animals from both treatments, at day 7 (initial) and day 35 (final), animals were weighted, total and standard body length recorded, and blood was drawn to evaluate steroid hormone plasma levels. Brains and gonads were removed at day 35 in order to assess monoamine concentrations, and to obtain gonadosomatic indexes, respectively.

2.3.2. Growth parameters and organosomatic indexes

We considered the following parameters: (1) relative length (rL): (final length − initial length) × 100/initial length, (2) relative weight (rW): (final weight − initial weight) × 100/initial weight, and (3) relative condition factor (rCF): (final CF-initial CF) × 100/initial CF. CF was calculated as (total weight/total length^3) × 100. Additionally, we calculated gonadosomatic indexes (GSI) as (100 * gonad weight/total body weight).

2.3.3. Steroid hormone plasma levels

In order to minimize possible circadian rhythm effects on hormonal concentrations, samples were collected between 1230 and 1430 h, at days 7 and 35. Blood samples (150–300 μl) were taken immediately after netting in less than 4 min [cortisol may rapidly increase due to fish manipulation (Fox et al., 1997)] by caudal vein puncture with EDTA pre-treated syringes (needle: 27 gauge × 1/2 inches), and collected in EDTA coated tubes. Plasma was separated by sample centrifugation at 3000 rpm for 15 min, and stored at −20 °C until assayed.

Steroid hormone [11-ketotestosterone (11-KT), testosterone (T), 17β-estradiol (E$_2$) and cortisol] levels were measured using ELISA. Working dilutions ranged from 1:2 to 1:10 for 11-KT, 1:2 for T and E$_2$, and were undiluted for cortisol. In all cases samples were assayed in duplicate and analyses were carried on samples whose coefficients of variation were below 20%, following the manufacturer's instructions. Intra-assay variation was 14.2% for 11-KT (detection limit: 1.3 pg/ml; Cayman Chemical Company, MI, USA), 8.38% for T (detection limit: 0.07 ng/ml; IBL International, Hamburg, Germany), 16.37% for E$_2$ (detection limit: 9.714 pg/ml; IBL International, Hamburg, Germany) and 15.17% for cortisol (detection limit: 2.46 ng/ml; IBL International, Hamburg, Germany). Inter-assay variation was 5.4%, 15.58%, 5.3% and 10.28%, respectively. Parallelism to standard curves was assessed by measuring hormone's concentration in serially diluted samples (4 different dilutions). Correlation coefficients were 0.99 for 11-KT, 0.99 for T, 0.98 for E$_2$ and 0.97 for cortisol.

2.3.4. Brain monoamines

On day 35, animals were anesthetized with 0.1% benzocaine and euthanized by decapitation. Brains were rapidly excised, and divided into anterior (olfactory bulbs + telencephalon + hypothalamus) and posterior (optic tecta + cerebellum + medulla oblonga + part of the spinal cord) portions (from now on, forebrain and hindbrain, respectively). Once weighted, samples were homogenized in 400 μl cold 4% (w/v) hydrochloric acid by mechanical disruption, and immediately stored at −80 °C until assayed.

Quantification of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA, 5-HT's major metabolite) content was determined by high performance liquid chromatography with electrochemical detection (HPLC-EC). To do so, homogenized samples were previously centrifuged at 10,000 rpm for 2 min, keeping the supernatants for the assay. In brief, HPLC-EC system consisted of a Phenomenex Luna 5-μm, C18, 150 × 4.60 mm column (Phenomenex, Torrance, CA, USA) and a LC-4C electrochemical detector with glassy carbon electrode (BAS, West Lafayette, IN, USA). The working electrode was set at +0.70 V with respect to a Ag/AgCl reference electrode. The mobile phase contained 0.76 M NaH$_2$PO$_4$·H$_2$O, 0.5 mM EDTA, 1.2 mM 1-octane sulfonic acid, and 5% acetonitrile, with pH adjusted to 2.8. Samples were quantified by comparison with standard solutions of known concentrations, and the detection limit was 1.5 ng/ml for 5-HT and 1.8 ng/ml for 5-HIAA. The ratio of 5-HIAA/5-HT was considered as an estimator of serotonergic activity (Dahlbom et al., 2012; Teles et al., 2013), and brain monoamine and metabolite levels were normalized to total tissue weight (Basic et al., 2013; Clotfelter et al., 2007; Winberg et al., 1997b).

2.3.5. Statistics

Statistical analyses were performed in Infostat 2010 (FCA, Universidad Nacional de Córdoba, Argentina). Serotonergic cellular areas among females and males were compared by one-way Analysis of Variance (ANOVA) with two nested factors. Cellular areas were correlated with body mass or total length, and since no significances were found ($p > 0.05$ for all the correlations; data not shown), body size was not considered as a relevant variable to account for possible differences in 5-HT-ir somata area between females and males. Eventual differences on growth parameters or GSI associated to differential feeding conditions were analyzed by means of a one-way ANOVA. In order to evaluate possible differences in hormonal profiles between animals fed either with TRP or CTL diets, comparisons were assed with data expressed as follows: (final hormonal level − initial hormonal level) × 100/initial hormonal level. As the final concentration is relativized to the initial one, the variability among animals is reduced, and results in a better approach with respect to considering absolute values, for evaluating how steroid plasma levels changed. One-way ANOVAs were carried out to elucidate variations in these hormonal data sets. Forebrain and hindbrain 5-HT and 5-HIAA levels, as well as monoaminergic activity, were also compared through ANOVAs (factors: feeding regime and sex). Finally, we performed some correlations between serotonergic activity and cortisol relative change.

When normality (Shapiro–Wilk test) and homoscedasticity (Levene test) assumptions were not fulfilled, log- or square root-transformations were performed. Additionally, values that were above two standard deviations from the mean were considered as outliers and therefore not included in the analyses. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Serotonergic brain system

The distribution pattern of 5-HT-ir cell bodies was similar among different animals, and no clear morphological dissimilarities appeared between sexes. *Chanchita's* brain serotonergic system was composed of several somata grouped in two major regions: the diencephalon and the brainstem. Diencephalic somata are present in the pretectal area and the hypothalamus, whereas brain stem somata are located in the raphe (Fig. 1a).

Pretectal 5-HT-ir cells were confined either on the dorsal or ventral periventricular nuclei, surrounding or nearby the fasiculus retroflexus. These cells correspond to the most anterior 5-HT-ir cells in *chanchita*'s brain, and were scarce in transverse sections (Fig. 1b, c′). The average cellular area did not differ between both sexes ($F = 0.57$; $p = 0.472$) (Table 1).

Hypothalamic 5-HT-ir cells were found within three nuclei: the posterior periventricular nucleus (NPPv), the nucleus of the lateral recess (NRL), and the nucleus of the posterior recess (NRP). Cell bodies at the NPPv are the most rostrally located, extending on both sides of the third ventricle (Fig. 1d–f). At the brain midline
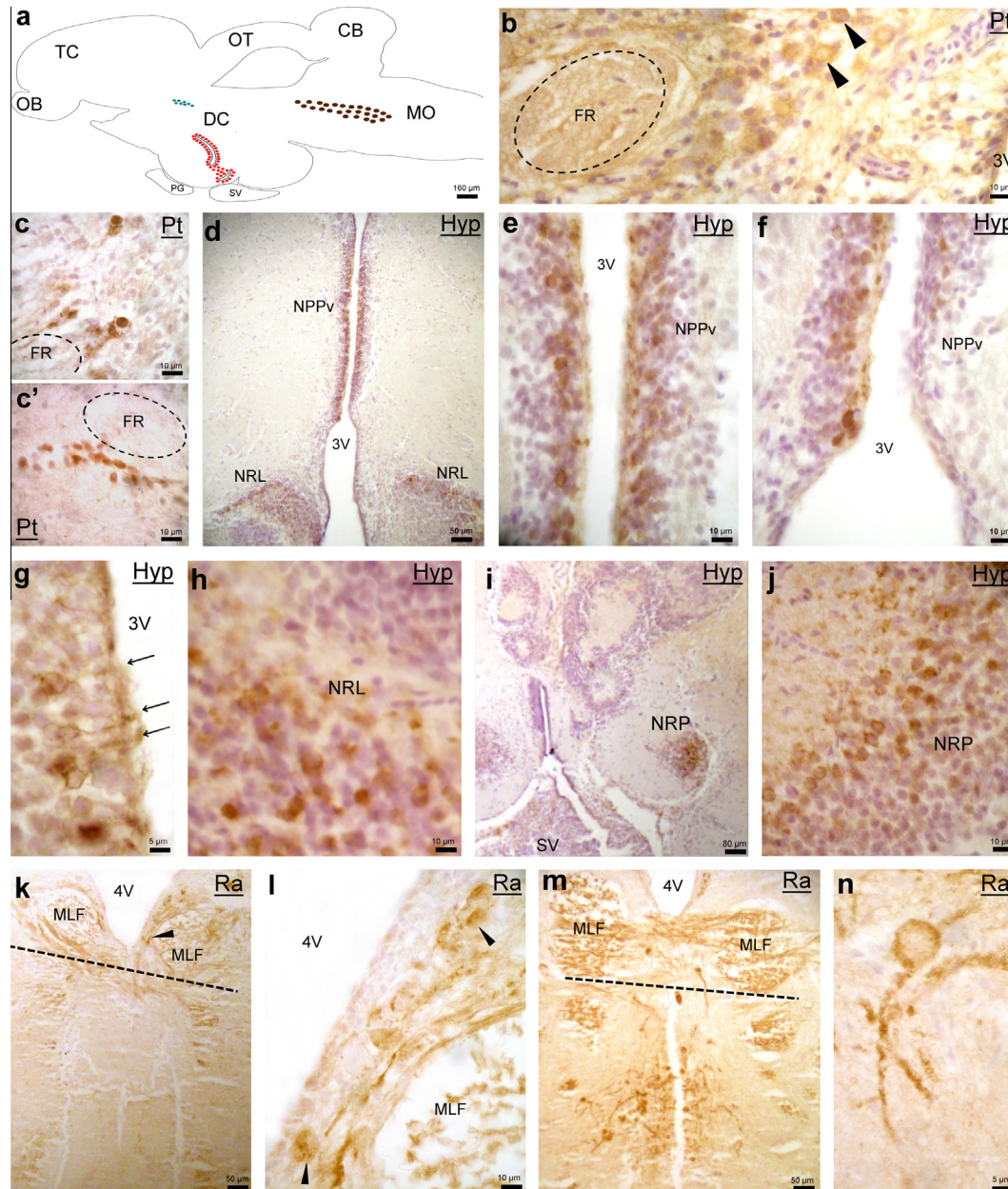

Fig. 1. Adult *chanchita* brain serotonergic system. (a) Schematic representation of the three main serotonergic populations: pretectal (blue), hypothalamic (red) and raphe (brown). CB: cerebellum; DC: diencephalon; MO: medulla oblonga; OT: optic tectum; PG: pituitary gland; SV: saccus vasculosus; TC: telencephalon. (b) Pretectal (Pt) cells (arrowheads) are scarce, located near the fasiculus retroflexus (FR) and at both sides and slightly apart from the third ventricle (3V). (c) Dorsal and (c′) ventral Pt cells. (d) Posterior periventricular nucleus (NPPv) 5-HT-ir cells are the most rostrally located hypothalamic (Hyp) cells, followed by those on the nucleus of the lateral recess (NRL). (e, f) NPPv 5-HT ir cells are intimately disposed on both sides of the 3V, and exhibit (g) several cellular processes (arrows) directed towards the ventricular lumen. (h) NRL 5-HT cell bodies show apart from the 3V. (i, j) The nucleus of the posterior recess (NRP) is just above the saccus vasculosus (SV) and contains the hindmost 5-HT-ir cells of the Hyp. (k, l) The most rostrally located raphe (Ra) 5-HT-ir somata are those corresponding to the dorsal ones (arrow head), below the fourth ventricle (4V) and surrounding dorsal and inner portions of the medial longitudinal fasciculi (MLF)-over the dashed line-. (m) Medial Ra 5-HT-ir cell bodies are situated ventral to the MLF, and aligned along the brain midline (below the dashed line). (n) Detail of a medial Ra 5-HT-ir cell.

Table 1
5-HT-ir cell areas (μm^2).

	Females	Males
PT	108.4 ± 32.25	106.3 ± 27.39
NPPv	10,321 ± 2042	9982 ± 1823
NRL	12,144 ± 3257	11,902 ± 2349
NRP	15,740 ± 3018	16,107 ± 3145
DR and MR	117.2 ± 24.12	104.3 ± 20.47

Average cellular area (means ± SEM) of pretectal (PT) and dorsal and medial raphe nuclei (DR and MR) of *chanchita* females and males. For hypothalamic nuclei, that is, posterior periventricular nucleus (NPPv), nucleus of the lateral recess (NRL), and nucleus of the posterior recess (NRP), total cellular area (means ± SEM) was computed for males and females. The statistical threshold was set at $p < 0.05$. $n = 5$ in all cases.

and ventral to the NPPv, NRL somata make their appearance, surrounding the lateral recesses (Fig. 1d and h). Finally, ventral to the NPPv and NRL, and just above the saccus vasculosus, NRP comes out as the hindmost 5-HT-ir cell nucleus of the hypothalamus (Fig 1i–j). Cell bodies from the three nuclei were small, densely grouped, and related to the ventricular system. In fact, they seemed to contact the cerebrospinal fluid, as evidenced by several cellular processes directed towards the ventricular lumen (Fig. 1g). None of whole hypothalamic cellular areas from the three nuclei differed between females and males (NPPv: $F = 0.21$; $p = 0.657$; NRL: $F = 1.20$; $p = 0.305$; NRP: $F = 0.74$; $p = 0.415$) (Table 1).

Within the raphe, 5-HT-ir cells had larger somata than those of the hypothalamus (Fig. 1k–n). Cell bodies were located at the dorsal (DR) and medial raphe (MR). Those in the DR, were just below the fourth ventricle and surrounded the dorsal and inner portions of the medial longitudinal fasciculi (MLF) (Fig. 1k, l). On the other hand, MR cell bodies, more posteriorly, were situated ventral to the MLF, and were aligned along the brain midline (Fig. 1m, n). No significant differences were observed between females and males raphe cellular areas ($F = 2.52$; $p = 0.151$) (Table 1).

3.2. Effects of trp-enriched diet

3.2.1. Weight, length, and gonadosomatic index

We evaluated growth parameters and gonadosomatic indexes in animals that received CTL or TRP diets during 4 weeks (Table S1). For females, no differences were present in rL ($F = 0.37$; $p = 0.557$) or rCF ($F = 0.03$; $p = 0.866$) with either of the diets. However, there was a trend towards higher rW (2.6 times) in females fed with TRP ($F = 4.90$; $p = 0.051$). On the other hand, GSI ($F = 0.01$; $p = 0.927$) did not vary between both experimental groups. In males, rL ($F = 1.01$; $p = 0.341$), rW ($F = 0.01$; $p = 0.962$) and rCF ($F = 0.08$; $p = 0.787$) were not differentially affected by the two diets, as well as GSI ($F = 0.27$; $p = 0.613$).

3.2.2. Cortisol plasma levels

Initial and final cortisol plasma levels from animals fed under CTL or TRP diets are presented in Fig. 2a. In males, plasma cortisol levels significantly decreased when they were fed with both CTL ($F = 5.29$; $p = 0.038$; 82.9 ± 25.5 ng/ml vs. 69.7 ± 13.4 ng/ml for initial and final, respectively) and TRP ($F = 8.37$; $p = 0.012$; initial: 84.3 ± 21.9 ng/ml; final: 53.4 ± 11.7 ng/ml) pellets. However, these comparisons do not account for the actual strength of hormonal change after 4 weeks of differential feeding. Consequently, and as stated on Section 2.3.5, results were also expressed as the relative variation (RV) with respect to the initial hormonal concentration, instead of just comparing the absolute means. When considering the RV, males fed in a TRP regime exhibited a 2.7 times higher change than those fed with CTL diets (-32 % for TRP and -12 % for CTL; $F = 4.84$; $p = 0.041$) (Fig. 2b).

In contrast to males, females did not show a significant reduction of their absolute cortisol levels in neither of the dietary
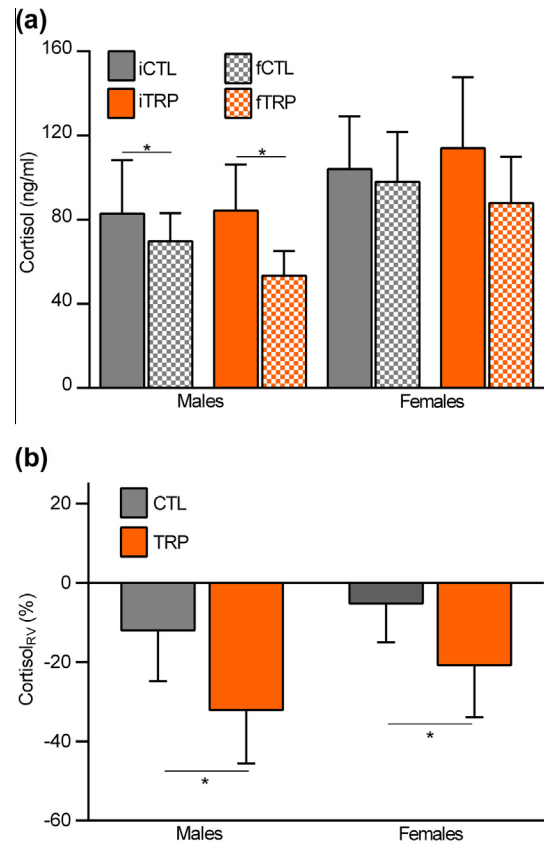

Fig. 2. *Chanchita* female and male (a) absolute plasma cortisol levels and (b) cortisol relative variation (cortisol$_{RV}$) after being fed under CTL (control) or TRP (8 times more L-tryptophan than CTL) regimens during 4 weeks. Data are presented as means ± SEM. Asterisks signal statistical differences, with significance set at $p < 0.05$. $n = 7$ for each group. f: final cortisol plasma levels; i: initial cortisol plasma levels.

regimes ($F = 0.13$, $p = 0.80$ for CTL; $F = 1.64$, $p = 0.22$ for TRP) (Fig. 2a). However, when taking into account the RV, and comparing it among females form different diets, TRP ones exhibited 4 times higher change than CTL ones (−20% vs. −5%, respectively; $F = 5.29$; $p = 0.037$) (Fig. 2b).

When comparing females with males, results showed that initial and final cortisol concentrations were significantly lower in males than females (initial: 83.61 ± 22.37 ng/ml vs 109.39 ± 27.98 ng/ml; $F = 4.71$; $p = 0.038$. Final: 61.58 ± 12.83 ng/ml vs. 93.02 ± 22.65 ng/ml; $F = 12.79$; $p = 0.001$). Finally, males and females did not significantly differ in their Cortisol RV within CTL ($F = 0.61$; $p = 0.45$) or TRP ($F = 1.57$; $p = 0.23$) treatments.

3.2.3. Sex steroid plasma levels

None of the reproductive steroid levels (T, 11-KT and E$_2$) varied after 4 weeks of distinct feeding regimes for both sexes, when comparing initial and final absolute values (Table S2). Relativized values are shown on Fig. 3. Neither 11-KT – Fig. 3a – (males: $F = 0.75$; $p = 0.414$; females: $F = 2.02$; $p = 0.214$) nor T – Fig. 3b – RV (males: $F = 0.19$; $p = 0.669$; females: $F = 0.43$; $p = 0.533$) were differentially affected by the dietary regime. In the same line, E$_2$ RV was not significantly affected by the dietary treatment in both males ($F = 2.78$; $p = 0.171$) and females ($F = 1.23$; $p = 0.310$) (Fig. 3c).

3.2.4. Monoaminergic outcome

Brain 5-HT and 5-HIAA levels are shown in Table 2. No significant differences were found on forebrain or hindbrain 5-HT and 5-HIAA concentrations between CTL and TRP fed males and females.
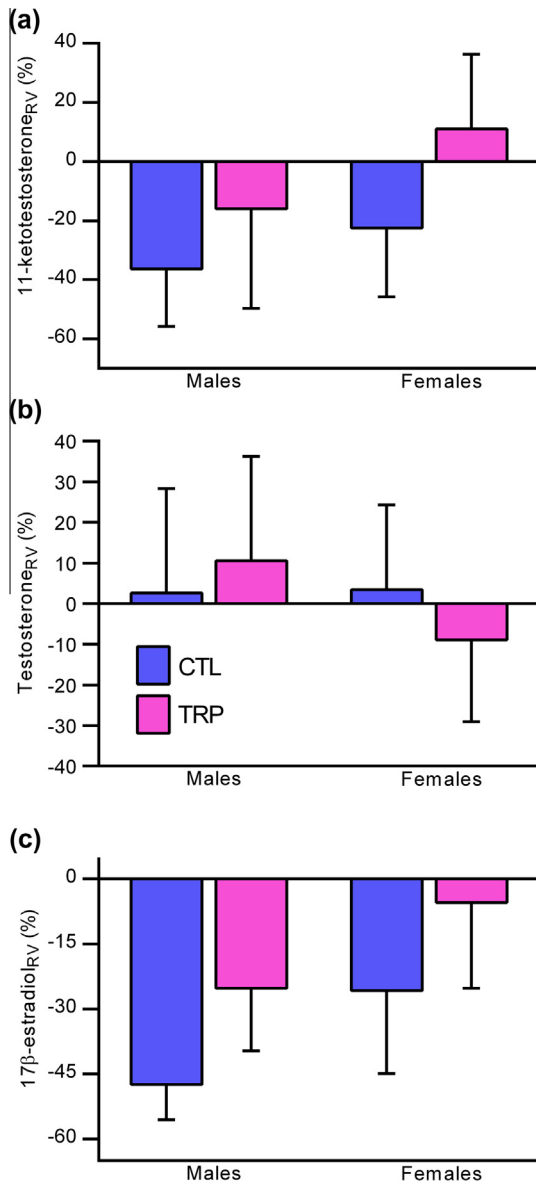
Fig. 3. (a) 11-ketotestosterone, (b) testosterone and (c) 17β-estradiol relative variations (X_{RV}) in *chanchita* females and males fed under CTL (control) or TRP (8 times more L-tryptophan than CTL) regimens during 4 weeks. Data are expressed as means ± SEM. Statistical significance set at $p < 0.05$. $n = 5$ for each group, except for TRP male's 11-ketotestosterone, where $n = 6$.
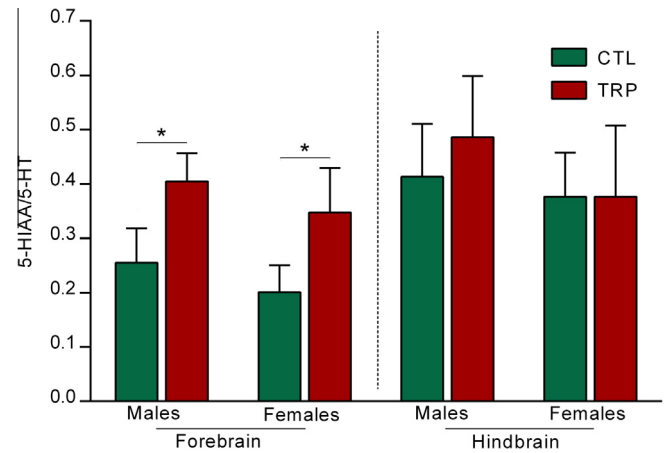

Fig. 4. Forebrain (olfactory bulbs + telencephalon + hypothalamus) and hindbrain (optic tecta + cerebellum + medulla oblonga + part of the spinal cord) serotonergic activity (5-HIAA/5-HT) from *chanchita* females and males fed under CTL (control) or TRP (8 times more L-tryptophan than CTL) regimens during 4 weeks. Data are presented as means ± SEM. Asterisks indicate statistical differences, with significance set at $p < 0.05$. $n = 6$ for each group.

When comparing 5-HT levels between females and males, no significant differences arouse neither on forebrain ($F = 0.02$; $p = 0.89$) nor hindbrain ($F = 0.01$; $p = 0.94$). In the same line, forebrain ($F = 0.93$; $p = 0.35$) and hindbrain ($F = 2.23$; $p = 0.15$) 5-HIAA content did not differ between sexes. Pooled male and female data showed no variation between CTL and TRP fed individuals in forebrain ($F = 0.50$; $p = 0.485$) and hindbrain ($F = 0.31$; $p = 0.580$) 5-HT levels, and in hindbrain 5-HIAA ($F = 0.67$; $p = 0.422$). In contrast, TRP fed animals had 50% more forebrain 5-HIAA concentrations than CTL ones ($F = 6.44$; $p = 0.019$) (Table 2).

There was a significant effect of TRP diets on both female's and male's forebrain serotonergic activity (Fig. 4). Females fed with TRP exhibited 1.8 times higher forebrain serotonergic activity than CTL ones (0.35 ± 0.08 vs. 0.20 ± 0.05, respectively; $F = 9.02$; $p = 0.012$), whereas in males fed with TRP supplemented pellets, forebrain serotonergic activity was 1.5 times higher (0.40 ± 0.05 vs. 0.26 ± 0.06, respectively; $F = 9.63$; $p = 0.008$) than those fed with CTL. These effects were still present when forebrain serotonergic activities from females and males were pooled together ($F = 18.18$; $p < 0.001$). On the contrary, hindbrain serotonergic activity was not significantly altered by feeding regime on females ($F = 0.37$; $p = 0.572$), males ($F = 0.93$; $p = 0.358$) or when sexes were pooled ($F = 0.42$; $p = 0.525$) (Fig. 4).

Table 2
Monoamine and parental metabolite brain levels (ng/ml of wet tissue).

		Forebrain		Hindbrain	
		CTL	TRP	CTL	TRP
Females	5-HT	1731 ± 416	1665 ± 315	812 ± 216	945 ± 183
		$F = 0.06$; $p = 0.812$		$F = 0.78$; $p = 0.407$	
	5-HIAA	351 ± 113	601 ± 228	298 ± 96.5	331 ± 111
		$F = 3.43$; $p = 0.093$		$F = 1.16$; $p = 0.315$	
Males	5-HT	1832 ± 450	1623 ± 378	879 ± 232	862 ± 164
		$F = 0.46$; $p = 0.510$		$F = 0.01$; $p = 0.922$	
	5-HIAA	467 ± 157	641 ± 130	364 ± 119	413 ± 88.4
		$F = 2.77$; $p = 0.131$		$F = 0.35$; $p = 0.566$	
Both	5-HT	1781 ± 439	1644 ± 346	845 ± 220	903 ± 176
		$F = 0.50$; $p = 0.485$		$F = 0.31$; $p = 0.580$	
	5-HIAA	409 ± 155	621 ± 186	331 ± 109	372 ± 86
		$F = 6.44$; $p = 0.019$		$F = 0.67$; $p = 0.422$	

Forebrain and hindbrain serotonin (5-HT) and 5-hydroxyindoleacid (5-HIAA) concentrations (means ± SEM) from *chanchita* females and males fed during 4 weeks either with CTL or TRP (8 times more L-tryptophan than CTL) diets. Bold values indicate statistical significance, set at $p < 0.05$. $n = 6$ for all experimental groups.

3.2.5. Serotonergic activity and cortisol$_{RV}$

Cortisol RV and forebrain serotonergic activity, the two parameters that significantly differed among feeding regimes, were negatively correlated. When pooling TRP + CTL data, a negative correlation was found between forebrain serotonergic activity and cortisol RV in both females ($R^2 = 0.45$; $F = 6.57$; $p = 0.034$, Fig. 5a) and males ($R^2 = 0.39$; $F = 5.92$; $p = 0.037$, Fig. 5b). Finally, when jointly evaluating females and males under both feeding regimes (pooling all data), the latter negative correlation was also present ($R^2 = 0.41$; $F = 13.46$; $p = 0.002$) (Fig. 5c).

4. Discussion

The current study provides the first insight into the distribution of serotonergic somata in *chanchita's* brain, and reveals interesting outcomes of an L-tryptophan enriched diet over growth, stress and sexual steroids, and serotonergic activity.
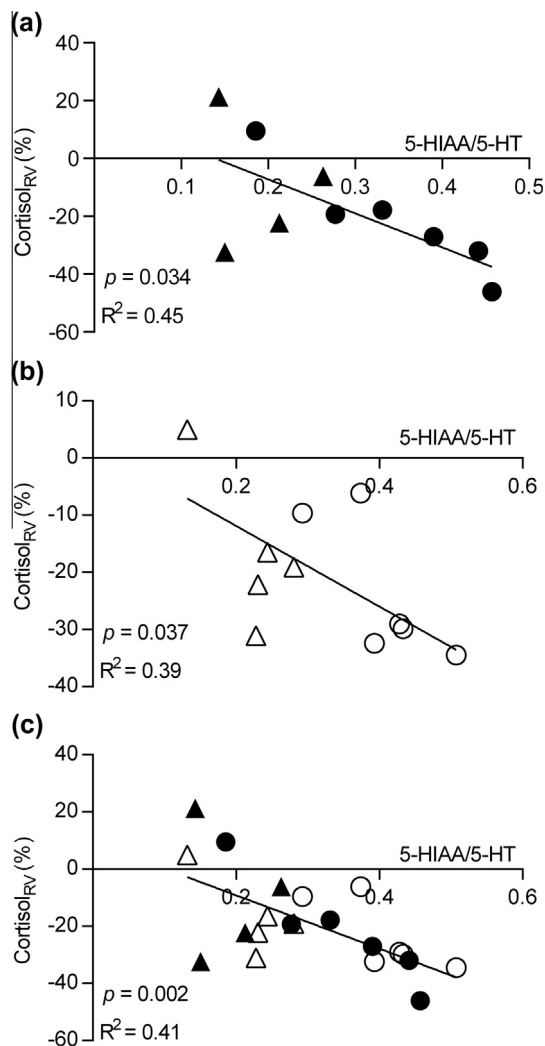

Fig. 5. Correlations between cortisol relative variation (cortisol$_{RV}$) and forebrain serotonergic activity (5-HIAA/5-HT) in *chanchita* females and males fed under CTL (control) or TRP (8 times more L-tryptophan than CTL) regimes during 4 weeks. Correlations were evaluated for (a) females (b) males and (c) females + males. Statistical significance was set at $p < 0.05$. Sample size ranged from 10 to 21, depending on the experimental groups correlated and the occurrence of outliers. Filled triangles: CTL females; filled circles: TRP females; unfilled triangles: CTL males; unfilled circles: TRP males.

4.1. Brain serotonergic system

Chanchita's brain serotonergic system is composed of several nuclei situated in three distinct zones: pretectal area, hypothalamus and raphe. Although trp hydroxylase is the unique specific marker for 5-HT-producing cells (Lillesaar, 2011), the antibody used in the present study spotted the usual pattern of serotonergic cells distribution exhibited by teleost fish. Nonetheless, we did not find 5-HT fibers projecting to the pituitary or 5-HT-ir cells in this gland, as has been shown in other teleost species (e.g., Corio et al., 1991; Lorenzi and Grober, 2012; Margolis-Kazan et al., 1985; Rodriguez-Gomez et al., 2000). This could be due to differences in sensitivity of the antisera and/or methods used in our research, or simply, because of the absence of those populations in *chanchita*.

Pretectal cells were the most anterior 5-HT-ir somata in *chanchita's* brain, as also reported in several teleost species amply used as animal models, such as the African cichlid *A. burtoni* (Loveland et al., 2014) and zebrafish *Danio rerio* (Kaslin and Panula, 2001). However, these cellular populations failed to appear in other species, including goby *Lythrypnus dalli* (Lorenzi and Grober, 2012) and Senegalese sole *Solea senegalensis* (Rodriguez-Gomez et al., 2000). As pretectal 5-HT-ir cell projections are involved in behaviors related to the integration of visual inputs with other sensory modalities (Wullimann, 1998), the aforementioned heterogeneity between species could implicate distinct visual requirements in particular habitats or social contexts.

Hypotalamic 5-HT-ir cells are present in practically all teleost fish studied to date (Corio et al., 1991; Kaslin and Panula, 2001; López and González, 2013; Lorenzi and Grober, 2012; Loveland et al., 2014; Oliveri et al., 2005; Parent and Northcutt, 1982), and in *chanchita* these somata were distributed within three nuclei: NPPv, NRL and NRP. NPPv cells, at least, showed processes that contacted the third ventricle. The role of hypothalamic 5-HT nuclei remains unclear; Vigh-Teichmann and Vigh (1989) suggested that brain cerebrospinal fluid (CSF)-contacting cells synthesize and release substances into the CSF, in response to information from the CSF or afferent fibers from numerous brain areas.

Increasing controversy has appeared over recent years concerning the anatomical identity and nomenclature of somata found within the hypothalamus (Lillesaar, 2011). Although in some studies alternative names have been attributed to the same populations, it is plausible that proximate species could present great differences. For example, *L. dalli's* hypothalamic populations are strikingly similar to those found in *chanchita* (Lorenzi and Grober, 2012), whereas in *A. burtoni*, phylogenetically closer to *C. dimerus*, NRL and NRP populations were not present (Loveland et al., 2014). Therefore, and regardless of the terminology applied, explicit divergences seem to be present in the morphological assembly of brain 5-HT-ir cells even in closely related species.

The hindmost 5-HT-ir cells in *chanchita* were located in the raphe, similarly to almost all other teleost fish (Lillesaar, 2011). However, Gaspar and Lillesaar (2012) had drawn attention to the high diversity of teleost raphe cells, even within each of 5-HT nuclei: multiple forebrain, midbrain, and brainstem inputs regulate these serotonergic populations (Dorocic et al., 2014), which in turn massively project throughout the brain and spinal cord (Ekström and Van Veen, 1984).

Once the brain serotonergic system was characterized, we wondered if there would be any difference between sexes at a morphological level. Not only at first glance *chanchita* males and females exhibited identical 5-HT-ir cell distribution, but when evaluating somata area, we found no significant differences between sexes in neither of the nuclei evaluated. Accordingly, in rodents, males and females did not differ in the size or volume of trp hydroxylase immunoreactive cells on either DR or MR (Jitsuki et al., 2009). From

a morphological perspective, Lorenzi and Grober (2012) found no significant difference on 5-HT-ir cell number neither within social status nor sexes in the teleost fish *L. dalli*. However, there is evidence on the existence of sex variation on brain serotonergic system in other teleosts, such as zebrafish (Dahlbom et al., 2012), and in rodents (Carlsson and Carlsson, 1988; Domínguez et al., 2003). Although we found no sex-based differences on 5-HT-ir soma size, we cannot rule out that other attributes, such as cellular number, nuclear area, differential receptor expression, and/or projections to distinct brain areas, could account for dissimilarities not evaluated in the present study. For example, women exhibit higher 5-HT 1A receptor and lower 5-HT transporter binding potentials than men in a broad range of brain areas (Jovanovic et al., 2008).

4.2. *L-Tryptophan-supplemented diet*

Feeding *chanchita* with an 8 times trp-enriched (TRP) diet during 4 weeks resulted in a significant decrease on their relative cortisol plasma levels and an augmented forebrain serotonergic activity, without differentially affecting body length and weight, or sex steroids plasma concentrations.

Absolute cortisol plasma levels significantly decreased in males fed with both CTL and TRP diets and, therefore, 4 weeks of isolation *per se* produced a decline on plasma cortisol. These results do not necessarily imply that all *chanchita* males would have accustomed to the novel context after 4 weeks, because cortisol concentrations may decrease even during chronic stress (Miller et al., 2007). Notwithstanding these reduced cortisol levels with both feeding regimes, relative cortisol decrease (i.e., relativizing to the initial concentration) was significantly stronger in males fed with TRP. In females, isolation itself did not produce any decline on cortisol plasma levels but, as for males, relative cortisol reduction was significantly superior in those fed with TRP pellets. In our experimental design, females had significantly higher initial and final cortisol levels than the corresponding concentrations in males.

Several studies have evaluated stress attributes on fish fed with trp-enriched diets in diverse scenarios. The general consensus is that trp down-regulates the stress response – i.e., reducing cortisol levels – after an acute-stressor, but not in undisturbed animals. For example, rainbow trout fed with supplementary dietary trp exhibited elevated plasma cortisol levels in a non-stressed context, but displayed a significant reduction on acute stress-induced plasma cortisol concentrations (Lepage et al., 2002, 2003). Recent studies, however, tend to indicate that trp may also act on undisturbed conditions. Seven days of a trp regime induced long-term inhibitory effects on the neuroendocrine stress response in Atlantic salmon *Salmo salar*, on both acute-stressed and non-stressed individuals (Basic et al., 2013). Martins et al. (2013) reported a reduction on plasma cortisol levels only in undisturbed Nile tilapia fed with 10 times trp-enriched pellets. In our experimental design, and at least for males, final cortisol plasma levels were in the same range of those present in territorial and non-territorial males within a social group (60–70 ng/ml) (Ramallo et al., 2015), and thus, are among the expected levels for males in a more natural scenario. However, it is not entirely correct to consider that isolated animals in the present work were subjected to undisturbed conditions, because *C. dimerus* is a gregarious species, which are known to get stressed when are deprived form conspecifics by isolation (Cacioppo et al., 2011; Earley et al., 2006). Instead, what can be assured is that our animals were not exposed to an acute stressor.

Trp-enriched diets had not only proven to reduce acute, but also chronic stress-derived consequences. For example, in fingerlings of the teleost fish *Cirrhinus mrigala*, trp supplementary diets mitigated 2 month of crowding stress (Tejpal et al., 2009). Diminished cortisol levels after consuming trp-enriched food have also been documented in mammals, such as pigs after social stress (Koopmans et al., 2005), weaning piglets (Liu et al., 2013), and humans (Capello and Markus, 2014). Moreover, women and men fed with depleted trp diets showed higher CSF corticotropin-releasing factor (CRF) (Tyrka et al., 2004). As exposed, the picture on trp effects is complex, and seems to depend on trp concentration, duration of feeding, and presence or absence of an acute or chronic stressor, among others. Furthermore, we cannot ignore the fact that stress parameters may also influence the brain serotonergic system, for example, by altering brain trp levels (Kennett et al., 1986).

After 4 weeks of trp supplementation, *chanchita* males and females showed higher forebrain but not hindbrain serotonergic activity, compared to CTL animals. Forebrain 5-HT-ir cells correspond to those in the pretectal area and hypothalamus. We cannot ascertain that the serotonergic activity enhancement produced by trp enrichment actually occurred in the latter populations, because raphe 5-HT-ir fibers are present all throughout the brain, including the forebrain (Kaslin and Panula, 2001). Despite this, aggression, reproduction and visuomotor functioning are regulated by 5-HT at the forebrain (Lucki, 1998; Wullimann, 1998), and thus, elevated serotonergic activity within this area might have hypothetically produced changes in those behaviors, a possibility that should be furthermore investigated.

Enhanced serotonergic activity may result from augmented 5-HIAA, lessened 5-HT, or a combination of both. When we analyzed males and females separately, TRP diets showed no effect on 5-HIAA and 5-HT concentrations. We decided to pool data from both sexes (but separately for TRP and CTL fed animals) given: (1) the morphological resemblance between male and female brain serotonergic systems and (2) the absence of differences in their 5-HT and 5-HIAA brain concentrations. When we grouped such data, we found that TRP-fed individuals had higher 5-HIAA forebrain concentrations than CTL ones. So raised 5-HIAA accounted for the elevated serotonergic activity at this portion of the brain.

As quoted early, trp depletion or enrichment have been employed as therapeutic wide-spread physiological methods for lowering (reviewed in Hood et al., 2005) and elevating (e.g., Attenburrow et al., 2003) 5-HT function in humans, respectively. trp-supplemented diets have also proven to increase brain 5-HT turnover in other mammals, such as rats (Mueller et al., 1976) and pigs (Sève, 1999). Heightened serotonergic signaling with trp enrichment has been consistently notified in rainbow trout *Oncorhynchus mykiss* (e.g., Johnston et al., 1990; Lepage et al., 2002, 2003). In other fish, like Mozmbique tilapia *Oreochromis mossambicus*, trp supplementation augmented brainstem 5-HIAA concentrations, but failed to alter serotonergic activity (Martins et al., 2013), while in Atalantic salmon *Salmo salar* fed with an enhanced trp diet, hypothalamic serotonergic activity was elevated (Basic et al., 2013).

The last-mentioned well-documented causal outcome of trp on 5-HT signaling is, however, not obvious: although trp is known to increase 5-HT synthesis (Fernstrom, 1983), the net result in *chanchita,* as in other species, was an elevation of 5-HIAA, with concomitant serotonergic activity enhancement. How does elevated brain trp availability lead to superior serotonergic activities, without affecting 5-HT levels with respect to non-supplemented trp diets? Higher brain concentration of trp seems not only to generate an increased 5-HT synthesis, but also to an enhanced release (Boadle-Biber, 1993). If this were the case, the excess of trp could exert an increase in speed or amount of released 5-HT, which would rapidly cause an augmented serotonergic signaling. Beyond these speculations, many other attributes must be necessarily considered: for example, (1) 5-HT's immediate precursor, 5-Hydroxytryptophan, is not enhanced in all brain areas where trp is elevated (Chaouloff et al., 1989) and (2) when 5-HT is elevated

in the extracellular matrix at the axonal terminals, several autoreceptors inhibit its synthesis and release (Adell et al., 2002).

In fish, as in other vertebrates, serotonin regulates the hypothalamic–pituitary-adrenal (HPA) axis (e.g. Lim et al., 2013; Medeiros and McDonald, 2013). It is generally accepted that 5-HT stimulates the latter axis in vertebrates (Fuller, 1992), and some authors have postulated that serotonin may not only act during sustained stress, but also at the beginning of the stress response, as an early signal (Gesto et al., 2013). The latter authors noticed that an acute stressor induced a rapid (within 15 s) increase in forebrain serotonergic activity, recovering its basal levels after a few hours. As 5-HT and cortisol are at least functionally related, we wondered if there could be any association between serotonergic outcome and cortisol plasma levels in chanchita individuals. Indeed, forebrain serotonergic activity negatively correlated with cortisol relative variation in females, males, and in both sexes pooled together. Consequently, trp may be responsible for the strength of cortisol relative decrease through an increased forebrain serotonergic activity.

Although the latter results seem to contradict the general idea that elevated 5-HT activity stimulates the HPA, it has also been proposed, as mentioned early in this section with respect to trp, that 5-HT reduces acute and even chronic stress-induced cortisol elevation. We cannot overlook, however, that the serotonergic system exhibits complex and reciprocal interactions with other leading monoamines – such as dopamine – and many other molecules involved in the HPA axis (Basic et al., 2013; Dinan, 1996; Höglund et al., 2001). For example, chanchita females fed with TRP supplementation exhibited higher forebrain concentrations of the main dopamine metabolite 3,4-Dihydroxyphenylacetic acid (unpublished data). Further studies are necessary to elucidate the mechanism through which trp enhances serotonergic activity and strengthens relative cortisol decrease.

Four weeks of a TRP regime had clear effects over cortisol plasma levels and serotonergic outcomes. For the consideration of a trp-enhanced diet as a stress mitigating technique, it should not adversely affect growth or reproductive physiology and behavior. We therefore analyzed how growth parameters changed after 4 weeks of TRP or CTL diets. No significant alterations were present in body weight and length, or condition factor relative changes. Interestingly, however, a strong trend towards higher relative body weight was present in females fed with TRP diets.

Not plenteous works have evaluated the effect of dietary trp supplementation on body growth parameters. In teleost fish, trp added to the food resulted in reduced weight and total length in O. mykiss (Papoutsoglou et al., 2005) and Epinephelus coioides (Hseu et al., 2003),but not in C. mrigala fingerlings (Tejpal et al., 2009). The same heterogeneous results were present on farmed birds fed with trp-enhanced diets, with favorable (Denbow et al., 1993) or detrimental (Rosebrough, 1996) effects over growth parameters. Isolation, independently of the treatment, was pernicious for chanchita females, as they showed negative relative condition factors. Juveniles of the cichlid fish Pelvicachromis taeniatus, grew faster when group reared vs. maintained in isolation (Hesse and Thünken, 2014).

Serotonin controls satiety and hunger via a broad range of receptors (reviewed in Feijó et al., 2011). Evidence predominantly indicates that elevated brain 5-HT signaling inhibits food intake, with concomitant body weight loss (e.g., Lam et al., 2010; Leibowitz and Alexander, 1998). In our study, augmented 5-HT activity with TRP food was not traduced in weight loss with respect to CTL animals. Due to the fact that, even though fish never stop growing, growth rates tend to decelerate with age (Katsanevakis, 2006), at least two characteristics of our experimental design

should not be overlooked: (1) we established a time lapse of 4 weeks between Initial and final growth parameters, time which could have been too short for the appearance of differences, and (2) we used adult specimens, whose growth rates are lower.

On another front, 5-HT and reproduction seem to be quite coupled (recently reviewed, in teleost fish, by Prasad et al., 2015). For example, 5-HT is implicated in such diverse issues as sexual preference (Zhang et al., 2013), stimulation of gonadotropin release (Khan and Thomas, 1992; Somoza et al., 1988; Somoza and Peter, 1991), and ovarian recrudescence and ovulation (Saligaut et al., 1992). Four weeks after being fed with a TRP regime – which enhanced forebrain serotonergic activity, chanchita specimens showed no differences neither on sex steroid plasma levels nor on gonadosomatic indexes, with respect to CTL ones. To the best of our knowledge, this is the first study to seek on the effects of an enriched-trp food on reproductive-related parameters. However, some investigations have evaluated the relationship between 5-HT and the latter hormones in fish. Elofsson et al. (2000) showed a negative correlation between optic tectum 5-HIAA/5-HT and T and 11-KT plasma levels in Arctic charr Salvelinus alpinus males. In the same line, A. burtoni males exhibited negative correlations between 5-HIAA/5-HT in the raphe and preoptic area and testes size (Loveland et al., 2014). These authors also reported a negative correlation between 5-HT type 1A receptor mRNA levels and 11-KT.

It seems, therefore, that high serotonin activity at some regions of the brain is related to lessen circulating sex steroid hormones and gonadal weight. Unchanged hormonal levels or gonadosomatic indexes with TRP pellets probably indicate that serotonin had no effect over those attributes in our experimental design. We must not leave out of consideration that serotonergic enhancement may not have been sufficient to produce changes at those hormones, and that there was a huge variability between animals in terms of their sex steroid plasma levels.

5. Conclusions

In the present work we showed that chanchita's brain serotonergic system is similar to that of other teleost fish, although some differences arouse with phylogenetically related species, and no sexual dimorphism seemed to exist at least at a morphological level or in 5-HT and 5-HIAA concentrations. On the other hand, an 8 times trp-enriched diet delivered during 4 weeks produced a significant reduction on relative cortisol plasma levels, and an enhancement on forebrain serotonergic activity, two attributes that were negatively correlated. We had also shown that trp did not affect growth parameters, gonadosomatic indexes, or sexual steroid plasma levels. Furthermore, trp fed females exhibited a solid trend towards higher relative body weights. Additional studies should be conducted in more realistic scenarios – including social interactions within animals fed with distinct trp concentrations , and considering temporal and regional segregation of serotonergic outcomes, along with other stress markers. The present results lead to consider trp supplementation as an effective "neurodietary" method to decrease the stress response, without disturbing growth, or sex steroid hormones.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.10.005.

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