Dietary L-tryptophan modulates agonistic behavior and brain serotonin in male dyadic contests of a cichlid fish

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Abstract
Although some studies have investigated the effects of dietary L-tryptophan on agonistic behavior, research on adult fish specimens is still lacking. Moreover, submissive behaviors have been generally overlooked. We focused on agonistic behavior between males of the cichlid fish Cichlasoma dimerus, in dyadic encounters held in a novel context after being fed or not with an L-tryptophan enriched diet (TRP) for 2 weeks. We arranged three different dyads: control/control (control conditions: not TRP enriched), control/TRP, and TRP/TRP. We also registered the response of the brain serotonergic system in four brain regions. TRP/TRP dyads showed higher latencies to first attack, lower overall aggression, and lower proportions of bites and passive copings (submissive display) compared to control/control. TRP dominant males performed fewer bites with respect to controls, and subordinate males opposed to TRP males showed fewer passive copings. Higher serotonergic activities were found in subordinates’ optic tectum and in the telencephalon and preoptic area/hypothalamus of TRP males. Altogether, results point out that dietary L-tryptophan reduced males’ motivation to attack and dominant aggression, which consequently influenced subordinate agonistic repertory. In addition, males within TRP/TRP dyads showed a switch in their behavioral agonistic repertory. These behavioral outcomes were probably due to modifications at brain serotonergic functioning.

Keywords Agonistic behavior · Brain · Cichlids · L-Tryptophan · Serotonin

Abbreviations
5-HIAA  5-Hydroxyindoleacetic acid
5-HT  Serotonin
Bs  Brainstem
CTL  Control dietary protocol
CT  CTL vs. TRP male
CC  CTL vs. CTL male
DOM  Dominant
Lt  Total body length
Ot  Optic tectum
Poa/Hyp  Preoptic area/hypothalamus
SUB  Subordinate
Tel  Telencephalon
Trp  L-Tryptophan
TRP  L-Tryptophan supplemented dietary protocol
TT  TRP vs. TRP male

Introduction
Animal conflicts are driven by competition for resources essential for their own survival (e.g., food and shelter), and others, not directly linked to their vitality, are related
to sexually selected traits (e.g., mating, parental care, etc.). Even though there is a high prevalence of inter- and within-individual behavioral plasticity (Stamps 2016), animals generally resolve their conflicts through a ritualized set of behaviors, performed in the context of agonistic encounters (Pellis and Pellis 2015). To illustrate this, for example, two animals may be brought together in a certain space, and this fact leads to the advent of ritualized agonistic behaviors. This simple protocol, in which two animals are joined to elicit a conflict, so-called dyadic contest (Kokko 2013), has been widely exploited experimentally (Ligon 2014; Crothers and Cummings 2015; Clinard et al. 2016).

As with every aspect of sociality, aggression is modulated by neuroendocrine (Nelson and Trainor 2007; Haller 2014; Angus et al. 2016), genetic (Filby et al. 2010) and epigenetic (Lenkov et al. 2015) factors, as well as being extremely dependent on context (Lehtonen et al. 2016) and life history (Veenema 2009; Haller et al. 2014). Among these factors, the neurotransmitter/neuromodulator serotonin (5-HT) seems to play a crucial role, because high brain serotonergic functioning is associated with low aggression (e.g., in mammals: Audero et al. 2013; Duke et al. 2013). In particular, different species of teleost fish have been frequently used to investigate the relationship between aggression and several physiological variables, such as brain serotonergic metabolism. Subordinates (SUBs) and dominants (DOMs) from different teleost fish species have shown chronic differences in serotonergic outcomes (Cubitt et al. 2008; Dahlbom et al. 2012; Loveland et al. 2014; Winberg and Thörnqvist 2016).

The above-mentioned intertwined relationship between brain serotonin and agonistic behavior has been studied not only by altering serotonergic functioning by pharmacological manipulations (e.g., in teleost fish: Dorelle et al. 2017; McDonald 2017), but also by incorporating the rate-limiting substrate for 5-HT’s synthesis, the amino acid l-tryptophan (Trp), into the diet. This is possible as the enzyme responsible for 5-HT’s synthesis from Trp has been reported to be half-saturated in normal conditions (Neff and Tozer 1968; Young and Gauthier 1981). Humans, for example, were tested with depleted or elevated Trp diets. In general, Trp depletion resulted in higher aggressiveness, whereas the contrary occurred with Trp enriched diets (e.g., Bjork et al. 1999; Young 2013), with analogous findings in domesticated animals (DeNapoli et al. 2000; Li et al. 2006). In teleost fish, few studies have evaluated the effect of Trp supplementation on the course of agonistic behavior, and these studies have focused on juveniles. In particular, these investigations have shown a reduction of aggression (Högland et al. 2005; Winberg et al. 2001; Wolkers et al. 2012, 2014), an increase in time until the first attack (Högland et al. 2005; Wolkers et al. 2012), and a change in the temporal appearance of different agonistic displays (Wolkers et al. 2014).

Since the mid-90s, cichlid fish have been gaining strength as biological models for the study of animal behavior and its neuroendocrine aspects. However, although the link between aggression and 5-HT has already been investigated in cichlid species (Loveland et al. 2014; Aguiar and Giaquinto 2018; Higuchi et al. 2018), studies on the effects of dietary Trp on agonistic behavior in this taxonomic group are lacking. Thus, and having this into account, we used a serially monogamous South American cichlid fish, Cichlasoma dimerus (Heckel 1840), to evaluate the relationship between dietary Trp, brain 5-HT, and agonistic behavioral outcomes. This species, locally known as chanchita, exhibits easily distinguishable social behaviors (Ramallo et al. 2014). Briefly, when a group of adult chanchitas is placed together, they promptly begin to display aggressive–submissive behaviors that derive in the establishment and posterior maintenance of a social hierarchy. In this scenario, and after 2–7 days, a single fish (generally a male) emerges as the most DOM within the group and the other—SUBs—occupy a defined position within the hierarchy. In our laboratory, we have already characterized and quantified C. dimerus agonistic interactions in diverse contexts (Alonso et al. 2012; Tubert et al. 2012; Ramallo et al. 2015; Scaia et al. 2018) and localized brain 5-HT immunoreactive cell bodies (Morandini et al. 2015).

In this context, the purpose of the present work was to investigate the effect of a 2-week enriched Trp diet on agonistic behavior. To do so, and after this dietary protocol, adult chanchita males were jointed in dyads in a novel aquarium for 1 h. We registered not only aggressive, but also submissive behaviors throughout the video recordings. In addition, we aimed to study the resultant pattern of brain 5-HT response in Trp and control fed males, as well as in DOMs and SUBs in four brain regions: telencephalon, pre-optic area/hypothalamus, optic tectum, and brainstem.

Materials and methods

Animals and housing

The specimens of chanchita used in this study were captured on the outskirts of the city of Esperanza (31° 24’ S, 60° 59’ W, Santa Fe, Argentina). Upon arriving at the animal housing center of Facultad de Ciencias Exactas y Naturales (Ciudad Autónoma de Buenos Aires, Argentina), animals were placed in groups of 8–10 individuals in 100 l aquaria. They were kept under standardized conditions of temperature (26 ± 2 °C) and photoperiod (14 h light: 10 h dark, with full spectrum illumination) for at least 1 month before starting experiments. Aquaria were arranged with gravel, plants, rocks, and slabs that intended to resemble the environment in which these fish live (Almirón et al. 2015). Each day, they were fed with commercial fish pellets (Traditional Peishe
Koi, Shulet, General Las Heras, Buenos Aires, Argentina). In total, 48 adult chanchita males were used for this study.

**Experimental setup**

Specimens that took part of the experiments were territorial males. These males were obtained from newly established group tanks of 80 l (same standardized conditions mentioned above), composed of 6–8 randomly chosen animals, and from which we simultaneously removed, after 2–4 days from the assembly of the tanks, the territorial individual from two different aquaria. These individuals occupied the highest position in their respective social hierarchy and were easily recognized, because they defended a territory and aggressively attacked other conspecifics within the aquarium (Ramallo et al. 2015).

The two territorial males resulting from different visually isolated group tanks were immediately and completely isolated from each other in 18 l aquaria, at the same conditions of temperature and photoperiod, and were ornamented with plants and gravel. During these days of mutual isolation, both animals were able to see a group of 2–3 chanchita juveniles located in an aquarium ahead (to reduce the detrimental effects of total isolation in this social species). Twenty-four dyads were conformed following this experimental methodology. During the first 3 days of isolation (acclimation period), we fed males with commercial pellets (Traditional Peihe Koi, Shulet, General Las Heras, Buenos Aires, Argentina). For the following 14 days (dietary period), fish received either a control diet (commercial fish pellets) (CTL protocol: 0.3 g of Trp/100 g) (n = 24) or an eight times supplemented Trp one (CTL/TRP protocol: 2.4 g of Trp/100 g). These two types of pellets only differed in the content of Trp. The supplemented diet was prepared in accordance with Wolkers et al. (2012), whose protocol was already employed in a previous work from our group (Morandini et al. 2015). Briefly, the preparation of the diet consisted of sprinkling commercial fish pellets 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 ºC. To avoid palatable effects, CTL parts, and 2.1 g of trp/100 g of food. Supplemented pellets were then dried at 37 ºC. To avoid palatable effects, CTL and stored at − 80 ºC until assayed. The content of 5-HT and 5-HIAA in each brain portion was measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC). For this, we first centrifuged the homogenized samples at 10,000 rpm for 2 min, keeping the supernatants for the assay. Protein content per μl of sample was obtained from homogenized corresponding to approximately 1.5% of their body mass at the first day of the acclimation period and at the first day of the dietary period.

Once the dietary period ended, both males were simultaneously placed in a novel aquarium and video recorded (see the following section for more details) to evaluate their agonistic interaction. As a result of the dyadic encounters, one male resulted DOM (winner) and the other SUB (loser). At the end of the video registrations, we immediately anesthetized both fish with 100 ml of 0.1% benzocaine (ref: E1501, Sigma-Aldrich, Saint Louis, MO, USA) in 2 l of water, determined their $M_b$, $L_s$, and $L_t$, and euthanized them by decapitation after opercula movement stopped. We rapidly dissected brains for the posterior evaluation of 5-HT and 5-hydroxyindoleacetic-5-HIAA- (5-HT’s main metabolite) concentrations. We calculated condition factors (100 × $W_f/L_t^3$) (Fulton 1904) at the start of dietary period (initial) and end of the contest (final) to verify that the general condition of males that consumed either a TRP or CTL diet was similar. This index has been broadly used in teleost fish studies (e.g. Muchlisin et al. 2010; Mir et al. 2012). There were no differences in condition factors between TRP and CTL males for each timepoint (initial: $F_{1,46}=2.03; p=0.161$; final: $F_{1,46}=2.32; p=0.135$; one-way ANOVAs).

**Behavioral analysis**

During the encounter, which took place in a 45 × 25 × 20 cm aquarium (with 21 l of water), animals were videotaped for 1 h (camera employed: HD Everio GZ-E100BU, JVC, Yokohama, Japan) between 12:00 and 14:00 h. A blind observer analyzed the videos. We evaluated two general temporal features of the agonistic encounters (latency to first attack and time of resolution) and the occurrence of seven agonistic interactions: five aggressive (tail hits, mouth holdings, chases, approaches, and bites) and two submissive (passive copings and retreats) (Table 1).

**Brain monoamines**

Brains were carefully and rapidly excised and divided into four major sections: telencephalon (Tel), preoptic area/ hypothalamus (Poa/Hyp), optic tectum (Ot), and brainstem (Bs). Each section was placed in a tube containing 250 μl of cold 4% (w/v) hydrochloric acid, promptly disrupted with a homogenizer (PRO200, PRO Scientific, Oxford, CT, USA), and stored at −80 ºC until assayed. The content of 5-HT and 5-HIAA in each brain portion was measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC). For this, we first centrifuged the homogenized samples at 10,000 rpm for 2 min, keeping the supernatants for the assay. Protein content per μl of sample was obtained from homogenized...
samples following Lowry et al. (1951), to which monoamine concentration was then normalized. The HPLC-EC system employed consisted of a Phenomenex Luna 5-μm, C18, 150 × 4.60 mm column (Phenomenex, Torrance, CA, USA) and an 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 an Ag/AgCl reference electrode. The mobile phase contained 0.5 mM EDTA, 0.76 M NaH₂PO₄·H₂O, 1.2 mM 1-octane sulfonic acid, and 5% acetonitrile, with pH of 2.8.

Samples were quantified by their comparison with standard solutions of known concentrations. 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 calculated and used as an estimator of serotonergic activity, in line with other studies in teleost fish (e.g., Teles et al. 2013; Loveland et al. 2014).

Statistics

Data were statistically processed with R-3.4.3. We compared latency to the first attack and time of resolution between the three types of encounters (CC, CT, and TT) by separate one-way analysis of variance (ANOVA), with type of dyad as factor, and corrected p values by the false discovery rate (FDR) (Benjamini et al. 2006). Normal distribution of residuals and variance’s homoscedasticity was fulfilled. We used post hoc contrasts to evaluate significant differences (Tukey’s test) when three or more independent means were compared with ANOVA.

To investigate whether the observed proportion of each agonistic display within CT and TT dyads was similar to that expected (we considered as “expected” the percentage of each agonistic display observed within CC dyads), we performed t tests. p values were corrected by FDR.

Table 1 Definitions of contest temporal attributes and aggressive and submissive displays observed in chanchita (Cichlasoma dimerus) male dyadic encounters

| Definition |
|---|
| Temporal attributes |
| Latency | Time elapsed since animals are placed in the same aquarium until either of them performed an aggressive display |
| Time of resolution | Period between the performance/recording of the first aggressive display until either fish performs five consecutive aggressive or submissive displays. At this point, the contest is considered resolved |
| Aggressive displays |
| Tail hit | Both animals lay parallel to each other, close to each other but not in direct physical contact, and slap their tail towards the other |
| Mouth holding | Two fish hold each other from the mouth, pulling and biting while holding |
| Chase | Fish moves rapidly towards opponent, chasing it for more than one body length (no body contact) |
| Approach | Fish swims quickly towards the opponent, moving less than a body length, but not contacting the other fish |
| Bite | Fish contacts opponent’s body, opens and closes the mouth |
| Submissive displays |
| Passive coping | Fish stays relatively immobile, slightly tilted up, in response to opponent’s approaches or bites |
| Retreat | Fish moves away, responding to opponent’s bites, approaches or chases |

To reduce the complexity of analyzing each behavioral display, we performed a prospective principal component analysis (PCA). For this, the individual frequency of each agonistic display was incorporated into the PCA, and we then analyzed the contribution of each behavior to the variance.

We evaluated if SUB and DOM males formed two statistically significant different groups when considering individual behavioral frequencies through a MANOVA, with the frequency of each agonistic display. To assess individual behavior, we compared approach and biting frequencies between DOM males by two-way ANOVAs, with dietary protocol of DOM fish (focal diet: TRP or CTL), and dietary protocol of the opponent (opponent diet: TRP or CTL) as factors. Analogously, we performed another set of two-way ANOVAs to study SUB behavior and evaluated the effect of their diet (focal diet: TRP or CTL) and opponent’s diet (TRP or CTL) on the frequency of retreats and passive copings. p values were corrected by FDR. Assumptions for parametric statistics were fulfilled.

Posteriorly, we studied if DOM and SUB differed in size. To do so, we carried out paired t tests, comparing DOM and SUB Lₜ (total length) and Mₜ (body weight) for each type of dyad. Then, we evaluated if those differences were the same or not among the three dyads. To do so, we calculated an index for Wₜ and Lₜ for each animal: (e.g., 100 × (DOM Lₜ − SUB Lₜ)/DOM Lₜ) and compared it for CC, CT and TT dyads through a one-way ANOVA. Parametric assumptions were fulfilled, and p values were corrected by FDR.

Finally, we performed two-way ANOVAs to evaluate brain concentrations of 5-HT, 5-HIAA, and serotonergic activity in the four brain regions: Tel, Poa/Hyp, Ot, and Bs, with diet and resultant rank as factors. We did not perform a repeated measure analysis because of missing values from
brain regions within each animal. To meet normality of the variables, data had to be square root transformed. \( p \) values were corrected by FDR.

We considered a data to be outlier when the studentized residuals were \( X \pm 3 \) and were thus excluded from the corresponding analyzes. Because of this, and missing values due to experimental circumstances, sample sizes were variable. Statistical significance was set at 0.05.

**Results**

**Contest temporal attributes**

There was an effect of the type of dyad on latency to the first attack (one-way ANOVA: \( F_{2,21} = 6.78, p = 0.012 \)). TT males took almost twice as long to perform the first attack with respect to males within CC (post hoc Tukey, 7.99 ± 0.87 min vs. 4.31 ± 0.74 min) and CT dyads (post hoc Tukey, 3.87 ± 0.55 min) (Fig. 1a). On the other hand, the time males took to resolve their conflicts did not differ among the three dyads (one-way ANOVA: \( F_{2,21} = 0.57, p = 0.573 \)) (Fig. 1b).

**Contest agonistic interactions**

The most frequent aggressive displays were on average bites (63.5%) followed by approaches (27.3%) and chases (3.6%), whereas tail hits (3.5%) and mouth holdings (2.1%) were quite infrequent, and if present, they were performed only at the beginning of the contest, in a period in which conflicts were still unresolved. With respect to submissive displays, retreats (61.3%) were more frequent than passive copings (38.7%).

The observed percentages of some agonistic displays in TT dyads were different from those expected (observed CC percentages) (Table 2). Within aggressive displays, TT bites showed a significant bias with respect to CC ones. Indeed, they were less representative than expected (TT: 53.9 ± 5.8% and CC: 72.1 ± 6.2% of bites; \( t_{14} = 3.02, p = 0.009 \)). Regarding submissive behaviors, TT retreats were more representative (or passive copings were less representative) than those of CC dyads (TT: 75.5 ± 4.4% of retreats and CC: 52.1 ± 8.3% of retreats; \( t_{14} = 2.65, p = 0.019 \)). Other agonistic interactions within TT and CT dyads did not differ from expected (CC dyads) (Table 2).

**Individual agonistic behavior**

All contests resulted in a clear winner (DOM) and loser (SUB). For the unique contest in which there could have been either a TRP or a CTL winner or loser, in seven out eight cases, CTL males initiated CT contests, and five out of eight were winners. The first two principal components (PC) of the PCA we performed, which considered inter-individual behavioral variation, incorporated 79% of the variance (69% PC1 and 10% PC2). DOM males are characterized by negative values of PC1 and SUB males by positive ones (Fig. 2). The PC1 had mainly the contribution of the four more frequent displays mentioned above: bites and approaches with negative loadings (−0.71 and −0.65, respectively), and retreats and passive copings with positive ones (0.70 and 0.71), with very little contribution of mouth holdings (0.05), tail hits (−0.08) and chases (0.16). For posterior analyzes, mouth holdings, tail hits, and chases were discharged.

We decided to divide the analysis between DOM and SUB (instead of considering rank as another factor), because they indeed showed clear behavioral biases: from 5919 agonistic
interactions, only 21 submissive displays were performed by DOM males, and the same happened with SUB males, which total aggressive displays were 35. In accordance, SUB and DOM males formed two statistically significant different groups when considering individual behavioral frequencies (MANOVA: Pillai, $F_{7,30} = 10.51$, $p < 0.0001$).

How was the individual behavior of DOM and SUB males, considering their own dietary protocol and the dietary protocol of their opponent? DOM males that followed a TRP dietary protocol, showed 3 times less frequencies of bites with respect to CTL males ($0.74 \pm 0.33$ bites/min vs. $2.31 \pm 0.31$ bites/min; $F_{1,22} = 8.91$, $p = 0.032$), whereas opponent’s diet had no significant effect on DOM frequency of bites ($F_{1,22} = 0.75$, $p = 0.390$; two outliers were removed from CTL data) (Fig. 3a). There was no significant interaction between focal and opponent diet for biting frequencies ($F_{1,22} = 0.01$, $p = 0.986$). For the frequency of approaches performed by DOM males, there was no effect of either focal ($F_{1,21} = 2.37$, $p = 0.224$) or opponent diet ($F_{1,21} = 2.98$, $p = 0.091$) (Fig. 3b), neither a significant interaction between both factors ($F_{1,21} = 1.97$, $p = 0.168$, respectively).

For SUB males, there was no significant effect of the diet received on their frequency of passive copings ($F_{1,21} = 0.17$, $p = 0.238$), but when their opponents received a TRP diet, they performed 2.4 times less passive coping displays per minute ($0.49 \pm 0.14$ vs. $1.17 \pm 0.18$; $F_{1,21} = 8.21$, $p = 0.028$) (Fig. 3c). There was no interaction between both factors ($F_{1,21} = 0.16$, $p = 0.692$). Finally, neither focal ($F_{1,22} = 0.04$, $p = 0.522$; one outlier removed from CTL data) nor opponent ($F_{1,22} = 3.22$, $p = 0.121$; two outliers removed from CTL data) diets had a significant effect on the frequency of retreats performed by SUB males (Fig. 3c), factors that did not significantly interact ($F_{1,22} = 0.07$, $p = 0.798$).

Social rank dependence on morphological attributes

In 3 out of 24 dyads, the SUB male was larger than the DOM male, two from CT and one from TT, and, also, in 3 out of 24, SUB males were heavier than DOM males, one from each type of dyad. We further investigated if body size differences between both males of each dyad could have determined which animal emerged as DOM. As shown in Table 3, DOM males were significantly larger and heavier than SUB males, independently of the type of dyad. In particular, DOM males were 11% larger than SUB males for CC and TT dyads ($p = 0.002$ for both comparisons), 5% larger for CT ones ($p = 0.03$). With respect to body weight, DOM males were 32% heavier than SUB for CC dyads ($p = 0.002$), 31% for CT dyads ($p = 0.03$), and 25% for TT ones ($p = 0.003$).
Finally, we compared these results among each dyad by performing a paired index per contest for $L_t$ and $W_b$. Results showed that the differences in $L_t$ and $W_b$ between DOM and SUB were not significantly different among the three dyads ($F_{1,21} = 0.70, p = 0.507$ for $L_t$ and $F_{1,21} = 0.20, p = 0.818$ for $W_b$).

### Brain 5-HT, 5-HIAA, and serotonergic activity

There was no interaction between diet and rank in none of the brain regions for 5-HT or 5-HIAA concentrations and brain serotonergic activity. Because of this, brain variables in SUB and DOM responded in a similar manner for TRP or CTL diets. Thus, we proceeded to study, separately, the effects of rank and type of diet on the concentration of brain 5-HT, [5-HT], brain 5-HIAA, [5-HIAA], and serotonergic activity (5-HIAA/5-HT).

There were no differences in [5-HT] between ranks for the Tel ($F_{1,36} = 0.13, p = 0.721$), Poa/Hyp ($F_{1,37} = 0.52, p = 0.474$), Bs ($F_{1,38} = 1.52, p = 0.226$), but for Ot, differences were marginally significant ($F_{1,40} = 4.05, p = 0.052$) (Fig. 4a). For [5-HIAA], SUB and DOM did not differ in Tel, Poa/Hyp or Ot (Tel: $F_{1,35} = 0.87, p = 0.357$; Poa/Hyp: $F_{1,39} = 1.03, p = 0.316$; Ot: $F_{1,38} = 2.41, p = 0.130$), but showed a marginally significant difference in the Bs ($F_{1,38} = 3.37, p = 0.075$) (Fig. 4b). Serotonergic activities,
Table 3 Comparison of total body length ($L_t$) and weight ($W_b$) between dominant (DOM) and subordinate (SUB) males of chanchita (Cichlasoma dimersus), for each type of dyad: CTL/CTL (CC), CTL/TRP (CT), and TRP/TRP (TT)

|       | DOM | SUB | Difference (95% CI) | t (df = 7) | p     |
|-------|-----|-----|---------------------|------------|-------|
| $L_t$ (cm) |     |     |                     |            |       |
| CC    | 10.2 ± 0.5 | 9.2 ± 0.4 | 0.7–1.8 | 5.66 | 0.002 |
| CT    | 10.1 ± 0.8 | 9.6 ± 0.7 | 0.1–1.2 | 2.89 | 0.03  |
| TT    | 10.9 ± 0.7 | 9.8 ± 0.5 | 0.8–2   | 5.51 | 0.002 |
| $W_b$ (g) |     |     |                     |            |       |
| CC    | 30.5 ± 6.9 | 23.1 ± 5.7 | 3.8–11.1 | 4.81 | 0.003 |
| CT    | 31.3 ± 6.8 | 23.9 ± 6.3 | 1.2–13.5 | 2.83 | 0.03  |
| TT    | 30.7 ± 6.7 | 24.5 ± 6.6 | 3.3–9   | 5.14 | 0.002 |

Data (mean ± SEM) were analyzed by paired $t$ tests between SUB and DOM, for each morphological attribute. Bold $p$ values (corrected by FDR) indicate statistical significance at $\alpha = 0.05$, $n = 8$ (replicates within each rank and type of dyad).

however, were higher in the Ot of SUB with respect to DOM (1.06 ± 0.08 vs. 0.79 ± 0.09; $F_{1,35} = 9.20$, $p = 0.032$), and did not differ in the other brains areas: Tel ($F_{1,35} = 0.21$, $p = 0.653$), Poa/Hyp ($F_{1,37} = 0.82$, $p = 0.370$), and Bs ($F_{1,38} = 0.37$, $p = 0.546$) (Fig. 4c).

We proceeded to compare CTL vs. TRP fed males. Having been fed with either a CTL or a TRP diet did not produce any clear effect on [5-HT] in either of the brain regions evaluated (Tel: $F_{1,35} = 0.11$, $p = 0.738$; Poa/Hyp: $F_{1,37} = 2.70$, $p = 0.109$; Ot: $F_{1,38} = 0.98$, $p = 0.328$; Bs: $F_{1,40} = 0.18$, $p = 0.67$) (Fig. 5a). On the other hand, [5-HIAA] was higher in two brain areas of TRP males: Tel ($7.95 \pm 0.44$ vs. $6.40 \pm 0.49$ pg/µg of protein; $F_{1,35} = 5.56$, $p = 0.025$) and Poa/Hyp ($4.36 \pm 0.30$ vs. $3.51 \pm 0.26$ pg/µg of protein; $F_{1,35} = 5.38$, $p = 0.026$), and did not differ in the other two regions (Ot: $F_{1,38} = 0.01$, $p = 0.915$; Bs: $F_{1,38} = 2.06$, $p = 0.160$) (Fig. 5b). Finally, higher serotonergic activities were found in the Tel ($1.03 \pm 0.09$ vs. $0.78 \pm 0.09$; $F_{1,35} = 4.43$, $p = 0.043$) and Poa/Hyp ($0.85 \pm 0.06$ vs. $0.62 \pm 0.06$; $F_{1,37} = 7.52$, $p = 0.0153$) of TRP males, but not in Ot ($F_{1,35} = 5.23$, $p = 0.195$) or Bs ($F_{1,38} = 2.28$, $p = 0.117$) (Fig. 5c).

Discussion

In this work, we showed that, in chanchita adult males, a 2-week TRP enriched diet modified their agonistic repertory in the context of dyadic encounters. Particularly, when both males underwent a TRP dietary protocol (TT), the latency to the first attack increased. TT contests were also characterized by a substantial diminution of the expected percentage of bites and passive copings, and dominant (DOM) males were less aggressive. Regarding individual behavior, DOM males fed with TRP performed fewer bites, and when subordinate (SUB) males were opposed to a TRP fed male, these showed less frequencies of passive copings (fish stays relatively immobile in response to opponent’s attacks). On the other hand, DOM and SUB males were equally represented in both types of diets, and body size difference was an important factor for determining dominance. Brain serotonergic outcomes were also modulated by both dietary protocol and rank. SUB males showed higher serotonergic activity ([5-HIAA]/[5-HT]) in their optic tectum (Ot). Finally, TRP fed males exhibited higher [5-HIAA] and [5-HIAA]/[5-HT] in the telencephalon and preoptic area/hypothalamus Poa/Hyp. To the best of our knowledge, all the studies on the effect of Trp diet on agonistic behavior in fish have focused on juveniles (reviewed in Hoseini et al. 2019), and thus, these are the first results on adult specimens.

Behavioral modulation

Males within TT dyads took twice as long to attack with respect to CT and CC ones. Because animal’s motivation to engage in a conflict strongly depends on the perceived value of the resource in dispute (Arnott and Elwood 2008), it is plausible to consider that Trp supplementation perhaps produced an alteration on that perception: TT males were not so urged to start fighting for territory or dominance. Alternatively, dietary Trp could have modified males’ capacity of adaptation to a novel environment, resulting in a longer recognition time of the new context. On the other hand, CTL fish within CT dyads may have taken the opportunity of engaging (earlier) in a dispute with respect to males from TT dyads, given that they might have assessed less motivated TRP opponents. In fact, in seven out eight cases, CTL males initiated CT contests.

CT and CC dyads did not differ in their latencies, in line with reports from rainbow trout dyadic encounters (Winberg et al. 2001). The absence of difference for chanchita, on the other side, is not completely in tune with all the previous studies in other fish species: for example, in Brycon amazonicus (Spix and Agassiz 1829), latencies were significantly higher when one fish was fed with Trp with respect to control fed fish (Wolkers et al. 2012). Still, it is important to note that these studies present some experimental differences with our work: (1) they used juveniles; (2) the sex was not distinguished; and (3) fish were not placed in a novel context. Instead, resident/intruder paradigms were employed. In general, resident fish underwent either a CTL or TRP protocol, and behavioral comparisons were inspected between both groups of residents when a CTL intruder was placed in their territory. Therefore, the TT group here evaluated has not been studied. In addition, because in the present work, no male had its own territory, dominance might have also

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been a resource for which males competed. In this sense, Serra et al. (2015) showed, for example, that the interaction between basal cortisol levels and aggression depended on the novelty or not of the arena in which B. amazonicus fought, suggesting that the context in which agonistic encounters are held is an important factor to account for. Dietary Trp concentration and duration are other factors of divergence between different studies; in effect, for example, latencies to the first attack were reported to be dependent on the dose of dietary Trp (Wolkers et al. 2012). Finally, interspecies differences due to divergent evolutionary pressures could also account for discrepancies between the present results and others regarding teleost species.

Conflict time of resolution did not differ between the three types of dyads. To have obtained differences in the time of resolution, SUB males had to have signaled their subordinate condition earlier or later. The fact that this has not occurred suggests that dietary Trp did not seem to have affected the temporal dynamic of conflict resolution, which may have been more dependent on the way in which each male perceived itself in relation to its opponent.

In TT contests, the expected (CC dyads) percentage of bites was reduced, as well as the relative percentage of passive copings (or, in other words, the expected relative percentage of retreats was higher). This is the first report of a modification in behavioral agonistic repertory due to dietary Trp, and suggests, along with the increase in latency, that chanchita males from TT contests decided to go through their conflict in a different way with respect to CC and CT males. In addition, DOM males from TT dyads were less aggressive than those from CC ones, an observation that is in line with other studies on teleost fish (Winberg et al. 2001; Lepage et al. 2005; Wolkers et al. 2012, 2014). Notably, Wolkers et al. (2014) found that aggression diminution was present during the first 20 min of the agonistic encounters. Because of this, it is possible that Trp effects in the present work could have been framed to certain lapse/s of the contests, and thus, the temporal evolution of the agonistic repertory should not be overlooked in future works.

The latter modifications were due to changes in the frequency of punctual agonistic interactions. TRP males that won the fight produced three times less frequencies of bites, which are the unique asymmetrical agonistic interactions that imply body contact in chanchita males’ dyadic encounters. Whereas focal diet affected
biting frequencies, opponent’s diet was associated with submissive displays. When SUB males, independently of their own diet, had a TRP opponent, they showed a 2.4 times reduction in the frequency of passive copings with respect to CTL opponents. The most obvious scenario is that SUB males opposed to TRP males performed fewer frequencies of passive copings because DOM TRP males were substantially less aggressive. In other words, due to the modulation of DOM males’ agonistic behavior, SUB males could have modified their own behavior, because this strongly depends on how DOM males act. It is within the framework of this social interaction that SUB males probably adjusted their behavior, implying that dietary Trp might have indirectly acted on SUBs’ agonistic outcome by directly modifying DOM males’ behavior. In line with this, a study on zebra fish has shown that fish not only evaluate their opponent in relation to themselves, but also assess contest outcome as it happens (Oliveira et al. 2016), constantly adjusting their own behavior.

As expected, DOM and SUB formed two distinct multivariate groups when considering individual agonistic displays. In all contests, there was a clear winner and loser, and both behavioral phenotypes were equally represented within the dietary protocols. Even though prior experience can modulate future contest outcomes (Lan and Hsu 2011; Dijkstra et al. 2012) and overcome size disadvantages (Beaugrand et al. 1991; Alcazar et al. 2014), winning or losing for chanchita males seemed to have strongly depended, but possibly not exclusively, on body size differences. In the three types of dyads, DOMs were significantly larger and heavier than their opponents, and these differences between DOM and SUB were not significantly different among the three dyads. Inter-individual size asymmetries are indeed major factors related to dominance in cichlid fish (Wazlavek and Figler 1989; Neat et al. 1998; Baduy et al. 2017). Altogether, these results suggest that there was no effect of the diet on the expected proportion (50/50) of TRP and CTL winners and losers, neither on body size dominance determination.

Although body size differences were markedly related to rank independently of the type of dyad (CC, CT, or TT), with heavier and/larger males becoming DOM, in 3 out of 24 dyads, larger and/or heavier males resulted SUB. Inter-individual variation within chanchita behavioral strategies are an important factor here overlooked that could explain these results. Indeed, fish behavioral coping styles/strategies (for

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**Fig. 5** Serotonin ([5-HT], 5-hydroxyindoleacetic acid ([5-HIAA]), and serotonergic activity ([5-HIAA]/[5-HT]) in four brain regions of control CTL and TRP fed Cichlasoma dimerus males: telencephalon (Tel) preoptic area/hypothalamus (Poa/Hyp), optic tectum (Ot) and brainstem (Bs). We performed two-way ANOVAs with rank (DOM or SUB) and type of diet (CTL or TRP) as class variables. Because of the absence of interaction between both factors for either of the variables studied, here we present data referred to diet (social rank effects are presented on the previous figure). *p* values were corrected by FDR. a CTL and TRP males did not differ on brain [5-HT] (Tel: *p* = 0.738; Poa/Hyp: *p* = 0.109; Ot: *p* = 0.328; Bs: *p* = 0.67). b On the other hand, [5-HIAA] was higher in the Tel (*p* = 0.025) and Poa/Hyp (*p* = 0.026) of TRP males, but did not differ either at the Ot (*p* = 0.915) or Bs (*p* = 0.160). c In the same line of [5-HIAA], higher [5-HIAA]/[5-HT] were present in the Tel (*p* = 0.043) and Poa/Hyp (*p* = 0.015) of TRP males, and not in the Ot (*p* = 0.195) or Bs (*p* = 0.117). Asterisks indicate statistical differences (*p* < 0.05) between CTL and TRP within a brain region. Data are expressed in box plots (5–95 percentiles). Within the box plot, crosses indicate mean values and transverse lines median values.
example, being proactive or reactive, or presenting different degrees of boldness), as well life experiences above-mentioned can predict aggression and rank acquisition (Överli et al. 2004), and overall contest behavior (Briffa et al. 2015), and they can even be modulated by slight differences in social context (Frost et al. 2007). Thus, unexplained behavioral outcomes in the present work could be due not only to different life experiences, but also to unexplored interactions between dietary Trp and inter-individual coping strategies.

**Serotonergic outcome**

Behavioral modulation as a result of dietary Trp was accompanied by brain serotonergic alterations. At first, we analyzed serotonergic profiles of DOM and SUB males, as there was no interaction between rank and diet. SUB and DOM males did not differ on [5-HT] or [5-HIAA] at any of the brain regions evaluated. However, there was a trend towards lower [5-HT] in the Ot of SUB males, and higher Bs [5-HIAA], also in SUBs. These results are difficult to interpret in isolation, because their physiological meaning is dependent on its relationship with their reciprocal molecule: 5-HIAA or 5-HT, as appropriate. Because of this, the ratio [5-HIAA]/[5-HT] is a better way to shed light on the activity of the serotonergic system at a specific brain region (Shannon et al. 1986). In the present study, SUB males exhibited 30% higher [5-HIAA]/[5-HT] at the Ot. These higher serotonergic activities in chanchita SUB males may alternatively be related to avoidance responses in which 5-HT is implicated at that brain area (Herrero et al. 1998). During agonistic encounters, the brain 5-HT system is activated in all socially challenged fish (Winberg et al. 1991; Wolkers et al. 2015), but higher activities seem to endure only in SUB (Summers et al. 2005). Indeed, SUB fish have shown higher serotonergic activities in varied brain areas compared to DOM (Winberg and Nilsson 1993; Dahlbom et al. 2012; Teles et al. 2013; Loveland et al. 2014). However, these studies have focused on consolidated (at least more than 1 day) social ranks.

In relation to the possible effects of diet, CTL and TRP males did not show differences on [5-HT] at any brain area. Nonetheless, TRP males exhibited higher [5-HIAA] at the Tel and Poa/Hyp, which were accompanied by higher [5-HIAA]/[5-HT] at those areas. In a previous work on this species, we showed that isolated males fed with the same Trp supplemented diet exhibited higher serotonergic activities at the forebrain, which included the Tel and Poa/Hyp (Morandini et al. 2015). These augmented serotonergic activities in both TRP DOM and SUB males could explain the demotivation to initiate an attack within TT dyads, and the reduction of TRP DOM aggression. Accordingly, high brain serotonergic activities have been related to suppression of aggression and an increase in latency to the first attack in fish (Winberg and Thörnqvist 2016). However, could the increase in [5-HIAA]/[5-HT] in the Tel and Poa/Hyp also explain the change in SUB behavior (higher proportion of retreats with respect to passive copings) when they were opposed to DOM TRP? Because there was no effect of dietary Trp on SUB agonistic behavior, the most plausible hypothesis is that the modulation of SUB behavior was not mediated by Trp; instead, and as stated earlier, SUB males could have changed their agonistic repertory because of TRP DOM males’ behavior. Nonetheless, further in the discussion, we will propose an alternative explanation regarding increase in [5-HIAA]/[5-HT] in the Tel and Poa/Hyp of both DOM and SUB under a Trp dietary protocol.

Not surprisingly, considering the extreme dependence of brain 5-HT on Trp physiological levels (Fernstrom and Wurtman 1971), elevated serotonergic activities with dietary Trp enrichment have already been registered in other teleost species (Johnston et al. 1990; Lepage et al. 2002, 2003; Basic et al. 2013). It is not possible to undoubtedly ascertain which 5-HT population was responsible for the serotonergic activation due to Trp treatment. Chanchita’s brain serotonergic system is composed of three populations, located at the pretectum, hypothalamus and brainstem (Morandini et al. 2015). To take one example, brainstem 5-HT fibers project almost all regions of the fish Danio rerio (Hamilton-Buchanan 1822) brain (Caslin and Panula 2001), including the forebrain, and thus, serotonergic activities are generally calculated with [5-HT] and [5-HIAA] from different brain sources. Consequently, we cannot finely relate brain serotonergic changes with behavioral modulations.

Regardless of which 5-HT population is implicated, it seems to be clear that 5-HT does not only play a key role on aggression, but also on animal coping strategies (Koolhaas et al. 2010). Passive and active coping styles have been extensively studied in fish (van Raaij et al. 1996; Överli et al. 2002; Castanheira et al. 2013): passive copings are related to reactive personalities, whereas the opposite occurs with escaping strategies.

Accordingly, chanchita DOM males under a TRP regime could be seen as less proactive (less aggressive) than CTLs, whereas the opposite could be said for SUB males opposed to TRP males, which showed a higher relative proportion of retreats with respect to passive copings when compared to the expected proportion (always within TT contests), suggesting that 5-HT may differentially modulate SUB and DOM agonistic repertory. Maruska et al. (2013) showed that social ascent or descent in males of the cichlid fish Astatotilapia burtoni ( Günther 1894) resulted in a differential pattern of immediate early genes brain expression, between emerging DOMs and SUBs. The authors evaluated brain nuclei implicated in the ‘social behavior network’ (Goodson 2005) and found that the brain areas implicated showed a high presence on 5-HT immunoreactive fibers (Caslin and
Panula 2001). In the same line, Teles et al. (2016) evidenced that loser and winner zebrafish presented specific patterns of gene expression at different brain areas. Therefore, it is plausible that the alteration of brain serotonergic system in TRP males could have distinctively affected DOM and SUB behaviors through the modulation of some forebrain areas that constitute the ‘social brain network’, thus exhibiting different patterns of brain activity (Pan et al. 2010).

In conclusion, dietary Trp reduced the motivation to attack, and modulated both aggressive and submissive behaviors, the latter of which have been generally unconsidered. In addition, Trp supplementation produced a change in males’ agonistic repertory, which would have been direct for DOM males, and indirect for SUB, which probably adjusted their behavior according to what they perceived from DOMs. Modifications at the brain serotonergic system could have potentially accompanied these behavioral outcomes. To the best of our knowledge, this was the first study on the effects of a Trp enriched diet on adult intra-sexual agonistic behavior in a teleost fish. Future studies in the same line on other teleost species would shed light on the present results and should also focus on female intra-sexual agonistic behavior.

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Compliance with ethical standards

Ethical standards All experiments were conducted in conformity with international standards on animal welfare, as well as being in accordance to institutional (Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) and national (Comité Nacional de Ética en la Ciencia y la Tecnología, MINCyT, Argentina) regulations. All procedures were in compliance with the Guide for Care and Use of Laboratory Animals (National Research Council 2011).

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