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Suppression of aggression in rainbow trout (*Oncorhynchus mykiss*) by dietary L-tryptophan

Svante Winberg[1,*], Øyvind Øverli[2] and Olivier Lepage[1]

[1]*Evolutionary Biology Centre, Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, SE-752 36, Sweden* and [2]*Evolutionary Biology Centre, Department of Animal Development and Genetics, Uppsala University, Norbyvägen 18A, SE-752 36, Sweden*

*e-mail: Svante.Winberg@ebc.uu.se

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Summary

Juvenile rainbow trout *Oncorhynchus mykiss* were isolated in individual compartments in observation aquaria and allowed to acclimate for 1 week, during which they were fed commercial trout feed. Thereafter, the fish were tested for aggressive behaviour using a resident/intruder test. Following this first resident/intruder test, the feed was exchanged for an experimental wet feed supplemented with 0.15 % or 1.5 % L-tryptophan (by wet mass). Controls received the same feed but without L-tryptophan supplementation. The fish were fed to satiety daily, and their individual feed intake was recorded. Aggressive behaviour was quantified again after 3 and 7 days of L-tryptophan feeding using the resident/intruder test. Feeding the fish L-tryptophan-supplemented feed for 3 days had no effect on aggressive behaviour, whereas feeding the fish L-tryptophan-supplemented feed for 7 days significantly suppressed aggressive behaviour in the fish, an effect seen at both levels of L-tryptophan supplementation. Fish fed L-tryptophan-supplemented feed showed elevated plasma and brain levels of L-tryptophan. The amino acid L-tryptophan is the precursor of serotonin, and supplementary dietary L-tryptophan was found to elevate levels of 5-hydroxyindoleacetic acid (5-HIAA) and the 5-HIAA/serotonin concentration ratio in the brain. Neither feed intake nor plasma cortisol level was significantly affected by dietary L-tryptophan. Central serotonin is believed to have an inhibitory effect on aggressive behaviour, and it is suggested that the suppressive effect of dietary L-tryptophan on aggressive behaviour is mediated by an elevation of brain serotonergic activity.

Key words: serotonin, brain, behaviour, fish, feed, stress, Salmonidae, *Oncorhynchus mykiss*.

Introduction

The organisation and function of the brain serotonergic system seem to be highly conserved across the vertebrate subphylum (Parent et al., 1984). Serotonin (5-hydroxytryptamine, 5-HT) has been reported to inhibit aggressive behaviour in various vertebrates, ranging from teleost fish to primates (Adams et al., 1996; Edwards and Kravitz, 1997; Larson and Summers, 2001). Social subordination, as well as other stressors including exposure to predators (Winberg and Nilsson, 1993), often results in a rapid activation of brain 5-HT neurotransmission, as indicated by increased brain concentrations of 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite) and/or elevated ratios of 5-HIAA to 5-HT concentration in the brain (Winberg et al., 1992a; Blanchard et al., 1993; Fontenot et al., 1995; Summers and Greenberg, 1995; Matter et al., 1998). It has been suggested that stress-induced activation of the brain 5-HT system mediates behavioural inhibition, e.g. the suppression of aggressive behaviour, inhibition of food intake and lowered spontaneous locomotor activity frequently observed in socially subordinate animals (Winberg et al., 1993a,b; Winberg and Nilsson, 1993; Øverli et al., 1998).

The amino acid L-tryptophan (TRP) is the precursor of 5-HT, and the first and rate-limiting step in the biosynthesis of 5-HT is the hydroxylation of TRP to 5-hydroxytryptophan, a reaction catalysed by the enzyme tryptophanhydroxylase (TPH) (for a review, see Boadle-Biber, 1993). In mammals, this enzyme is not saturated by its substrate TRP *in vivo*. In addition, TPH does not appear to be subjected to any inhibition by 5-HT, the end product of the reaction pathway. Consequently, an elevation of brain TRP levels results in an increase in the rate of 5-HT synthesis.

Further, it appears that brain TRP levels are remarkably sensitive to the supply of the amino acid from the circulation (Fernstrom and Wurtman, 1972). The major factor regulating TRP uptake into the mammalian brain is a transport carrier located at the blood–brain barrier, a carrier that transports not only TRP but also several other large neutral amino acids (LNAAs), including tyrosine, phenylalanine, leucine,

isoleucine and valine, into the brain (Fernstrom and Wurtman, 1972).

Our understanding of the control of brain 5-HT synthesis in teleost fish and in other non-mammalian vertebrates is still limited. However, it has been suggested that the rate of brain 5-HT synthesis may also be restricted by TRP availability in fish (Johnston et al., 1990; Aldegunde et al., 1998, 2000). Aldegunde et al. (1998) reported that, as in mammals, a stereospecific and saturable carrier, also transporting tyrosine and possibly other LNAAs, mediates TRP uptake into the brain of rainbow trout.

In mammals, it seems that the 5-HT released by nerve activity is the newly synthesised fraction, whereas a large fraction of the intraneuronal 5-HT pool appears to be metabolised to 5-HIAA without being released (Lookingland et al., 1986). This observation has raised the question of whether TRP administration results in an increase in the functional release of 5-HT or whether it results only in an elevation of the rate of intraneuronal 5-HT metabolism. However, a number of studies, using *in vivo* microdialysis, have now shown that exogenous TRP not only elevates the rate of 5-HT synthesis but also the rate of 5-HT release in rats (for a review, see Boadle-Biber, 1993). To our knowledge, there are no studies on the effects of exogenous TRP on 5-HT release in teleost fish.

TRP-supplemented feed has been reported to inhibit aggressive behaviour in chickens (Shea et al., 1990). In the present study, we report the effects of dietary supplemental TRP on brain serotonergic activity and aggressive behaviour in isolated juvenile rainbow trout. Specifically, it was hypothesised that increased dietary TRP intake would stimulate brain serotonergic activity and suppress aggressive behaviour in these fish.

Materials and methods

Experimental feed

Experimental wet feed was prepared from 1 kg fillets of Baltic herring and 1 kg of shrimp. Grounded herring fillets and shrimp were mixed with 1 l of water and 100 g of gelatine (Kebo Lab, Sweden) and split into portions (Table 1). Two of these portions were supplemented with TRP at 1.5 or 15.0 g kg^{-1} feed. The feed was stored at −20 °C.

The energy, water content and concentration of free TRP determined by analysis are presented in Table 1. The water content of the experimental feed was approximately 15 times higher than that of the commercial trout feed pellets. The free TRP concentrations, expressed as free [TRP] per gram of dry feed, were similar in the commercial trout feed and the control wet feed. Moreover, the energy contents, given as the calorimetric heat of combustion in the dry feed, of the commercial trout feed and the experimental wet feeds were similar.

Fish

Experimental fish were juvenile (2-year-old) rainbow trout *Oncorhynchus mykiss* (Walbaum), weighing 184.5±41.1 g and 94.6±13.9 g (means ± S.D.) in experiments 1 (1.5 % TRP) and 2 (0.15 % TRP), respectively. Prior to the experiment, the fish had been kept indoors in a 1 m^3 holding tank at a density of approximately 0.02 kg l^{-1} for more than than 1 month. The light/dark regime was continuously and automatically adjusted to conditions at latitude 51°N. When in the holding tank, fish were hand-fed with commercial trout pellets (Ewos ST40) at 1–2 % of their body mass per day.

The resident/intruder test

The level of aggression of individual fish was determined by introducing a small conspecific (approximately 50 % of the body mass of the resident fish) into the compartment of an isolated experimental fish. The behaviour of such pairs was recorded on video for 1 h, after which the intruder was removed. Each intruder was used only once. From video recordings, the latency to first attack and the number of aggressive acts performed by the resident fish during six consecutive 5 min periods, starting from the time of the first attack, were recorded.

Experimental protocol

During the acclimation period, the fish were fed commercial trout pellets (Ewos ST40) once a day to satiety. The feed intake of individual fish was quantified by counting the number of pellets consumed. Following 1 week of acclimation, the fish were subjected to a resident/intruder test (as described above), after which the feed was exchanged for

Tabel 1. *The amount of L-tryptophan added, the total concentration of free L-tryptophan and the water and energy content of one commercial trout feed and three experimental wet feeds*

Feed	TRP added (g kg^{-1} wet feed)	Free TRP (mg g^{-1} dry feed)	Water content (%)	Energy content (MJ g^{-1} dry feed)
Ewos ST40		0.22	5.2	23.35
Control		0.22	82.0	22.42
0.15 % TRP	1.5	8.38	80.2	24.00
1.5 % TRP	15.0	74.30	81.4	22.64

TRP, L-tryptophan.
See Materials and methods for more details.

experimental feed with or without (control) supplemental TRP. Two levels of TRP supplementation were used, 1.5 % and 0.15 % TRP (mass/wet mass). The fish were fed experimental feed by hand once a day, and the amount of feed consumed by individual fish was quantified by weighing the feed container before and after feeding. The resident/intruder test was repeated twice, after receiving TRP supplemented feed for 3 and 7 days. Following the final resident/intruder test (after being fed TRP-supplemented feed for 7 days), the fish were killed, and blood samples and brain tissues were collected. Blood samples and brain tissue were also collected from fish that had been held visually isolated and fed TRP-supplemented (1.5 or 0.15 %) or control feed for 7 days but not subjected to any resident/intruder test (non-tested fish). The experiments were performed in two successive rounds using TRP supplementation of 1.5 % (experiment 1) and 0.15 % (experiment 2). Separate controls were included in each experiment.

Blood and brain tissue sampling

Following the final resident/intruder test, the fish were anaesthetised (500 mg l^{-1} ethyl-*m*-aminobenzoate methanesulphonate), and blood (approximately 1 ml) was collected from the caudal vasculature using a syringe pre-treated with heparin. Blood samples were rapidly transferred to Eppendorf tubes and were centrifuged at 1500 ***g*** for 10 min at 4 °C. Following centrifugation, the blood plasma was separated, divided into samples, frozen on dry ice and stored at −80 °C. Following blood sampling, the fish were killed by decapitation, and the brain was rapidly removed (within 2 min) and divided into telencephalon (excluding the olfactory bulbs), hypothalamus (excluding the pituitary gland) and brain stem (including the medulla and part of the spinal cord but excluding the optic tectum and cerebellum). Each brain part was wrapped in aluminium foil, frozen in liquid nitrogen and stored at −80 °C.

Assays

The frozen brain samples were homogenised in 4 % (w/v) ice-cold perchloric acid (PCA) containing 0.2 % EDTA and 40 ng ml^{-1} epinine (deoxyepinephrine, the internal standard) using a Potter–Elvehjem homogenizer (brain stem) or an MSE 100 W ultrasonic disintegrator (telencephalon and hypothalamus).

Brain [5-HT] and [5-HIAA] were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection, as described by Øverli et al. (1999). Plasma, brain and feed [TRP] were analysed using the same HPLC system but with the oxidizing potential set at 600 mV.

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

The energy content of the experimental wet feed was quantified using a bomb calorimeter (Leco, AC 100, Leco Corp., Michigan, USA).

Statistical analyses

All data are presented as means ± standard error of the mean (S.E.M.) unless stated otherwise. The data on aggression and attack latency in intruder tests were analysed together using a repeated-measures multivariate analysis of varaince (MANOVA) with experiment and treatment as class variables (treatment nested within experiment). Similarly data on plasma [TRP] and [cortisol] and on brain [TRP] and [5-HT] and the [5-HIAA]/[5-HT] ratio from the two experiments were analysed together using a MANOVA with experiment, treatment (treatment nested within experiment) and aggression test (tested or not tested, effect of aggression test nested within treatment and experiment) as class variables. Contrast analysis was used to examine *post-hoc* differences between aggression-tested fish fed 1.5 % TRP and aggression-tested controls in experiment 1, and between aggression-tested fish fed 0.15 % TRP and controls in experiment 2. In addition, *post-hoc* differences between the two separate control groups were also examined using contrast analysis. To fulfil the assumption of normal distribution, all data were log-transformed prior to statistical analyses. All statistical analyses were performed using SAS statistical software.

Results

Aggressive behaviour

There was a significant interaction between time and treatment (MANOVA, treatment nested within experiment, $F_{4,88}$=3.99, P=0.005), and feeding the fish TRP-supplemented feed for 7 days had a significant effect on the number of aggressive acts recorded in the intruder test (treatment nested within experiment, $F_{2,47}$=5.17, P=0.0097), TRP-supplemented fish performing fewer aggressive acts against the intruder than controls (Fig. 1A,B). Moreover, fish receiving TRP-supplemented feed showed significantly lower levels of aggression on day 7 than on day 0 (treatment nested within experiment, $F_{2,44}$=5.57, P=0.007) and day 3 (treatment nested within experiment, $F_{2,44}$=5.78, P=0.0059). Thus, both levels of TRP supplementation had similar effects on aggressive behaviour, and the contrast analysis showed that, following 7 days of TRP supplementation, fish receiving 0.15 % TRP (P=0.0370) and fish receiving 1.5 % TRP (P=0.0213) differed significantly from their respective controls (Fig. 1A,B).

Prior to switching to experimental feed (test 1, day 0), controls and TRP-supplemented fish did not differ in the number of aggressive acts performed against the intruder (Fig. 1A,B). Moreover, 3 days of TRP supplementation (test 2) had no significant effect on the number of aggressive acts performed against the intruder (Fig. 1A,B).

There was no significant difference between the two experiments in the number of aggressive acts performed against the intruder on day 0, day 3 or day 7.

Attack latency was not significantly affected by feeding the fish supplementary TRP, and there was no significant differences between treatment groups on day 0, day 3 or day 7 (Fig. 2A,B). There was a non-significant trend towards a time

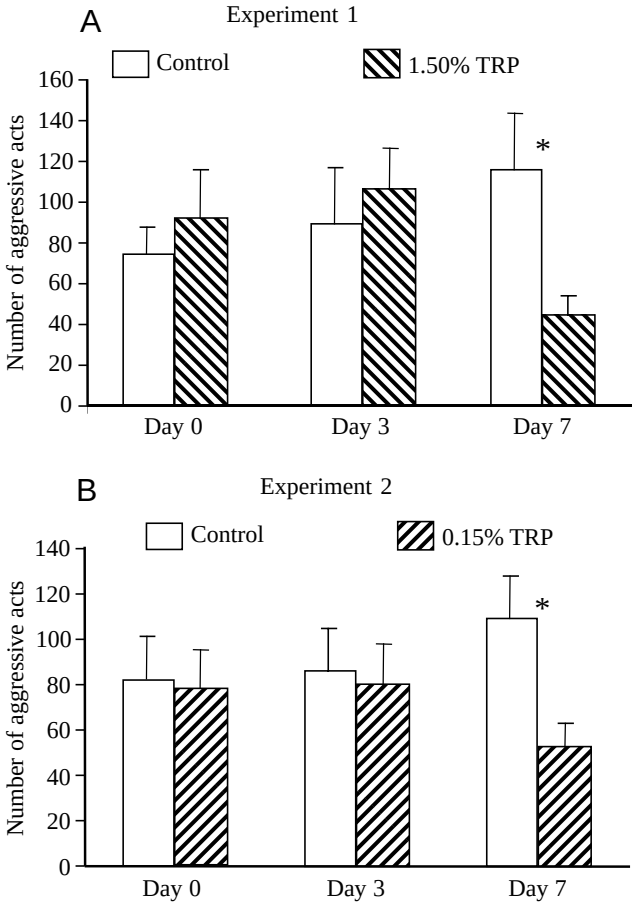

Fig. 1. The number of aggressive acts performed during repeated 30 min resident/intruder tests by isolated juvenile rainbow trout before (day 0) and after 3 and 7 days of receiving an experimental wet feed supplemented with (A) 1.5 % (experiment 1) or (B) 0.15 % (experiment 2) L-tryptophan (TRP). In both experiments, the control fish were fed experimental wet feed without supplementary tryptophan. Values are means + S.E.M. (N=12 in all groups). *P<0.05 (repeated-measures MANOVA followed by contrast analysis, see text for further details).

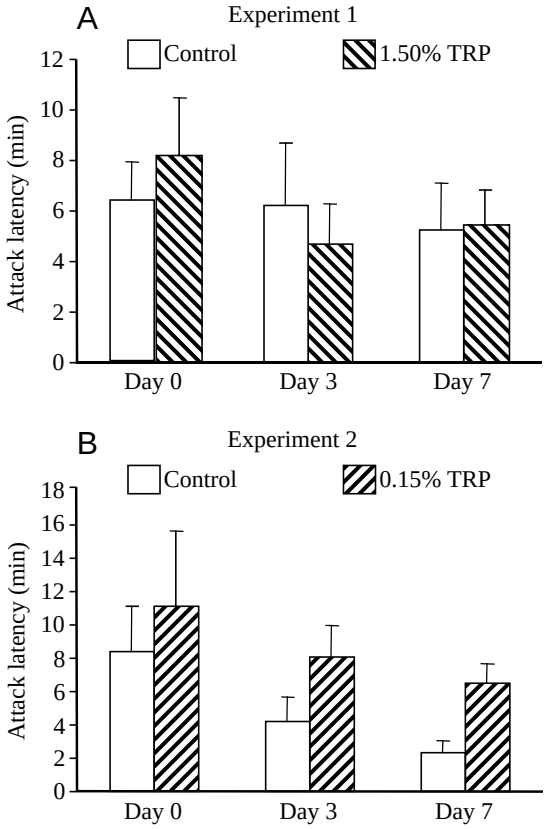

Fig. 2. Attack latency of isolated juvenile rainbow trout in repeated 30 min resident/intruder tests performed before (day 0) and 3 and 7 days after feeding the fish with an experimental wet feed supplemented with (A) 1.5 % (experiment 1) or (B) 0.15 % (experiment 2) L-tryptophan (TRP). In both experiments, the control fish were fed the same experimental wet feed but without supplementary tryptophan. Values are means + S.E.M. (N=12 in all groups).

effect ($F_{2,42}$=3.01, P=0.060) on attack latency, attack latency tending to be reduced in repeated tests, but no interaction between time and experiment or between time and treatment.

Feed intake

During acclimation to the experimental aquaria, feed intake gradually increased to reach a high constant level after 4 days (Fig. 3). When switching to experimental feed, control feed as well as feed supplemented with 0.15 or 1.5 % TRP, feed intake temporarily decreased, but it increased rapidly again to reach the level observed for commercial trout feed at the end of the acclimation period (Fig. 3). There was no difference in feed intake between fish receiving control feed and feed supplemented with 0.15 or 1.5 % TRP (Fig. 3).

Blood plasma [TRP] and [cortisol]

Feeding the fish TRP-supplemented feed had a significant effect on plasma [TRP] (treatment nested within experiment, $F_{2,44}$=145.48, P<0.0001), whereas subjecting the fish to the resident/intruder test did not have any significant effect on plasma [TRP] (Fig. 4). There was also a significant difference in plasma [TRP] between the two experiments ($F_{1,44}$=97.91, P<0.0001). Contrast analysis showed that fish fed feed supplemented with 1.5 % TRP displayed a significantly elevated plasma [TRP] compared with controls (P<0.001, Fig. 4). Similarly, fish feed the 0.15 % TRP feed also showed a significantly higher plasma [TRP] than controls (P=0.0082), but there was no significant difference in plasma [TRP] between the two different control groups (P=0.2774; Fig. 4).

There were no significant effects of feeding the fish TRP-supplemented feed on plasma [cortisol], and there was no significant difference in plasma [cortisol] between the two experiments. However, plasma [cortisol] was significantly affected by the resident/intruder test (effect of resident/intruder test nested within treatment and experiment, $F_{4,44}$=3.07, P=0.0281; Fig. 5).

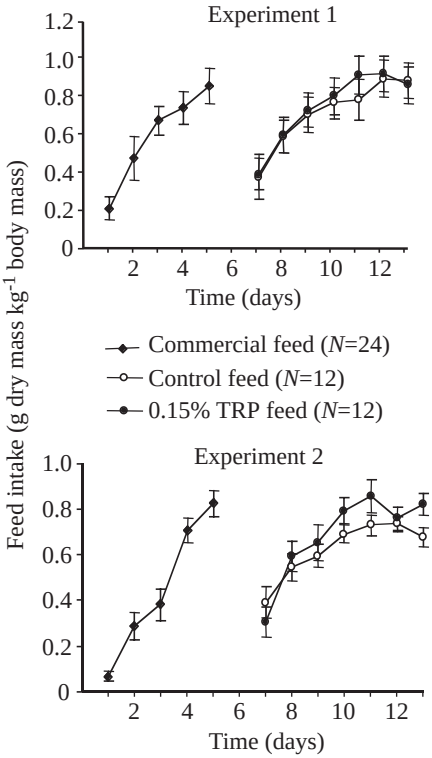

Fig. 3. Feed intake, determined as the amount of feed (dry mass) consumed per kilogram of body mass, of isolated juvenile rainbow trout after being transferred to observation aquaria. On day 7, the commercial trout feed was exchanged for an experimental wet feed supplemented with 1.5 % (experiment 1) or 0.15 % (experiment 2) L-tryptophan (TRP). In both experiments, the control fish were fed the same experimental wet feed but without supplementary tryptophan. Feed intake was not registered on day 6. Values are means ± S.E.M.

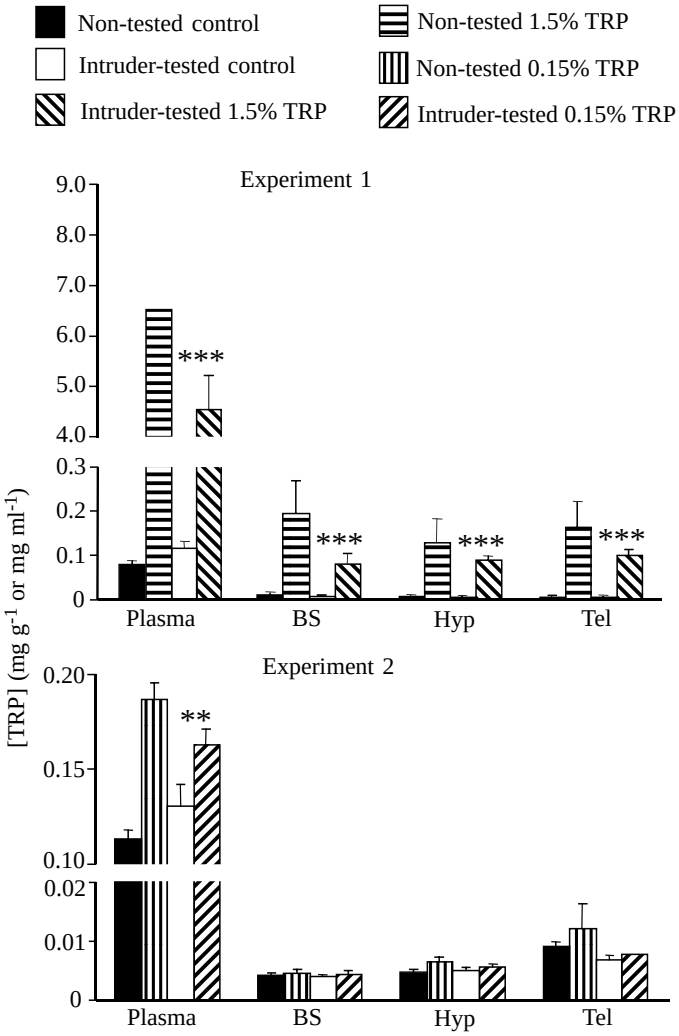

Fig. 4. The amount of L-tryptophan (TRP) in the blood plasma, brain stem (BS), hypothalamus (Hyp) and telencephalon (Tel) of isolated juvenile rainbow trout fed an experimental wet feed supplemented with 1.5 % (experiment 1) or 0.15 % (experiment 2) L-tryptophan (TRP) for 7 days. In both experiments, the control fish were fed the same experimental wet feed but without supplementary tryptophan. Intruder-tested fish were subjected to resident/intruder tests (see text for details). Values are means + S.E.M. (intruder-tested, N=10–12; non-tested, N=6–12). ***P<0.001, **P<0.01 (repeated-measures MANOVA followed by contrast analysis, see text for further details).

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

Feeding the fish TRP-supplemented feed (treatment nested within experiment) had significant effects on [TRP] in the telencephalon ($F_{2,44}$=45.47, P<0.0001), hypothalamus ($F_{2,44}$=71.10, P<0.0001) and brain stem ($F_{2,44}$=39.87, P<0.0001), whereas subjecting the fish to the resident/intruder test (effect of resident/intruder test nested within treatment and experiment) had no significant effect on [TRP] in any of these brain parts (Fig. 4). The contrast analysis revealed that TRP concentrations in the telencephalon (P<0.0001), hypothalamus (P<0.0001) and brain stem (P<0.0001) of fish receiving 1.5 % TRP-supplemented feed were significantly elevated compared with controls (Fig. 4). However, in fish receiving feed supplemented with 0.15 % TRP, the difference in [TRP] compared with controls did not reach the level of statistical significance in the telencephalon (P=0.8618), hypothalamus (P=0.1905) or brain stem (P=0.4216) (Fig. 4). There were significant differences between the two experiments in [TRP] in the telencephalon ($F_{1,44}$=27.90, P<0.0001), hypothalamus ($F_{1,44}$=85.12, P<0.0001) and brain stem ($F_{1,44}$=129.48, P<0.0001), and the contrast analysis showed that there was a significant (P=0.0045) difference in brain stem [TRP] between the two control groups, whereas telencephalic (P=0.1290) and hypothalamic (P=0.5208) [TRP] did not differ significantly between the controls used in the two experiments (Fig. 4).

There were no significant effects of dietary TRP supplementation on [5-HT] (treatment nested within experiment) in the telencephalon, hypothalamus or brain stem (Table 2). Subjecting the fish to the resident/intruder test (effect of resident/intruder test nested within treatment and experiment) did not affect [5-HT] in these brain parts either (Table 2). However, there was a significant difference between the two experiments in [5-HT] in the telencephalon

Table 2. *The concentration of 5-HT and 5-HIAA in the brain stem, hypothalamus and telencephalon of isolated juvenile rainbow trout fed an experimental wet feed supplemented with 1.5 % (experiment 1) or 0.15 % (experiment 2) L-tryptophan for 7 days and subjected (tested) or not (non-tested) to resident/intruder tests*

		Brain stem		Hypothalamus		Telencephalon	
Group	Resident/intruder test	[5-HT] (ng g^{-1})	[5-HIAA] (ng g^{-1})	[5-HT] (ng g^{-1})	[5-HIAA] (ng g^{-1})	[5-HT] (ng g^{-1})	[5-HIAA] (ng g^{-1})
Control	Non-tested	229±16	45±3	3062±264	336±75	1018±120	315±34
Experiment 1	Tested	228±14	38±2	3283±202	272±23	1066±46	288±22
Supplemented with 1.5 % TRP	Non-tested	262±25	70±3	3092±172	725±98	1049±84	364±19
Experiment 1	Tested	283±17	64±3	3173±170	534±55	116±37	331±19
Control	Non-tested	243±18	61±3	2167±122	101±24	89±119	193±9
Experiment 2	Tested	307±24	75±6	2012±229	112±22	918±122	236±18
Supplemented with 0.15 % TRP	Non-tested	212±25	60±7	2488±143	132±16	923±99	208±25
Experiment 2	Tested	302±19	78±4	2058±263	167±33	712±102	250±45

5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; TRP, L-tryptophan.
Values are means ± S.E.M. (N=6–12).
See text for more details and statistical analyses.
Values are presented as ng g^{-1} wet mass.

($F_{1,44}$=7.82, P=0.0082) and hypothalamus ($F_{1,44}$=25.18, P<0.0001), but not in the brain stem ($F_{1,44}$=0.01, P=0.9145). According to the contrast analysis, hypothalamic [5-HT] in controls was significantly higher in experiment 1 than in experiment 2 (P=0.0004), but no such difference was detected in telencephalic [5-HT] (Table 2).

There were significant effects of feeding the fish TRP-supplemented feed (treatment nested within experiment) on [5-HIAA] in the hypothalamus ($F_{2,44}$=4.84, P=0.0136) and brain stem ($F_{2,44}$=8.03, P=0.0013), but not in the telencephalon ($F_{2,44}$=0.49, P=0.6137) (Table 2). The resident/intruder test (effect of resident/intruder test nested within treatment and experiment) had significant effects on brain stem [5-HIAA] ($F_{4,44}$=2.97, P=0.0318), but not on telencephalic ($F_{4,44}$=1.14, P=0.3519) or hypothalamic ($F_{4,44}$=0.69, P=0.6009) [5-HIAA] (Table 2). There were significant differences between the two experiments in [5-HIAA] in all these brain parts (telencephalon, $F_{1,44}$=13.81, P=0.0007; hypothalamus, $F_{1,44}$=54.48, P<0.0001; brain stem, $F_{1,44}$=8.34, P=0.0064), with fish in experiment 2 (effects of 0.15 % TRP) generally showing a higher brain [5-HIAA] than fish in experiment 1 (effects of 1.5 % TRP) (Table 2). The contrast analysis showed that brain stem 5-HIAA concentrations were significantly higher in fish fed 1.5 % TRP than in controls (P=0.0006), whereas there was no significant difference in brain stem [5-HIAA] between fish fed 0.15 % TRP and controls (P=0.9961). Furthermore, hypothalamic 5-HIAA concentrations were significantly higher in fish fed 0.15 % TRP than in controls (P=0.0190), and a trend towards higher hypothalamic [5-HIAA] compared with controls was also observed in fish fed 1.5 % TRP (P=0.0628). Brain stem [5-HIAA] was significantly lower (P<0.001), whereas hypothalamic [5-HIAA] was significantly higher (P<0.001), in aggression-tested controls in experiment 1 than in experiment 2 (Table 2).

Feeding the fish TRP-supplemented feed had significant effects (treatment nested within experiment) on [5-HIAA]/[5-HT] ratios in the hypothalamus ($F_{2,47}$=16.92, P<0.0001) and brain stem ($F_{2,47}$=7.51, P=0.0015), whereas there was no significant effect on telencephalic [5-HIAA]/[5-HT] ratio (Fig. 6). Subjecting the fish to the resident/intruder test also had significant effects (effect of resident/intruder test nested within treatment and experiment) on hypothalamic ($F_{4,47}$=2.79, P=0.0371) and telencephalic ($F_{4,47}$=2.67, P=0.0436) [5-HIAA]/[5-HT] ratios, whereas [5-HIAA]/[5-HT] ratios in the brain stem ($F_{4,47}$=1.44, P=0.2370) were not affected (Fig. 6). Furthermore, there was a significant difference between the two experiments in [5-HIAA]/[5-HT] ratios in the hypothalamus ($F_{1,47}$=59.13, P<0.0001) and brain stem

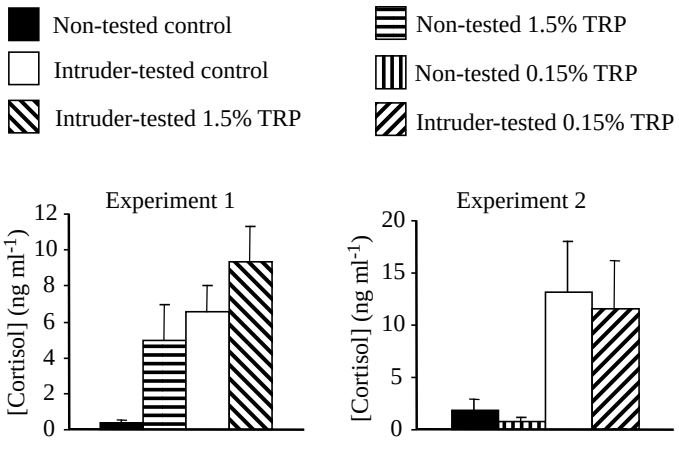

Fig. 5. Plasma levels of cortisol in isolated juvenile rainbow trout fed an experimental wet feed supplemented with 1.5 % (experiment 1) or 0.15 % (experiment 2) L-tryptophan (TRP) for 7 days. In both experiments, the control fish were fed the same experimental wet feed but without supplementary tryptophan. Intruder-tested fish were subjected to resident/intruder tests (see text for details). Values are means + S.E.M. (intruder-tested, N=10–12; non-tested, N=6–12).

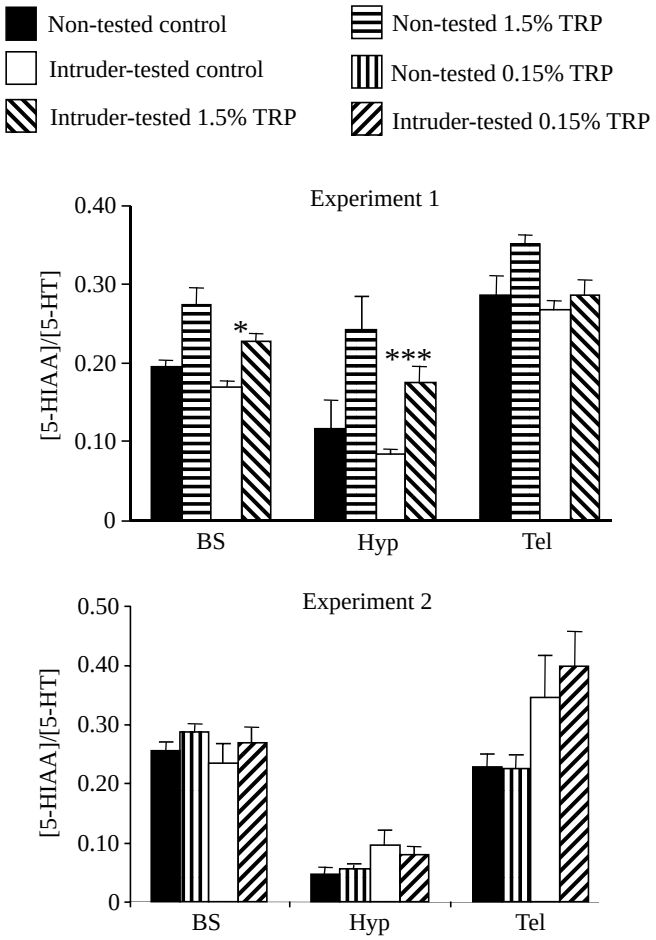

Fig. 6. The [5-hydroxyindoleacetic acid]/[serotonin] ([5-HIAA]/[5-HT]) ratios in the brain stem (BS), hypothalamus (Hyp) and telencephalon (Tel) of isolated juvenile rainbow trout fed an experimental wet feed supplemented with 1.5% (experiment 1) or 0.15% (experiment 2) L-tryptophan (TRP) for 7 days. In both experiments, the control fish were fed the same experimental wet feed but without supplementary tryptophan. Intruder-tested fish were subjected to resident/intruder tests (see text for details). Values are means + S.E.M. (intruder-tested, N=10–12; non-tested, N=6–12). ***P<0.001, *P<0.05 (repeated-measures MANOVA followed by contrast analysis, see text for further details).

($F_{1,47}$=12.33, P=0.0010), but not in the telencephalon. Fish in experiment 1 (effects of 1.5% TRP) showed higher hypothalamic but lower brain stem [5-HIAA]/[5-HT] ratios than fish in experiment 2 (effects of 0.15% TRP) (Fig. 6). The contrast, the analysis revealed that, among fish subjected to the resident/intruder test, fish receiving 1.5% TRP showed significantly higher hypothalamic (P<0.0001) and brain stem (P=0.0134) [5-HIAA]/[5-HT] ratios than controls. There were also significant differences in brain stem [5-HIAA]/[5-HT] ratios (P=0.0002) between aggression-tested controls in experiments 1 and 2, whereas the contrast analysis did not reveal any significant difference (P=0.1082) in hypothalamic [5-HIAA]/[5-HT] ratios between the two control groups (Fig. 6).

Discussion

The results of this study show that dietary supplementation with TRP suppresses aggressive behaviour in juvenile rainbow trout. The amino acid TRP is the precursor of 5-HT, and supplementary dietary TRP elevated [5-HIAA] and [5-HIAA]/[5-HT] ratios in the brain. Plasma and brain [TRP] were drastically elevated in fish receiving the highest level of TRP supplementation. The lower level of TRP supplementation had more modest effects on plasma and brain [TRP], and only the elevation in plasma [TRP] reached the level of statistical significance. Similarly, there were only relatively small effects of the low TRP dose on brain [5-HIAA]/[5-HT] ratios, and only hypothalamic [5-HIAA]/[5-HT] ratios were significantly elevated. Still, both the high and the low dose of TRP had very similar effects on aggressive behaviour.

There was no difference between the two experiments in the number of aggressive acts performed or on attack latency in intruder tests. However, there were differences between the two experiments in plasma [TRP], as well as in [TRP], [5-HT] and [5-HIAA] and in [5-HIAA]/[5-HT] ratios in certain brain areas. This variation between the two experiments may reflect growth and developmental changes in the fish; the fish in experiment 2 (0.15% TRP) were twice as large as the fish in experiment 1 (1.5% TRP). Winberg et al. (1992b) reported similar experimental effects in a study on the effects of stress and starvation on brain serotonergic activity in Arctic charr, a study that was performed in three experimental rounds, with fish differing in size. However, since separate control groups were included in each of the two experiment in the present study, and a nested design was used in statistical analysis, experimental effects should not affect any of the conclusions drawn from the data generated in the present experiment.

The central 5-HT system is believed to have an inhibitory effect on aggressive behaviour in a variety of vertebrates (Edwards and Kravitz, 1997; Larson and Summers, 2001), including teleost fish (Adams et al., 1996). Dietary intake of TRP, the amino acid precursor of 5-HT, has been reported to suppress aggressive behaviour in feed-restricted male chickens (*Gallus domesticus*) (Shea et al., 1990). The inhibitory effect of dietary TRP on aggressive behaviour in male chickens was dependent on dominance status, being more obvious in dominant birds (Shea et al., 1991), and appeared to be mediated by brain 5-HT (Shea Moore et al., 1994).

Notably, feeding the fish TRP-supplemented feed for 3 days had no effect on aggressive behaviour, whereas feeding the fish TRP-supplemented feed for 7 days suppressed aggressive behaviour in the fish, an effect seen at both levels of TRP supplementation. The effect of elevated dietary TRP intake on 5-HT synthesis and release could be expected to be very rapid. Thus, the fact that the effect of TRP on aggressive behaviour was first manifest after feeding the fish TRP-supplemented feed for 1 week suggests that other mechanisms may be involved. Similarly, the anti-depressive effect of specific 5-HT re-uptake inhibitors (SSRIs), such as fluoxetine (Prozac), is evident only after long-term treatment (Mongeau et al., 1997).

A compensatory response at pre- and/or postsynaptic receptors as a result of prolonged 5-HT transporter blockade is generally suggested as the mechanism involved in the therapeutic actions of SSRIs. For instance, long-term treatment with SSRIs is believed to result in a desensitisation of somatodendritic autoreceptors, in turn causing an upregulation of 5-HT neurotransmission (Mongeau et al., 1997).

In the mammalian brain, the 5-HT somatodendritic autoreceptors are of the 5-HT_{1A}-receptor type, a type also identified in the salmonid brain (Winberg and Nilsson, 1996). The delay in onset of the suppressive effect of TRP on aggressive behaviour observed here could suggest altered 5-HT receptor mechanisms. It is suggested that the effect of dietary TRP on aggressive behaviour could be mediated by a downregulation of autoreceptor sensitivity, which in turn could result in an upregulation of 5-HT neurotransmission, especially since brain TRP availability is also increased. However, the lag time for the effect TRP may also be related to the fact that long- and short-term brain 5-HT activation seem to have differential effects on aggressive behaviour, only long-term activation of the brain 5-HT system resulting in inhibition of aggressive behaviour. Notably, in response to stressful agonistic interactions, socially dominant animals show a rapid, but short-lived, elevation of 5-HT activity in certain brain areas without any concomitant inhibition in aggressive behaviour (Summers et al., 1998; Øverli et al., 1999).

In mammals, central 5-HT is believed to have an inhibitory effect on appetite and food intake (Samanin and Garattini, 1996), and intracerebroventricular injections of 5-HT have been reported to inhibit feed intake in the goldfish (*Carassius auratus*). However, the inhibitory effect of 5-HT on feed intake in goldfish appeared to be mediated by corticotropin releasing factor (CRF) (de Pedro et al., 1998). The brain 5-HT system is also believed to have a stimulatory effect on the hypothalamic/pituitary/adrenal (HPA) axis in mammals (Chaouloff, 1993; Dinan, 1996) and on hypothalamic/pituitary/interrenal axis activity in rainbow trout (Winberg et al., 1997). Serotonin appears to exert its stimulatory effects on the mammalian HPA axis at the level of the hypothalamus, by stimulating CRF release (Dinan, 1996). However, in the present study, feeding the fish TRP-supplemented feed, which appeared to stimulate brain 5-HT activity, had no effect on either feed intake or plasma [cortisol]. When the standard trout feed was exchanged for experimental feed either with or without supplemental TRP, the fish showed a transient decrease in intake. Thereafter, the fish rapidly increased their intake and, if calculated as the amount of wet feed consumed, it greatly exceeded that observed when the fish were fed commercial trout pellets (data not shown). However, if expressed as the amount of dry feed consumed, feed intake reached the same level as observed when feeding the fish commercial trout pellets. If calculated on a dry mass basis, the energy contents of our experimental feed and the commercial trout pellets used were similar, suggesting that the fish were adjusting their food intake according to the energy content of the feed.

Fish subjected to the resident/intruder stress displayed a small but significant elevation of plasma [cortisol] compared with non-tested fish, a pattern observed in both controls and TRP-supplemented fish. In no case did we observe the intruder performing any aggressive behaviour against the resident fish. Still, the slight increase in plasma [cortisol] of aggression-tested fish may reflect stress since the intruder represented a challenge to the resident fish, even if it did not show any aggressive behaviour. Brain [5-HIAA] and [5-HIAA]/[5-HT] ratios were also affected by the resident/intruder test, but there was no clear pattern across different brain areas or experimental groups. An elevation in brain [5-HIAA] and [5-HIAA]/[5-HT] ratios is a stress response that has been reported for a variety of teleost species in response to different stressors (Winberg and Nilsson, 1993).

Fish receiving TRP-supplemented feed showed both suppression of aggressive behaviour and elevated brain [5-HIAA]/[5-HT] ratios, suggesting that the effects of dietary TRP on aggression were mediated by a stimulation of brain 5-HT activity. Nevertheless, the suppression of aggressive behaviour induced by elevated dietary TRP may be mediated through mechanisms other than the central 5-HT system. For instance, a TRP-induced stimulation of 5-HT synthesis could elevate rates of melatonin synthesis and secretion, since 5-HT is the precursor of melatonin. Elevated plasma melatonin levels following TRP treatment have been reported in humans (Hajak et al., 1991), rats (Yaga et al., 1993) and chickens (Heuther et al., 1992). Furthermore, the 5-HT-releasing drugs fenfluramine and methylenedioxymethamphetamine elevate plasma melatonin levels in rats, suggesting that 5-HT availability may be a factor in melatonin production in rats (Huether et al., 1993). A possible mechanism may be precursor load (with 5-HT) of the low, daytime levels of pineal *N*-acetyltransferase (Huether et al., 1993).

Thus, in addition to its effects on the 5-HT system, increasing dietary TRP content may affect melatonin production in fish. Munro (1986) showed that intracranial injection of melatonin reduced aggressive responsiveness in the cichlid *Aequidens pulcher*. Furthermore, in the same study, he also found that intracranial injections of 5-HT reduced aggressive behaviour in this species, but that this effect was inhibited if 5-HT was administrated together with *S*-adenosyl homocysteine, a substance that inhibits the conversion of 5-HT to melatonin.

Dietary supplementation of TRP could be an interesting aquaculture management strategy, especially during periodic feeding restrictions, which are frequently utilised to control the rate of production of fish and to obtain fish of a certain size at predetermined time intervals. Feeding restrictions entail higher levels of competition for feed and may result in disproportionate feed acquisition and heterogeneous growth (McCarthy et al., 1992; Jobling and Koskela, 1996; Damsgård et al., 1997). In addition, the results of the present study show that dietary TRP has effects that appear to be similar to those of SSRIs, drugs commonly used to treat affective disorders in humans. There are reports showing that diets rich in TRP or carbohydrates elevate brain TRP availability by increasing the

plasma concentration ratio of TRP to the sum of other LNAAs and improve mood in vulnerable human subjects (Markus et al., 1999; Markus et al., 2000), even though in other studies such evidence has been weak and inconsistent (Bellisle et al., 1998).

In conclusion, the results from the present study show that supplemental dietary L-tryptophan suppresses aggressive behaviour in juvenile rainbow trout. This behavioural effect of TRP is likely to be mediated by a stimulation of brain serotonergic activity, but the slow time course of the effect suggests that effects on 5-HT receptor mechanisms may be involved. Furthermore, possible effects of TRP on circulating melatonin levels cannot be excluded as the mechanism of action. Decreasing aggressive behaviour in fish-rearing units by providing feed with increased dietary TRP could be a promising aquaculture management strategy. Since plasma, and thereby brain, TRP levels are correlated with the amount of feed ingested, the effects of dietary TRP will be most pronounced in dominant individuals, which consume the larger part of the feed offered and are also the most aggressive. Thereby, the tendency to develop strong dominance hierarchies, resulting in stress, reduced disease resistance and highly variable growth rates, may be diminished.

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References

Adams, C. F., Liley, N. R. and Gorzalka, B. B. (1996). PCPA increases aggression in male firemouth cichlids. *Pharmacology* **53**, 328–330.

Aldegunde, M., Garcia, J., Soengas, J. L. and Rozas, G. (1998). Uptake of tryptophan into brain of rainbow trout. *J. Exp. Zool.* **282**, 285–289.

Aldegunde, M., Soengas, J. L. and Rozas, G. (2000). Acute effects of L-tryptophan on tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Zool.* **286**, 131–135.

Bellisle, F., Blundell, J. E., Dye, L., Fantino, M., Fletcher, R. J., Lambert, J., Roberfroid, M., Specter, S., Westenhöver, J. and Westerterp-Plantenga, M. S. (1998). Functional food science and behaviour and psychological functions. *Br. J. Nutr.* **80 (Suppl. 1)**, S173–S193.

Blanchard, D. C., Sakai, R. R., McEwen, B., Weiss, S. M. and Blanchard, R. J. (1993). Subordination stress: behavioral, brain and neuroendocrine correlates. *Behav. Brain Res.* **58**, 113–121.

Boadle-Biber, M. C. (1993). Regulation of serotonin synthesis. *Prog. Biophys. Mol. Biol.* **60**, 1–15.

Chaouloff, F. (1993). Physiopharmacological interactions between stress hormones and central serotonergic systems. *Brain Res. Rev.* **18**, 1–32.

Damsgård, B., Arnesen, A. M., Baardvik, B. M. and Jobling, M. (1997). State-dependent feed acquisition among two strains of hatchery-reared Arctic charr. *J. Fish Biol.* **50**, 859–869.

De Pedro, N., Pinillos, M. L., Valenciano, A. I., Alonso-Bedate, M. and Delgado, M. J. (1998). Inhibitory effect of serotonin on feeding behavior in goldfish: Involvement of CRF. *Peptides* **19**, 505–511.

Dinan, T. G. (1996). Serotonin and the regulation of hypothalamic–pituitary–adrenal axis function. *Life Sci.* **58**, 1683–1694.

Edwards, D. H. and Kravitz, E. A. (1997). Serotonin, social status and aggression. *Curr. Opin. Neurobiol.* **7**, 812–819.

Fernstrom, J. D. and Wurtman, R. J. (1972). Brain serotonin content: regulation by plasma neutral amino acids. *Science* **178**, 414–416.

Fontenot, M. B., Kaplan, J. R., Manuck, S. B., Arango, V. and Mann, J. J. (1995). Long-term effects of chronic social stress on serotonergic indices in the prefrontal cortex of adult male cynomolgus macaques. *Brain Res.* **705**, 105–108.

Hajak, G., Huether, G., Blanke, J., Blömer, M., Freyer, C., Poeggeler, B., Reimer, A., Rodenbeck, A., Schulz-Varszegi, M. and Rüther, E. (1991). The influence of intravenous L-tryptophan on plasma melatonin and sleep in men. *Pharmacopsychiatry* **24**, 17–20.

Heuther, G., Poeggeler, B., Adler, L. and Rüther, E. (1993). Effects of indirectly acting 5-HT receptor agonists on circulating melatonin levels in rats. *Eur. J. Pharmacol.* **238**, 249–254.

Heuther, G., Poeggeler, B., Reimer, A. and George, A. (1992). Effect of tryptophan administration on circulating melatonin levels in chicks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. *Life Sci.* **51**, 945–953.

Jobling, M. and Koskela, J. (1996). Interindividual variations in feeding and growth in rainbow trout during restricted feeding and in a subsequent period of compensatory growth. *J. Fish Biol.* **49**, 658–667.

Johnston, W. L., Atkinson, J. L., Hilton, J. W. and Were, K. E. (1990). Effect of dietary tryptophan on plasma and brain tryptophan, brain serotonin and brain 5-hydroxyindoleacetic acid in rainbow trout. *J. Nutr. Biochem.* **1**, 49–54.

Larson, E. and Summers, C. H. (2001). Serotonin reverses dominant social status. *Behav. Brain Res.* (in press).

Lookingland, K. J., Shannon, N. J., Chapin, D. S. and Moore, K. E. (1986). Exogenous tryptophan increases synthesis, storage and interneural metabolism of 5-hydroxytryptamine in the rat hypothalamus. *J. Neurochem.* **47**, 205–212.

Markus, C. R., Olivier, B., Panhuysen, G. E. M., van der Gugten, J., Alles, M. S., Tuiten, A., Westenberg, H. G. M., Fekkes, D., Koppeschaar, H. F. and de Haan, E. H. F. (2000). The bovine protein α-lactalbumin increases the plasma ratio of tryptophan to the other large neutral amino acids and in vulnerable subjects raises brain serotonin activity, reduces cortisol concentration and improves mood under stress. *Am. J. Clin. Nutr.* **71**, 1536–1544.

Markus, C. R., Panhuysen, G., Jonkman, L. M. and Bachman, M. (1999). Carbohydrate intake improves cognitive performance of stress-prone individuals under controllable laboratory stress. *Br. J. Nutr.* **82**, 457–467.

Matter, J. M., Ronan, P. J. and Summers, C. H. (1998). Central monoamines in free-ranging lizards: differences associated with social roles and territoriality. *Brain Behav. Evol.* **51**, 23–32.

McCarthy, I. D., Carter, C. G. and Houlihan, D. F. (1992). The effect of feeding hierarchy on individual variability in daily feeding of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.* **41**, 257–263.

Mongeau, R., Blier, P. and de Montigny, C. (1997). The serotonergic and noradrenergic systems of the hippocampus: their interactions and the effects of antidepressant treatments. *Brain Res. Rev.* **23**, 145–195.

Munro, A. D. (1986). Effects of melatonin, serotonin and naloxene on aggression in isolated cichlid fish (*Aequidens pulcher*). *J. Pineal Res.* **3**, 257–262.

Olsen, Y. A., Falk, K. and Reite, O. B. (1992). Cortisol and lactate levels of Atlantic salmon *Salmo salar* developing infectious anaemia (ISA). *Dis. Aquat. Org.* **14**, 99–104.

Øverli, Ø., Harris, C. A. and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant–subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain Behav. Evol.* **54**, 263–275.

Øverli, Ø., Winberg, S., Damsgård, B. and Jobling, M. (1998). Food intake and spontaneous swimming activity in Arctic charr (*Salvelinus alpinus* L.): Role of brain serotonergic activity and social interaction. *Can. J. Zool.* **76**, 1366–1370.

Parent, A., Poitras, D. and Dubé, L. (1984). Comparative neuroanatomy of central monoaminergic systems. In *Handbook of Chemical Neuroanatomy* (ed. A. Björklund and T. Hökfelt), pp. 409–439. Amsterdam, New York, Oxford: Elsevier.

Samanin, R. and Garattini, S. (1996). Pharmacology of ingestive behaviour. *Thérapie* **51**, 107–115.

Shea, M. M., Douglass, L. W. and Mench, J. A. (1991). The interaction of dominance status and supplemental tryptophan on aggression in *Gallus domesticus* males. *Pharmacol. Biochem. Behav.* **38**, 587–591.

Shea, M. M., Mench, J. A. and Thomas, O. P. (1990). The effect of dietary

tryptophan on aggressive behavior in developing and mature broiler breeder males. *Poultry Sci*. **69**, 1664–1669.

Shea Moore, M. M., Kuenzel, W. J. and Mench, J. A. (1994). A technique for cannulating cisterna magna and sampling cerebrospinal fluid from socially housed birds. *Poultry Sci*. **73**, 556–563.

Summers, C. H. and Greenberg, N. (1995). Somatic correlates of adrenergic activity during aggression in the lizard, *Anolis carolinensis*. *Horm. Behav*. **28**, 29–40.

Summers, C. H., Larson, E. T., Summers, T. R., Renner, K. J. and Greenberg, N. (1998). Regional and temporal separation of serotonergic activity mediating social stress. *Neurosci.* **87**, 489–496.

Winberg, S. and Lepage, O. (1998). Elevation of brain 5-HT activity, POMC expression and plasma cortisol in socially subordinate rainbow trout. *Am. J. Physiol.* **43**, R645–R654.

Winberg, S. and Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress, with particular reference to fish. *Comp. Biochem. Physiol. C* **106**, 597–614.

Winberg, S. and Nilsson, G. E. (1996). Multiple high-affinity binding sites for [^{3}H]serotonin in the brain of a teleost fish, the Arctic charr (*Salvelinus alpinus*). *J. Exp. Biol*. **199**, 2429–2435.

Winberg, S., Nilsson, A., Hylland, P., Söderstrom, V. and Nilsson, G. E. (1997). Serotonin as a regulator of hypothalamic–pituitary–interrenal activity in teleost fish. *Neurosci. Lett.* **230**, 113–116.

Winberg, S., Nilsson, G. E., McCarthy, I. D., Carter, C. G. and Houlihan, D. F. (1993a). Feeding rank and brain serotonergic activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Exp. Biol.* **179**, 197–211.

Winberg, S., Nilsson, G. E. and Olsén, K. H. (1992a). Changes in brain serotonergic activity during hierarchic behaviour in Arctic charr (*Salvelinus alpinus* L.) are socially induced. *J. Comp. Physiol. A* **170**, 93–99.

Winberg, S., Nilsson, G. E. and Olsén, K. H. (1992b). The effect of stress and starvation on brain serotonin utilization in Arctic charr (*Salvelinus alpinus*). *J. Exp. Biol.* **165**, 229–239.

Winberg, S., Nilsson, G. E., Spruijt, B. M. and Höglund, U. (1993b). Spontaneous locomotor activity in Arctic charr measured by computerized imaging technique: role of brain serotonergic activity. *J. Exp. Biol.* **179**, 213–232.

Yaga, K., Reiter, R. J. and Richardson, B. A. (1993). Tryptophan loading increases daytime serum melatonin levels in intact and pinealectomised rats. *Life Sci*. **51**, 945–953.