The Journal of Experimental Biology 206, 3589-3599

doi:10.1242/jeb.00614



# Time-course of the effect of dietary L-tryptophan on plasma cortisol levels in rainbow trout *Oncorhynchus mykiss*

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*Accepted 15 July 2003*

## Summary

**Isolated juvenile rainbow trout were fed a feed supplemented with L-tryptophan (TRP) for 3, 7 or 28 days, after which they were either sampled directly (undisturbed) or subjected to a standardised stressor prior to sampling. Controls (stressed and undisturbed) received the same feed but without any supplementary TRP. Stress resulted in a significant elevation of plasma [cortisol] in fish fed control feed and in fish fed TRP-supplemented feed for 3 and 28 days. However, fish fed TRP-supplemented feed for 7 days did not show any significant elevation of plasma [cortisol] in response to stress. Plasma levels of adrenocorticotropin followed the same general pattern as cortisol. Plasma and brain [TRP] were elevated in fish fed TRP-supplemented feed. The amino acid TRP is the precursor of the monoamine neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) and the brain 5-HT system is known to be involved in the control of the hypothalamic–pituitary–interrenal (HPI) axis. Fish fed TRP-supplemented feed showed elevated levels of 5-hydroxyindoleacetic acid (5-HIAA, a major 5-HT metabolite) in the hypothalamus and optic tectum. However, TRP treatment did not appear to result in any effects on brain dopaminergic activity and the effects on brain norepinephric activity do not support a role of norepinephrine in mediating the effects of TRP on HPI axis reactivity in rainbow trout.**

Key words: serotonin, brain, fish, rainbow trout, *Oncorhynchus mykiss*, feed, stress, Salmonidae, aquaculture.

## Introduction

The essential amino acid L-tryptophan (TRP) is the precursor of the monoamine neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). The first and rate-limiting step in the 5-HT biosynthesis is the hydroxylation of TRP to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH) (reviewed by Boadle-Biber, 1993). This enzyme is not saturated by its substrate, TRP, *in vivo* and is not subjected to inhibition by 5-HT, the endproduct of the reaction pathway. Thus, brain TRP availability is a major determinant of the 5-HT synthesis rate, and elevated dietary intake of TRP elevates brain TRP levels, which in turn increase the rate of brain 5-HT biosynthesis in fish (Johnston et al., 1990; Aldegunde et al., 1998, 2000; Winberg et al., 2001; Lepage et al., 2002) as well as in mammals (Fernstrom, 1983; Fernstom and Wurtman, 1972; Boadle-Biber, 1993). In mammals, it has been confirmed, using *in vivo* micro dialysis and voltametry, that elevated dietary intake of TRP results in increased functional release of 5-HT (reviewed by Boadle-Biber, 1993).

Brain 5-HT is involved in the regulation of the hypothalamus–pituitary–adrenocortical (HPA) axis in mammals (Chaoulof, 1993; Dinan, 1996) as well as in the control of the hypothalamus–pituitary–interrenal (HPI) axis in fish (Winberg et al., 1997; Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2002). Stressors, like social subordination, handling and predator exposure, usually produce a rapid activation of the brain serotonergic system, revealed by an increase in brain levels of the major serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and/or elevated brain [5-HIAA]/[5-HT] ratios (an index of 5-HT activity) (Winberg et al., 1992; Winberg and Nilsson, 1993). In several studies, brain [5-HIAA]/[5-HT] ratios have been found to correlate with plasma [cortisol] (Winberg and Lepage, 1998; Øverli et al., 1999) and adrenocorticotropin (ACTH) levels (Höglund et al., 2000), suggesting that brain 5-HT has a stimulatory action on the HPI axis. However, the role of the brain 5-HT system in the control of the HPI axis is still not clear. For instance, 8-OH-DPAT, a selective 5-$HT_{1A}$ receptor agonist, may have either stimulatory or inhibitory effects on HPI axis activity in rainbow trout, depending on the dose and context. In undisturbed fish 8-OH-DPAT stimulates the HPI axis (Winberg et al., 1997; Höglund et al., 2002), whereas if administrated at low doses to stressed fish, 8-OH-DPAT has the opposite effect, suppressing the stress-induced elevation of plasma [ACTH] and [cortisol] (Höglund et al., 2002).

In a previous study we similarly showed that feeding rainbow trout TRP-supplemented feed for 7 days results in a slight but significant elevation of basal plasma levels of cortisol, but at the same time causes a significant reduction in the stress-induced elevation of plasma cortisol concentrations (Lepage et al., 2002). These effects were suggested to occur as a consequence of the elevated 5-HT activity caused by dietary TRP supplementation, although it is not clear through what mechanisms 5-HT modulates HPI axis activity. Winberg et al. (2001) reported that dietary supplementation with TRP for 7 days also results in an inhibition of aggressive behaviour in rainbow trout, whereas 3 days of TRP supplementation have no effect on aggressive behaviour. An anti-aggressive effect of the brain 5-HT system has been reported in a number of vertebrates (Raleigh et al., 1991; Blanchard et al., 1991, 1993; Deckel, 1996; Deckel and Jevitts, 1997; Edwards and Kravitz, 1997; Larson and Summers, 2001) including teleost fish (Adams et al., 1996; Winberg and Nilsson, 1993), and the suppression of aggressive behaviour induced by elevated dietary TRP is believed to be mediated by elevated brain 5-HT activity.

Norepinephrine (NE) and dopamine (DA) are also important in the control of neuroendocrine release factors at the level of the hypothalamus and pituitary. For instance, in teleost fish, the central NE system has been suggested to stimulate HPI axis activity (Øverli et al., 1999; Höglund et al., 2000). DA, on the other hand, might have effects that are to some extent opposite to those of 5-HT (Winberg and Nilsson, 1992), and L-dopa treatment, which elevates brain DA activity, has been reported to induce social dominance (Winberg and Nilsson, 1992) and to counteract the stress-induced elevation of plasma [cortisol] and brain 5-HT activity in Arctic charr *Salvelinus alpinus* (Höglund et al., 2001).

These results suggest that brain catecholaminergic systems are interacting with the 5-HT system and that NE and DA may modulate the effect of 5-HT on the HPI axis. Moreover, catecholamines are synthesised from L-tyrosine, another essential large neutral amino acid (LNAA), competing with TRP for uptake to the brain (Fernstrom, 1983). Thus, elevated dietary intake of TRP may also affect brain NE and DA activity.

In the present study we report the effects of feeding rainbow trout TRP-supplemented feed for 3, 7 and 28 days on plasma levels of cortisol and ACTH as well as on brain NE, DA and 5-HT activity. The effects of elevated dietary intake of TRP were studied in both stressed and undisturbed fish.

## Materials and methods

### *Experimental animals*

For the experiment, juvenile 2 year-old rainbow trout *Oncorhynchus mykiss* Walbaum, weighing 144.36±40 g (mean ± s.e.m., *N*=89) were used. Prior to the experiment, fish were kept indoors in a 1 $m^3$ holding tank, at a rearing density of approximately 0.02 $kg^{-1}$, for more than 1 month. The holding tank was continuously supplied with aerated Uppsala tapwater at 8–11°C and the light/dark regime was continuously and automatically adjusted to latitude 51°N conditions. Fish were hand-fed with commercial trout pellets (EWOS ST40) at 1–2% of body mass per day.

### *Experimental protocol*

The experiment was performed in two consecutive rounds, each round including 48 individuals kept in eight 250 litre glass aquaria continuously supplied with aerated Uppsala tapwater (0.8l $min^{-1}$, 8–10°C). Light (12 h:12 h light:dark) was provided by a 30 W Lumilux daylight fluorescent tube placed 100 mm above the water surface of each aquarium. Each aquarium was divided into four individual 65 litre compartments by removable PVC walls.

At the start of the experiment, fish were selected from the holding tank, weighed and transferred to individual compartments within the experimental aquaria. The first week after transfer to social isolation, fish were hand-fed with commercial feed (EWOS ST40) until satiation. Individual feed intake was quantified by counting the number of pellets consumed. For quantification of fed intake, individual fish were fed with one pellet at the time until the fish rejected three pellets in a row. Pellets not consumed were removed from aquarium. Following this week of acclimation, commercial feed was exchanged for an experimental feed, supplemented with TRP to a level (3.57 g total TRP $kg^{-1}$ dry feed) corresponding to 8 times the TRP content of the commercial feed (0.44 g total TRP $kg^{-1}$ dry feed), but otherwise identical to this feed. A similar number of fish were fed with a control feed, not supplemented with TRP. Fish were fed once a day to satiation, or at maximum until the fish consumed a number of pellets corresponding to 1.5% of the body mass, for 3, 7 or 28 days, and individual feed intake was quantified. At the end of the experimental feeding period, half of the fish fed TRP-supplemented feed and half of the fish that received control feed, were exposed to a standardised stressor for 2 h. The stressor consisted of lowering the water level in the aquaria until the dorsal fin of the fish was exposed above the water surface. The remaining fish were left undisturbed and served as non-stressed controls. Following stress, fish were killed, and blood and brain tissue samples collected.

### *Blood and brain tissue sampling*

Upon sampling, fish were rapidly netted and anaesthetised in 500 mg $l^{-1}$ ethyl-*m*-aminobenzoate methanesulphonate. Blood (1 ml approximately) was collected from the caudal vasculature with a heparinized syringe and kept on ice. Fish were then decapitated, and the brain was rapidly (within 2 min) removed and dissected into four different regions: telencephalon (excluding the olfactory bulb), hypothalamus (excluding the pituitary gland), brain stem (including the medulla and part of the spinal cord but excluding the cerebellum), and the optic tectum. Each brain part was wrapped in aluminium foil, frozen in liquid nitrogen and stored at –80°C. Finally, following centrifugation at 27 000 ***g*** for 10 min, plasma portions were frozen and kept at –80°C.

### *Assays*

The frozen brain samples were homogenized in 400 µl of sodium acetate buffer (0.1 mol $l^{-1}$, pH 5), containing 0.1 mg $ml^{-1}$ pargyline (a monoamineoxidase inhibitor; Sigma P-8013), using a Potter–Elvenhjem homogeniser (brain stem and optic tectum) or an MSE 100 W ultrasonic disintegrator (telencephalon and hypothalamus).

After centrifugation (27 000 ***g***, 10 min), 8 µl of ascorbic acid oxidase (Sigma A-0157; 100 units/800 µl $H_2O$) was added to the supernatant, which was then left for 10 min at room temperature. Thereafter, 200 µl of 4% (w/v) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 40 ng $ml^{-1}$ epinine (deoxyephinephrine, used as an internal standard) was added. Following centrifugation (27 000 ***g***, 10 min), the samples were rapidly frozen and stored at –80°C.

Brain [5-HT], [5-HIAA], [DA], [DOPAC] (3,4-dihydroxyphenylacetic acid), [NE] and [MHPG] (3-methoxy-4-hydroxyphenylglycol) were quantified using high-performance liquid chromatography with electrochemical detection (HPLC-EC), as described by Øverli et al. (1999).

Plasma and brain [TRP] were analysed using the same HPLC system but with the oxidizing potential set at 600 mV (Lepage et al., 2002).

Cortisol analysis was performed directly on rainbow trout plasma without extraction, using a validated radioimmunoassay (RIA) modified from Olsen et al. (1992), as described by Winberg and Lepage (1998).

Plasma ACTH concentrations were determined using a validated heterologous radioimmunoassay (Balm and Pottinger, 1993). In brief, standards (0–160 pg; NIBBS hACTH1-39, Herts, UK) or unknowns (25 µl) were incubated together with antibody (IgG-ACTH-1; Campro Scientific, Veenendaal, The Netherlands) for 72 h at 4°C. Radio-labelled ACTH (3-[$^{125}$I]iodotyrosyl2)ACTH1-39; Amersham IM 216, Buckinghamshire, UK; 74 TBq/mmol; 4000 c.p.m./tube) was added to each tube and these were incubated for a further 24 h. Immunoprecipitation was achieved by adding 100 µl of a sheep anti-rabbit antiserum (SAR-IgG; Sigma R-9754) solution containing rabbit IgG (Sigma I-5006) to each tube, vortex mixing and then incubating at room temperature for 20 min. A 1 ml portion of ice-cold PEG solution (7.5% polyethylene glycol; PEG 6000) was added to each tube and tubes were centrifuged. The supernatants were aspirated and the activity remaining in the pellets counted in a liquid scintillation counter (Packard Tri-Carb 1900TR; Illinois, USA) using gammavials (Zinsser Analytic, Berkshire, UK). A standard curve (3-parameter hyperbolic decay) was fitted and the unknowns interpolated.

### *Statistics*

All values are means ± standard error of the mean (S.E.M.). Since there was no difference between fish fed control feed for 3, 7 and 21 days, either in stressed or undisturbed fish, in any of the parameters analysed, data from these groups were pooled to create two groups, one consisting of stressed fish fed control feed for 3, 7 and 21 days following acclimation and the other of undisturbed fish fed control feed during the same time periods.

The effects of feeding TRP-supplemented feed (controls fed TRP-supplemented feed for 0 days, and TRP-supplemented fish fed TRP-supplemented feed for 3, 7 or 21 days) and stress on [cortisol], [TRP], [5-HT], [5-HIAA], [5-HIAA]/[5-HT], [DA], [DOPAC], [DOPAC]/[DA], [NE], [MHPG] and [MHPG]/[NE] were analysed using a two-way analysis of variance (2-way ANOVA) followed by the least significance difference (LSD) *post-hoc* test. Correlations were tested using Spearman rank-correlation coefficients. All statistical analyses were performed using STATISTICA software.

## Results

### *Feed intake*

After transfer to the individual chambers of the experimental aquaria, feed intake increased progressively (Fig. 1). When switching to the experimental feed, control feed and TRP-supplemented feed, feed intake remained at the same level as observed for commercial feed and continued to increase until day 10 (day 3 after switching to experimental feed) when it reached 1.5% of the body mass (Fig. 1). There was no difference in feed intake between fish receiving control feed and feed supplemented with TRP.

### *Plasma [TRP], [ACTH] and [cortisol]*

Exposing the fish to the standardised stressor had a significant effect on plasma [cortisol] ($F_{1,77}$=49.63, $P$<0.0001), fish subjected to stress showing elevated plasma [cortisol] as compared to non-stressed fish (Fig. 2A). Feeding the fish TRP-supplemented feed had no significant effects on plasma [cortisol] by itself. There was, however, a significant ($F_{3,77}$=2.75, $P$=0.0485) interaction between TRP

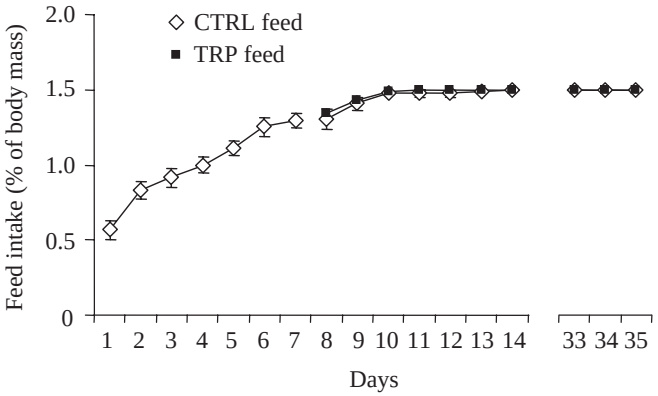


Fig. 1. Feed intake, as percentage of body mass, of isolated juvenile rainbow trout after being transferred to observation aquaria. On day 7, the commercial feed was exchanged for a feed supplemented with L-tryptophan to a level eight times (TRP feed) that of the non-supplemented control feed (CTRL feed). Fish were fed to satiation, or to a maximum of 1.5% of the body mass. Values are means ± S.E.M. (days 1–7, $N$=96; days 8–10, $N$=48; days 11–17, $N$=32; days 18–35, $N$=16).

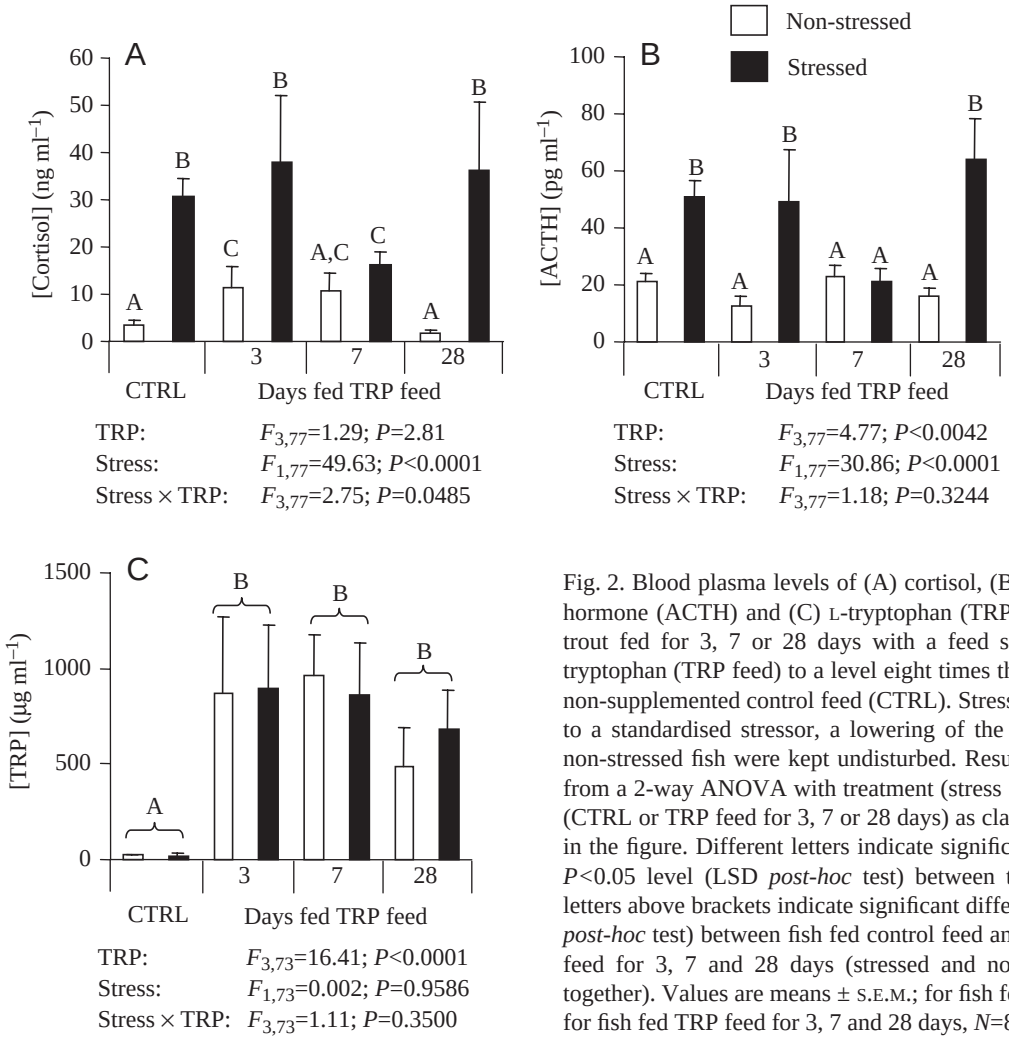


Fig. 2. Blood plasma levels of (A) cortisol, (B) adrenocorticotropic hormone (ACTH) and (C) L-tryptophan (TRP) in isolated rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results (*F*- and *P*-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Different letters indicate significant differences at the $P<0.05$ level (LSD *post-hoc* test) between the groups. Different letters above brackets indicate significant differences ($P<0.05$, LSD *post-hoc* test) between fish fed control feed and TRP-supplemented feed for 3, 7 and 28 days (stressed and non-stressed fish taken together). Values are means ± S.E.M.; for fish fed CTRL feed, $N=24$; for fish fed TRP feed for 3, 7 and 28 days, $N=8$.

supplementation and stress. In fish fed TRP-supplemented feed for 7 days there was no significant difference in plasma [cortisol] between stressed and non-stressed fish (Fig. 2A). Thus, in fish fed TRP-supplemented feed for 7 days, exposure to the stressor did not result in any elevation of plasma [cortisol]. By contrast, in fish fed TRP-supplemented feed for 3 or 28 days stress resulted in an elevation of plasma [cortisol] no different from that seen in fish fed control feed (Fig. 2A).

In non-stressed fish, feeding with TRP-supplemented feed for 3 days resulted in significantly higher plasma [cortisol] as compared to non-stressed fish fed control feed ($P=0.0233$). There was, however, no significant difference in plasma [cortisol] of non-stressed fish fed control feed and non-stressed fish fed TRP-supplemented feed for 7 or 28 days (Fig. 2A).

The effects observed for plasma [ACTH] mirrored those for plasma [cortisol] (Fig. 2B). Exposing the fish to the standardised stressor had a significant effect on plasma [ACTH] ($F_{1,77}=30.86$, $P<0.0001$), fish subjected to stress showing elevated plasma [ACTH] as compared to non-stressed fish (Fig. 2B). There was also a significant effect ($F_{3,77}=4.77$, $P=0.0042$) of feeding the fish TRP-supplemented feed on plasma [ACTH] but no significant interaction ($F_{3,77}=1.18$, $P=0.3244$) between stress and TRP supplementation.

As expected, feeding the fish TRP-supplemented feed resulted in a significant effect on plasma [TRP] ($F_{3,73}=16.41$, $P<0.0001$), with fish fed TRP-supplemented feed for 3, 7 and 28 days showing elevated plasma [TRP] ($P=0.0015$, $P<0.0001$, $P<0.0001$, respectively) (Fig. 2C). There was, however, no significant effect of stress on plasma [TRP]. Correlations were found between plasma [TRP] and [ACTH] ($r=0.208$; $P=0.0396$) and between [ACTH] and [cortisol] ($r=0.348$; $P=0.0010$).

### *Brain [TRP], [5-HT], [5-HIAA] and [5-HIAA]/[5-HT] ratios*

Subjecting the fish to stress had a significant effect on [TRP] only in the brain stem ($F_{1,79}=3.99$, $P=0.0492$), where stress resulted in elevated [TRP] (Fig. 3D). As expected feeding the fish TRP-supplemented feed had a significant effect on [TRP] in the telencephalon ($F_{3,76}=6.06$, $P=0.0009$), hypothalamus ($F_{3,79}=7.09$, $P=0.0003$) and optic tectum ($F_{3,76}=3.73$, $P=0.0146$). Fish fed TRP-supplemented feed for 3 ($P=0.0014$), 7 ($P=0.0008$) and 28 days ($P=0.0003$) showed elevated hypothalamic [TRP] as compared to fish fed control feed

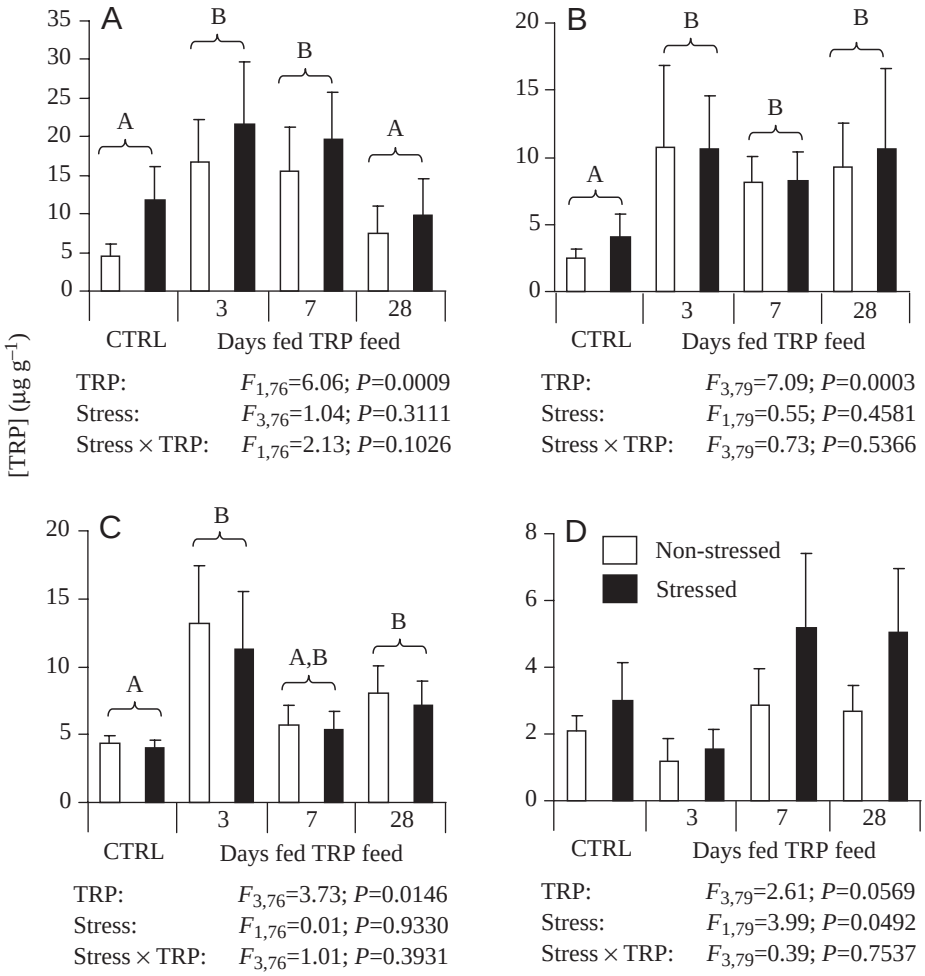


Fig. 3. The amount of L-tryptophan (TRP) in (A) the telencephalon, (B) hypothalamus, (C) optic tectum and (D) brain stem of isolated juvenile rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results (*F*- and *P*-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Different letters above brackets indicate significant differences ($P<0.05$, LSD *post-hoc* test) between fish fed control feed and TRP-supplemented feed for 3, 7 and 28 days (stressed and non-stressed fish taken together). Values are means ± S.E.M.; for fish fed CTRL feed, $N=24$; for fish fed TRP feed for 3, 7 and 28 days, $N=8$.

(Fig. 3B). Telencephalic [TRP] was elevated in fish fed TRP-supplemented feed for 3 ($P=0.0182$) and 7 days ($P=0.0001$), whereas [TRP] in telencephalon of fish fed TRP-supplemented feed for 28 days did not differ from telencephalic [TRP] of fish fed control feed (Fig. 3A). In the optic tectum the increase in [TRP] of fish fed TRP-supplemented feed appeared most pronounced at 3 days (Fig. 3C). There was no significant effect of TRP-supplemented feed on brain stem [TRP] ($F_{3,79}=2.61$, $P=0.0569$), even though brain stem [TRP] showed a tendency towards an increase after feeding the fish TRP-supplemented feed for 7 and 28 days (Fig. 3D). There was no significant interaction effect between stress and TRP supplementation on [TRP] in any part of the brain.

Stress had a significant effect on [5-HIAA] in the telencephalon ($F_{1,74}=22.34$, $P<0.0001$), stressed fish showing elevated [5-HIAA] (Fig. 4A). Stress also tended to elevate hypothalamic [5-HIAA] in a similar way (Fig. 4B), even though this effect did not reach the level of statistical significance ($F_{1,72}=3.57$, $P=0.0688$). Feeding the fish TRP-supplemented feed also had a significant effect on [5-HIAA], but only in the hypothalamus ($F_{3,72}=3.26$, $P=0.0264$) and optic tectum ($F_{3,70}=2.84$, $P=0.0441$) (Fig. 4B,C). Fish receiving TRP-supplemented feed for 7 days displayed a significant elevation of [5-HIAA] in the hypothalamus ($P=0.0410$) as compared to fish fed TRP-supplemented fed for 28 days (Fig. 4B). In the optic tectum [5-HIAA] was significantly higher in fish fed TRP-supplemented feed for 3 and 7 days ($P=0.0045$, $P=0.0039$, respectively) than in fish fed control feed, while fish fed TRP-supplemented feed for 28 days did not show any significant elevation of [TRP] in this brain part (Fig. 4C). In the brain stem neither stress nor TRP supplementation had any effect on [5-HIAA] (Fig. 4D), and there were no interaction effects between stress and TRP supplementation in any brain area.

Stress affected [5-HT] only in the brain stem ($F_{1,73}=4.90$, $P=0.0300$), stressed fish showing slightly elevated [5-HT] as compared to non-stressed fish (Fig. 5D). In the optic tectum there was a significant ($F_{3,70}=3.26$, $P=0.0264$) interaction effect between stress and TRP supplementation on [5-HT] (Fig. 5C), an effect not seen in other brain areas. In the optic tectum, stressed fish fed TRP feed for 3 days showed higher [5-HT] than stressed fish fed control feed ($P=0.0108$, Fig. 5C).

Stress also had a significant effect on telencephalic [5-HIAA]/[5-HT] ratios ($F_{1,73}=22.01$, $P<0.0001$), stressed fish showing elevated telencephalic [5-HIAA]/[5-HT] ratios. A similar trend was observed in the hypothalamus ($F_{1,72}=2.66$, $P=0.0540$) and brain stem ($F_{1,66}=2.69$, $P=0.0640$), even though in these brain areas the effect did not reach the level of

statistical significance (Fig. 6). Feeding the fish TRP-supplemented feed had no significant effect on [5-HIAA]/[5-HT] ratios in any brain area, even though [5-HIAA]/[5-HT] showed a tendency towards an increase in the optic tectum of fish fed TRP-supplemented feed ($F_{3,70}$=2.67, $P$=0.0544) (Fig. 6C). There was no significant interaction effect between stress and TRP supplementation on [5-HIAA]/[5-HT] ratios in any brain area.

*Brain [DOPAC], [DA] and [DOPAC]/[DA] ratios*

Neither stress nor feeding TRP-supplemented feed had any significant effects on either [DA] or [DOPAC] in any brain area (data not shown). There was however an effect of feeding the fish TRP-supplemented feed on hypothalamic [DOPAC]/[DA] ($F_{3,70}$=3.42, $P$=0.0219), fish fed TRP-supplemented feed for 28 days displaying a [DOPAC]/[DA] ratio of 0.064±0.025 as compared to a ratio of 0.108±0.030 in fish fed control feed ($P$=0.0048).

*Brain [NE], [MHPG] and [MHPG]/[NE] ratios*

There was no effect of either stress or TRP-supplemented feed on [NE] in any brain area (data not shown) but there was an effect of TRP supplementation on [MHPG] in the optic tectum ($F_{3,70}$=3.52, $P$=0.0195) and an interaction effect between stress and TRP supplementation on [MHPG] in the optic tectum ($F_{3,70}$=3.27, $P$=0.0262) and brain stem ($F_{3,67}$=3.00, $P$=0.0365) (Fig. 7). Optic tectum [MHPG] was elevated in both non-stressed and stressed fish fed TRP-supplemented feed for 7 days, as compared to levels in fish fed control feed ($P$=0.0006) or TRP-supplemented feed for 3 and 28 days ($P$=0.0010, $P$=0.0003, respectively) (Fig. 7A). In the brain stem [MHPG] was elevated in stressed fish fed TRP-supplemented feed for 3 days as compared to stressed fish fed control feed ($P$=0.0389) (Fig. 7B). Neither stress nor feeding TRP-supplemented feed had any significant effects on [MHPG]/[NE] in any brain area (data not shown).

## Discussion

The results of the present study show that feeding the fish a TRP-supplemented feed for 7 days results in a decrease in post-stress plasma cortisol levels. Feeding the fish this TRP-supplemented feed for 3 days had no effect, however, on post-stress plasma [cortisol], even though basal plasma [cortisol]

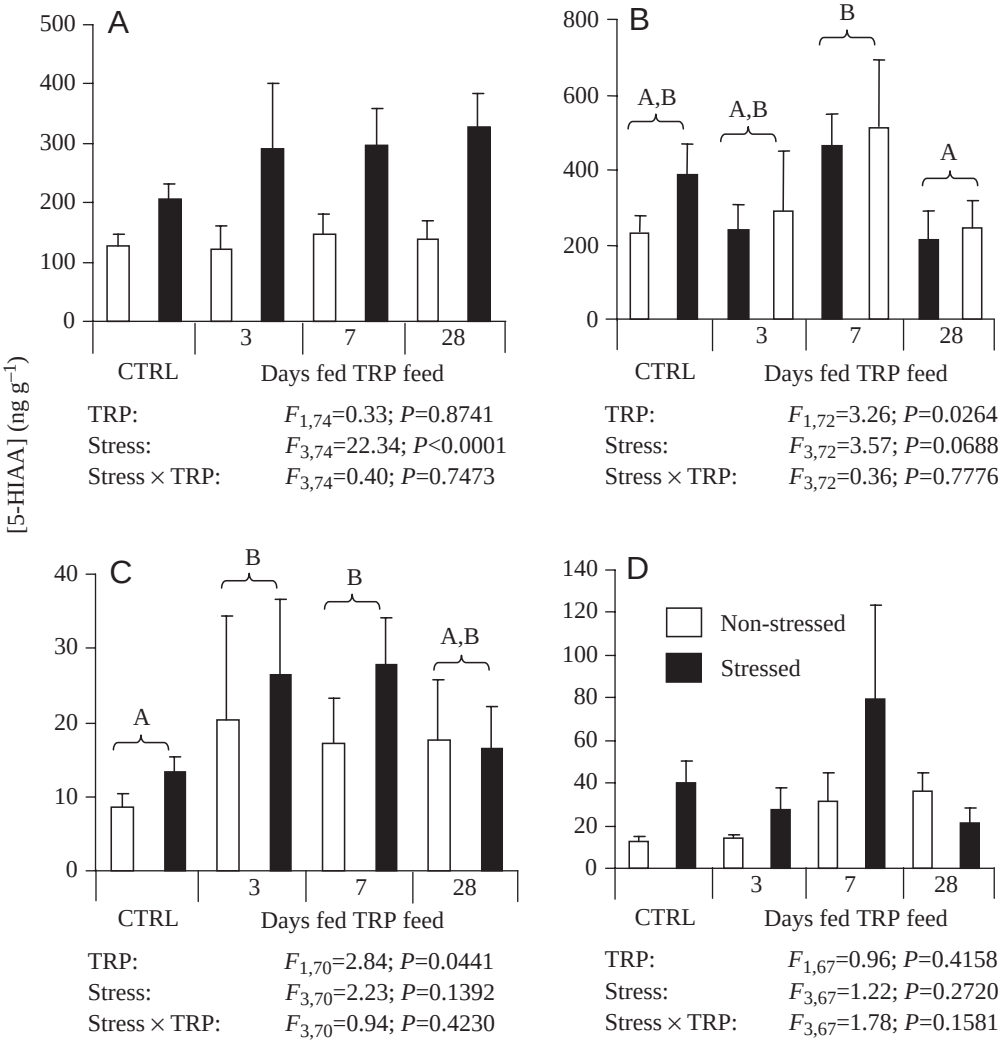


Fig. 4. The [5-HIAA] in (A) the telencephalon, (B) hypothalamus, (C) optic tectum and (D) brain stem of isolated juvenile rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results ($F$- and $P$-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Different letters above brackets indicate significant differences ($P$<0.05, LSD *post-hoc* test) between fish fed control feed and TRP-supplemented feed for 3, 7 and 28 days (stressed and non-stressed fish taken together). Values are mean ± S.E.M.; for fish fed CTRL feed; $N$=24, for fish fed TRP feed for 3, 7 and 28 days, $N$=8.

was slightly elevated as compared to fish fed control feed. Following 28 days of elevated dietary intake of TRP there was no effect on either post-stress or basal plasma [cortisol].

Lepage et al. (2002) similarly reported that feeding rainbow trout feed supplemented with three different levels of TRP, corresponding to two, four and eight times the TRP content of commercial trout feed, for 7 days resulted in a dose-dependent suppression of the stress-induced elevation of plasma [cortisol], along with a dose-dependent elevation of basal plasma [cortisol]. The elevation of basal plasma [cortisol] could suggest that the suppression of the stress-induced elevation of plasma [cortisol] observed in fish fed TRP-supplemented feed for 7 days is an effect of elevated negative feedback by cortisol. However, in the present study, feeding the fish TRP-supplemented feed resulted in elevated basal plasma [cortisol] only after 3 days, whereas the effect on post-stress plasma [cortisol] were observed first after feeding the fish this feed for 7 days, at a time when basal plasma [cortisol] were not elevated. This observation argues against the suggestion that reduced post-stress plasma [cortisol] in fish fed TRP-supplemented feed is an effect of elevated negative feedback of cortisol.

In the present study, plasma levels of ACTH correlated with plasma cortisol levels and the effects of stress and elevated dietary intake of TRP on plasma [ACTH] closely mirror the effects on plasma [cortisol]. This suggests that the effect of elevated dietary intake of TRP on plasma [cortisol] is mediated through effects on the HPI axis, at a level upstream of the interrenal tissue.

Treatments elevating plasma [TRP] and/or plasma [TRP]/[LNAA] ratios have also been reported to counteract stress-induced elevations of plasma [cortisol] in mammals, including humans (Morméde and Dantzer, 1979; Markus et al., 1998, 1999, 2000a,b), an effect which is believed to be mediated by brain 5-HT. In the present study, both plasma and brain [TRP] were elevated in fish fed TRP-supplemented feed. Fish fed this feed also showed elevated [5-HIAA] in the hypothalamus and optic tectum, but there were no significant effects of TRP on [5-HT] or [5-HIAA]/[5-HT] ratios in any of the brain areas analysed. Lepage et al. (2002) reported that feeding the fish TRP-supplemented feed for 7 days resulted elevated brain and plasma [TRP] along with a suppression of post-stress plasma [cortisol], but only small and not quite significant effects on brain [5-HIAA] and [5-HIAA]/[5-HT]

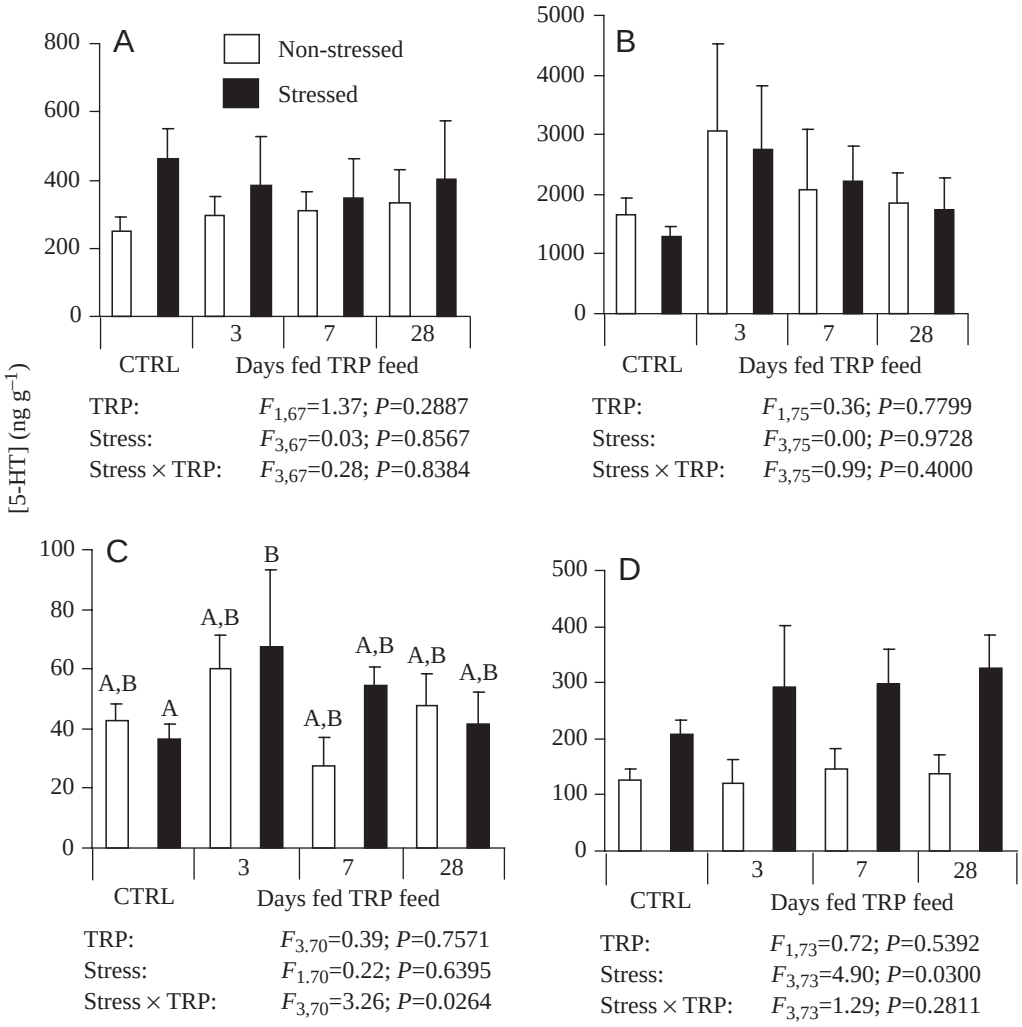


Fig. 5. The [5-HT] in (A) the telencephalon, (B) hypothalamus, (C) optic tectum and (D) brain stem of isolated juvenile rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results (*F*- and *P*-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Different superscript letters indicate significant differences at the $P<0.05$ level (LSD *post-hoc* test) between the groups. Values are mean ± S.E.M.; for fish fed CTRL feed, $N$=24; for fish fed TRP feed for 3, 7 and 28 days, $N$=8.

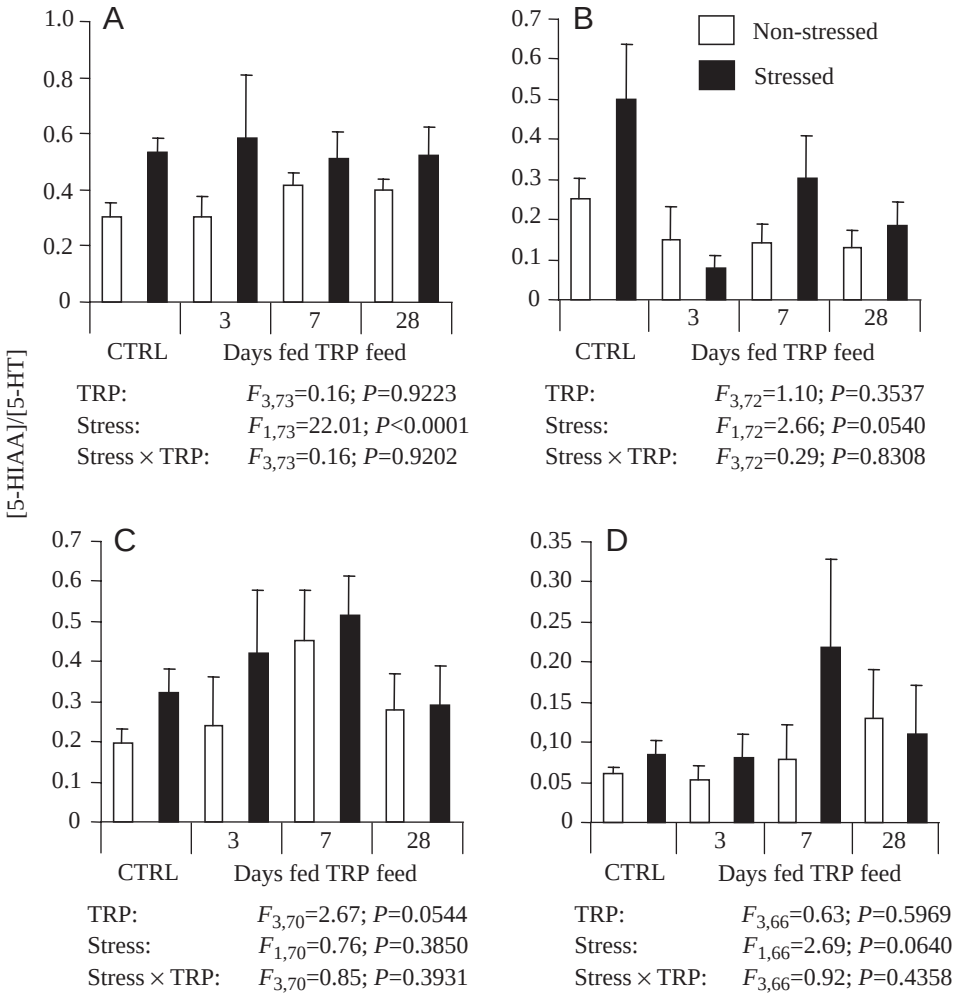


Fig. 6. The [5-HIAA]/[5-HT] ratios in (A) the telencephalon, (B) hypothalamus, (C) optic tectum and (D) brain stem of isolated juvenile rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results ($F$- and $P$-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Values are mean ± S.E.M.; for fish fed CTRL feed, $N$=24; for fish fed TRP feed for 3, 7 and 28 days, $N$=8.

ratios. In the study by Winberg et al. (2001) the lowest level of dietary supplementation of TRP (8.38 mg TRP $g^{-1}$ dry feed, about four times higher than the dose in the present study) also had modest effects on brain [5-HIAA] and [5-HIAA]/[5-HT] ratios in fish fed this feed for 7 days, but pronounced effects on aggressive behaviour. Still, it could not be excluded that the effects of TRP-supplemented feed on HPI axis reactivity and aggression in rainbow trout are mediated by the brain 5-HT system. However, the time course of the effect of TRP on aggression and HPI axis reactivity suggests that mechanisms other than a direct effect on 5-HT synthesis and release are involved.

The effect of elevated dietary TRP intake on 5-HT synthesis and release could be expected to be very rapid, but in the present study the effects of TRP on post-stress plasma [cortisol] were manifested first after feeding the fish TRP-supplemented feed for 7 days. Similarly, in the study by Winberg et al. (2001), the effects of elevated dietary TRP on aggression in rainbow trout were observed after feeding the fish TRP-supplemented feed for 7 days, but not after feeding the fish this feed for 3 days. Interestingly, the time course of the anti-depressive effects of specific 5-HT re-uptake inhibitors (SSRI), such as fluoxetine (Prozac), is strikingly similar, the anti-depressive effects of these drugs occurring only after long-term treatment (Mongeau et al., 1997). Moreover, the effects of SSRI and TRP on stress responses and aggression appear to be similar. Larson and Summers (2001) showed that a 1-week treatment with the SSRI, setralin, reduces aggressive behaviour and reverses dominant social status in the lizard *Anolis carolinensis*. As seen with elevated dietary intake of TRP, short-term treatment with SSRI activates the HPA axis, whereas long-term treatment has the opposite effect, desensitising the HPA axis in rats (Jensen et al., 1999). An effect on the densities and transduction mechanisms of post- and/or pre-synaptic 5-HT receptors, resulting in a delayed elevation of 5-HT post-synaptic effects in certain brain regions, has been suggested as a mechanism involved in mediating the effects of long-term SSRI treatment (Mongeau et al., 1997; Nutt et al., 1999).

Central 5-HT interacts with the brain NE system, and one possible mechanism through which 5-HT could suppress HPI axis activity is by inhibiting central NE activity (Aston-Jones et al., 1991; Engberg, 1992). However, fish fed TRP-supplemented feed for 7 days showed significantly higher [MHPG] in the optic tectum than fish fed control feed or TRP-supplemented feed for 3 or 28 days. If anything, this would

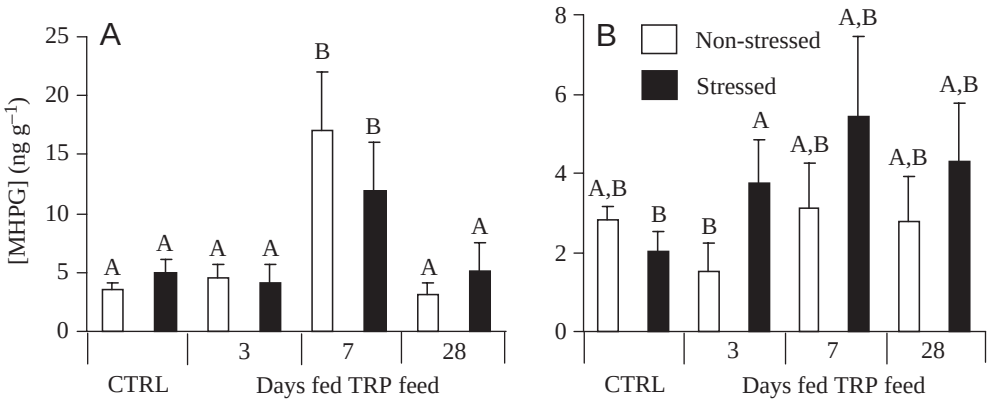


Fig. 7. The [MHPG] in (A) the optic tectum and (B) brain stem of isolated juvenile rainbow trout fed for 3, 7 or 28 days with a feed supplemented with L-tryptophan (TRP feed) to a level eight times the TRP content of the non-supplemented control feed (CTRL). Stressed fish were exposed to a standardised stressor, a lowering of the water level, whereas non-stressed fish were kept undisturbed. Results ($F$- and $P$-values) from a 2-way ANOVA with treatment (stress or no stress) and feed (CTRL or TRP feed for 3, 7 or 28 days) as class variables are given in the figure. Different superscript letters indicate significant differences at the level of $P$<0.05 (LSD *post-hoc* test) between the groups. Values are mean ± S.E.M.; for fish fed CTRL feed, $N$=24; for fish fed TRP feed for 3, 7 and 28 days, $N$=8.

argue against the hypothesis that the effects of elevated dietary intake of TRP on stress responsiveness are a result of a 5-HT-mediated inhibition of brain NE activity.

Elevated dietary intake of TRP may also have a more direct effect on the synthesis and release of DA and NE since the amino acid precursor of DA and NE is tyrosine, a large neutral amino acid (LNAA), which enters the brain *via* the same LNAA transport carrier as TRP (Wurtman et al., 1974; Fernstrom, 1983; Boadle-Biber, 1993). Thus, a rise in blood levels of TRP may competitively inhibit tyrosine uptake into the brain (Wurtman et al., 1974). However, Lepage et al. (2002) showed an increase in brain [TRP] but no concomitant decrease in brain levels of other LNAAs in rainbow trout fed TRP-supplemented feed for 7 days. In the present study, feeding the TRP-supplemented feed had an effect on hypothalamic [DOPAC]/[DA], but only after 28 days, when fish fed TRP-supplemented feed showed lowered hypothalamic [DOPAC]/[DA] ratios.

Since 5-HT is the precursor of melatonin, elevated dietary intake of TRP may also increase plasma levels of melatonin, and elevated plasma [melatonin] following TRP treatment have been reported in humans (Hajak et al., 1991), rats (Yaga et al., 1993) and chickens (Heuther et al., 1992). Melatonin is a hormone best known for its role in synchronising circadian rhythms, but which has also been reported to affect aggressive behaviour and post-stress plasma [cortisol]. For instance, Munro (1986) showed that intracranial injections of melatonin suppressed aggressive responsiveness in the cichlid *Aequidens pulcher*, and in mammals, melatonin has been reported to exert a glucocorticoid antisecretagogue effect (Xu et al., 1995; Rao et al., 2001).

In the present study the effect of dietary TRP on the stress-induced elevation of plasma [cortisol] was not observed after feeding the fish TRP-supplemented fed for 28 days, suggesting that long-term dietary TRP may activate compensatory mechanisms, normalizing brain [TRP] and cortisol release. Notably, following 28 days of dietary supplementation of TRP, [TRP] in the telencephalon and optic tectum no longer differed from that of fish fed control feed. In mammals, the TRP catabolising enzyme indoleamine-2,3-deoxygenase, which is present in the brain, is induced by TRP (Gal, 1974). In the present study, there was also a tendency towards decreased plasma [TRP] in undisturbed fish fed TRP-supplemented feed for 28 days as compared to undisturbed fish fed this feed for 3 and 7 days. A decline in plasma [TRP] following prolonged dietary supplementation of TRP could be related to an activation of the enzyme tryptophan pyrrolase in the liver (Chaouloff, 1993). Tryptophan pyrrolase is another TRP catabolising enzyme, which in the rat liver is regulated by the circulating concentration of its substrate (Feigelson and Greegard, 1962). However, Brown and Dodgen (1968) found that administration of repeated doses of TRP into channel catfish failed to induce the liver enzyme, and Walton et al. (1984) did not find any relationship between plasma TRP levels and the activity of hepatic TRP pyrrolase in rainbow trout.

In conclusion, the results from the present study confirm that supplemental dietary L-tryptophan has an effect on stress responses in rainbow trout. Fish fed TRP-supplemented feed for 7 days show reduced post-stress plasma [cortisol], whereas feeding the fish this feed for 3 or 28 days has no effect on post-stress plasma [cortisol]. Thus, the time courses of the effects of elevated dietary TRP on aggressive behaviour (Winberg et al., 2001) and post-stress plasma [cortisol] are similar. Both these effects of dietary TRP could be mediated by the brain 5-HT system, and, if so, probably through effects on 5-HT receptor densities and receptor mechanisms resulting in a delayed elevation of 5-HT post-synaptic effects. Elevated, dietary intake of TRP does not appear to have any direct effects on the synthesis and release of DA, and the results of the present study do not support the hypothesis that the suppressive effect of dietary TRP on HPI axis reactivity is mediated by 5-HT inhibition of brain NE activity. However, possible effects

of TRP on circulating melatonin levels cannot be excluded as the mechanism of action.

## List of abbreviations

| | |
|---|---|
| 5-HIAA | 5-hydroxyindoleacetic acid |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 8-OH-DPAT | a selective 5-$HT_{1A}$ receptor agonist |
| ACTH | adrenocorticotropin |
| DA | dopamine |
| DOPAC | 3,4-dihydroxyphenylacetic acid |
| HPA | hypothalamus–pituitary–adrenocortical |
| HPI | hypothalamic–pituitary–interrenal |
| HPLC-EC | high-performance liquid chromatography with electrochemical detection |
| LNAA | large neutral amino acid |
| LNAA | large neutral amino acid |
| LSD | least significance difference |
| MHPG | 3-methoxy-4-hydroxyphenylglycol |
| NE | norepinephrine |
| PCA | perchloric acid |
| RIA | radioimmunoassay |
| SSRI | specific 5-HT re-uptake inhibitors |
| TPH | tryptophan hydroxylase |
| TRP | L-tryptophan |

This study was supported by grants from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Carl Trygger Foundation, the FACIAS foundation and Ewos International Ltd.

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