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Serotonin decreases aggression via 5-HT$_{1A}$ receptors in the fighting fish *Betta splendens*

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Abstract

The role of the monoamine neurotransmitter serotonin (5-HT) in the modulation of conspecific aggression in the fighting fish (*Betta splendens*) was investigated using pharmacological manipulations. We used a fish's response to its mirror image as our index of aggressive behavior. We also investigated the effects of some manipulations on monoamine levels in the *B. splendens* brain. Acute treatment with 5-HT and with the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT both decreased aggressive behavior; however, treatment with the 5-HT$_{1A}$ receptor antagonist WAY-100635 did not increase aggression. Chronic treatment with the selective serotonin reuptake inhibitor fluoxetine caused no significant changes in aggressive behavior and a significant decline in 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations. Treatment with the serotonin synthesis inhibitor *p*-chlorophenylalanine resulted in no change in aggression, yet serotonergic activity decreased significantly. Finally, a diet supplemented with L-tryptophan (Trp), the precursor to 5-HT, showed no consistent effects on aggressive behavior or brain monoamine concentrations. These results suggest a complex role for serotonin in the expression of aggression in teleost fishes, and that *B. splendens* may be a useful model organism in pharmacological and toxicological studies.

Keywords: *Betta splendens*; Serotonin; Aggression; 8-OH-DPAT; WAY-100635; Fluoxetine; *p*-chlorophenylalanine; L-tryptophan

1. Introduction

The neurotransmitter serotonin (5-HT) is involved in modulating the responses to many stressors, including conspecific aggression. Serotonin plays a primarily inhibitory role in the expression of aggression (Edwards and Kravitz, 1997; Nelson and Chiavegatto, 2001; Summers et al., 2005b) and has been shown to influence the dynamics of agonistic interactions (Huber et al., 1997). The actions of 5-HT appear to differ, however, under situations of chronic versus acute stress (Stoddard et al., 2003; Summers and Winberg, 2006). Both dominant and subordinate individuals, for example, experience increased metabolism of serotonin following territorial encounters, but only in subordinates does this turnover remain elevated (Overli et al., 1999; Summers et al., 1998). Manipulations of serotonin levels have also been shown to reverse stable dominance ranks (Larson and Summers, 2001; Summers et al., 2005a).

Serotonin (5-hydroxytryptamine, or 5-HT) is produced in a two-step process from dietary L-tryptophan, which is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Aldegunde et al., 2000). Neurons containing serotonin are located primarily in the raphe nuclei of the brainstem, whose projections extend to various brain regions in mammals, including the hippocampus, cerebral cortex, amygdala, hypothalamus and pituitary. Once released into synapses, serotonin binds to a diverse class of receptors (Hoyer et al., 2002). Following stimulation of the postsynaptic neuron, 5-HT

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is reabsorbed by the presynaptic neuron, where some of it is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme monoamine oxidase. Serotonin turnover, or serotonergic activity, is often reported as the ratio of 5-HIAA:5-HT (Buchanan et al., 1994; Dias and Crews, 2006; Koutoku et al., 2003; Winberg et al., 1992).

Due to the importance of serotonin in the response to stress and expression of mood in humans, numerous pharmaceuticals have been developed to stimulate or inhibit serotonergic activity. These include serotonin receptor agonists and antagonists, selective serotonin reuptake inhibitors (SSRIs), and serotonin synthesis inhibitors. Not surprisingly, drugs that enhance serotonin action (agonists and SSRIs) decrease aggression in a range of animal models (Deckel, 1996; Fuller, 1996; Haug et al., 1990; Perreault et al., 2003; Sperry et al., 2003). Conversely, drugs that suppress serotonin action (antagonists and synthesis inhibitors) have been shown to increase aggression or counteract the effects of agonists in a range of vertebrates (Adams et al., 1996; Buchanan et al., 1994; de Boer et al., 1999, 2000).

Fishes show the same general relationship among dominance, aggression and serotonin as other vertebrates (Lepage et al., 2005; Overli et al., 1999, 2004; Winberg et al., 1992, 1997b). Agonistic encounters activate serotonin circuits in dominant and subordinate fish; serotonin metabolism declines in dominants and remains elevated in subordinates. Relatively few studies, however, have manipulated serotonin levels or the binding activity at serotonin receptors in fishes (Adams et al., 1996; Perreault et al., 2003; Stoddard et al., 2003; Winberg et al., 2001). Such questions have tremendous practical importance, as pharmaceuticals are released into waterways via sewage effluent at biologically-relevant levels (Brooks et al., 2005; Foran et al., 2004). Other forms of environmental contamination are also known to affect the serotonergic systems of fishes (Ronan et al., 2007; Sloman et al., 2005; Tsai et al., 1995).

We used *Betta splendens*, commonly called Siamese fighting fish, in our experiments. This species is a useful model in the study of aggression because their agonistic displays are highly stereotyped and have been well described from the perspectives of ethology (Clayton and Hinde, 1968; Simpson, 1968), energetics (Haller, 1991; Haller and Wittenberger, 1988) and neuromuscular anatomy (Gorlick, 1989, 1990; Ma, 1995a,b). A stereotaxic atlas of this species has been published (Marino-Neto and Sabbatini, 1988) as well as several brain ablation studies (Hollis and Overmier, 1982; Marino-Neto and Sabbatini, 1983). *B. splendens* has been used to study the behavioral effects of ergot drugs, particularly D-lysergic acid diethylamide or LSD (Arbit, 1957; Evans et al., 1956; Mueller, 1959; Turner, 1956), which is a 5-HT_{5A} receptor agonist, but little is known about the serotonergic regulation of aggression in this species.

The goal of this research was to investigate the extent to which activation or inhibition of the serotonergic system affects male–male aggression in *B. splendens*. To address the question of activation, we used intramuscular injections of serotonin (5-HT), the 5-HT_{1A} and 5-HT_7 receptor agonist 8-OH-DPAT, the selective serotonin reuptake inhibitor fluoxetine (FLX), as well as dietary administration of the serotonin precursor L-tryptophan (Trp). To address the question of serotonergic inhibition, we used intramuscular injections of the selective serotonin receptor 5-HT_{1A} antagonist WAY-100635 (WAY) and the serotonin synthesis inhibitor *p*-chlorophenylalanine (pCPA). For a subset of FLX, pCPA and Trp fish, we also measured brain levels of serotonin and its primary metabolite 5-hydroxyindoleacetic acid (5-HIAA), as well as dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), in order to better understand the relationship between monoaminergic activity and aggression in this species.

2. Methods

2.1. Study organisms

All protocols described below were conducted with the approval of Amherst College's Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. We used sexually mature male *B. splendens* of the domesticated strain obtained from a local distributor. Fish were housed individually in 2-l containers, kept on a 12:12 light:dark cycle and fed 1–2 times daily with dried chironomid larvae unless otherwise specified. We used municipal tap water subjected to reverse osmosis, which was then reconstituted to a conductivity of 100–150 μS and warmed to 27 °C. The mean body mass of fish used in these experiments was 1.66±0.02 g. Fish were typically housed in the laboratory for 3–4 weeks prior to their use in experiments, and no fish was used for more than one experiment.

2.2. Serotonin manipulations

All drugs were purchased from Sigma-Aldrich (St. Louis, USA). We used similar protocols for the 5-HT, 8-OH-DPAT and WAY experiments. Fish were randomly assigned to treatment groups and acclimated for 24 h in 7-l polycarbonate tanks, after which they were presented with a 25.4×15 cm mirror for 10 min. Mirror image stimulus tests are a standard protocol for eliciting aggression in fishes, particularly *B. splendens*. Results from mirror tests are generally significant predictors of responses to other stimuli in this species, such as videotaped or live males, as well as of dominance in dyadic interactions (Clotfelter et al., 2006; Earley et al., 2000; Verbeek et al., 2007). The duration (in seconds) of opercular displays (Clayton and Hinde, 1968; Simpson, 1968) was recorded; we selected opercular displays because they are known to be associated with fight outcome (Evans, 1985; Simpson, 1968). In a subset of experiments, we also report the latency (in seconds) to respond to the mirror. All mirror tests and all injections (see below) were performed between 1200 and 1800 EST.

Following the mirror test, fish received a single intramuscular injection from a 26-gauge Hamilton syringe. Experimental fish received injections of the drug dissolved in 4 μl of teleost saline (Hoar and Hickman, 1975), while control fish received 4 μl of the saline vehicle only. Dosages listed below were calculated based on previous studies (on fishes, where possible)

and extrapolated to an approximate body mass of 1.5 g (de Boer et al., 1999; Lopez-Mendoza et al., 1998; Perreault et al., 2003; Sperry et al., 2003; Stoddard et al., 2003; Winberg et al., 2001). Within an experiment, fish typically varied in mass by less than 10%. Thus, we used the same dosage for all fish. To account for these minor differences in size, we included body mass as a covariate in our analyses (see below). We injected fish with 0, 10, 100 or 1000 nmol solutions of 5-HT; 0, 1.5 or 4.5 mmol solutions of 8-OH-DPAT; or 0 or 2.3 mmol solutions of WAY. In addition, one group was sequentially injected with 2.3 mmol WAY and 1.5 mmol 8-OH-DPAT, a treatment we implemented because some studies suggest that WAY-100635 is potentiated by serotonin agonists (de Boer et al., 1999; Lopez-Mendoza et al., 1998). We chose 8-OH-DPAT and WAY because they are agonists and antagonists, respectively, of 5-HT_{1A} receptors, which are important in regulating aggression. One hour following these injections, we re-evaluated aggressive behavior in each fish with another mirror test.

In the FLX study, we injected fish daily for 14 days with either 0 or 4.3 mmol solutions of FLX (Perreault et al., 2003; Semsar et al., 2004). We assessed aggression before and after the injection period using mirror tests as described above. In the pCPA study, fish were injected daily for 3 days with 0 or 100 mmol pCPA solutions (Dias and Crews, 2006) and subjected to mirror tests at the beginning and end of the injection period. The final mirror test was performed 24 h after the last FLX or pCPA injection.

We administered L-tryptophan (Trp) via the diet using the following recipe: spray-dried white fish meal (200 g), wheat flour (202 g), vegetable oil (9 g), vitamins (5 g), water (569 g), and Trp (1.5 g in one experimental group and 15 g in the other). The control diet was identical except that Trp was replaced with a similar quantity of water. To confirm the accuracy of the Trp supplementation diets, the Trp content of one randomly-selected 20 mg sample from each diet was measured using a Waters AccQTag amino acid analysis HPLC system (Waters Corp.), which revealed concentrations of 0.33 g/kg (control), 2.8 g/kg and 26.6 g/kg. Thus, we hereafter refer to the experimental treatment groups as 10X Trp and 100X Trp, respectively, to indicate their approximate Trp concentration compared to the control diet. Fish were fed these diets twice daily for 7 days, and mirror tests were conducted at the beginning and end of this period.

2.3. Analysis of monoamines

Brains were rapidly (<3 min) removed and dissected into two regions: telencephalon (hereafter forebrain) and caudal midbrain and hindbrain (hereafter hindbrain) (Winberg et al., 1993). The tissue was weighed (±0.1 mg) and stored at −80 °C until analysis. Brain removal took place between 1200 and 1800 EST. Serotonin (5-HT), its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using high performance liquid chromatography (HPLC) with electrochemical detection (Emerson et al., 2000; Summers et al., 1998). Briefly, the samples were sonicated in 10 w/v 25 mM sodium acetate buffer (pH 5) containing α-methyl dopamine (internal standard), and centrifuged twice at 15,000 ×*g* for 2 min. The supernatant was removed and 45 μl was injected into a chromatographic system (Waters Associates, Inc.) and analyzed electrochemically with an LC-4B potentiostat (Bioanalytical Systems). The electrode potential was set at +0.6 V with respect to an Ag/AgCl reference electrode. The pellet was dissolved in 100 μl of 0.2 N NaOH and protein content was assayed.

2.4. Statistics

To examine the behavioral effects of each drug, we calculated standardized residuals from regressions of post-treatment behavior on pre-treatment behavior and fish body mass to account for the effects of these independent variables. There was no significant collinearity between the two covariates ($p>0.22$ for all). These residuals were used as dependent variables in independent *t*-tests and one-way ANOVAs (with Dunnett's post-hoc comparisons) to compare among treatment groups. Figures present the post-treatment behavioral data, not the residuals. For monoamine analyses we report actual means (±SE). Statistical tests were conducted using SPSS v. 14.0. All

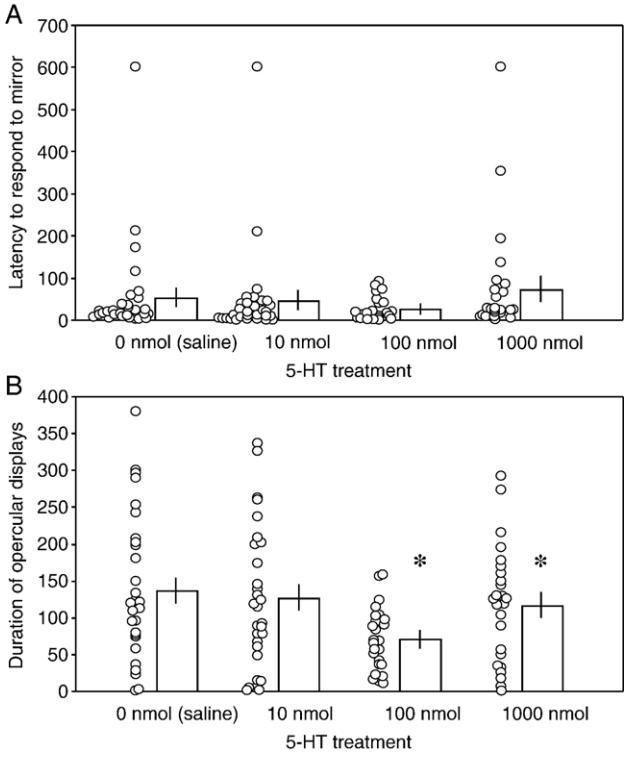

Fig. 1. The effects of intramuscular injection of serotonin (5-HT) on the latency to respond to a mirror stimulus (A) and the duration of agonistic displays (B) by male *B. splendens* (both in seconds). Latency to respond was not significantly affected by 5-HT ($F(3,97)=1.55$, $p=21$), but the duration of the response was significantly decreased ($F(3,97)=2.82$, $p=0.043$). Dunnett's post-hoc tests showed that these differences were significant for both the 100 nmol and the 1000 nmol treatment groups relative to the control fish. Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text). *$p<0.05$.

tests were two-tailed and differences were considered significant at $p<0.05$.

3. Results

3.1. Serotonin (5-HT)

We injected 21, 30, 25 and 25 *B. splendens* with 0, 10, 100 or 1000 nmol solutions of 5-HT, respectively. In the mirror test 1 h following the injection, there was no effect of serotonin treatment on their latency to respond to the mirror (ANOVA, $F(3,97)=1.55$, $p=0.21$; Fig. 1A), but the duration of attacks exhibited was significantly reduced in a roughly dose-dependent fashion ($F(3,97)=2.82$, $p=0.043$; Fig. 1B). A Dunnett's post-hoc comparison revealed that the responses by fish in the 100 nmol and 1000 nmol treatment groups were significantly less than that of control fish ($p=0.031$ and $p=0.041$, respectively).

3.2. 8-OH-DPAT

The serotonin receptor agonist 8-OH-DPAT showed results similar to 5-HT. We injected 10 *B. splendens* each with 0, 1.5 or 4.5 mmol solutions of 8-OH-DPAT. Aggressive behavior was highly reduced in both 8-OH-DPAT treatment groups. Both latency to attack ($F(2,27)=29.79$, $p<0.001$; Fig. 2A) and the duration of attacks ($F(2,27)=12.33$, $p<0.001$; Fig. 2B) were significantly affected. Post-hoc tests revealed that aggressive behavior in fish injected with both doses of 8-OH-DPAT was significantly reduced relative to controls ($p<0.001$ for both).

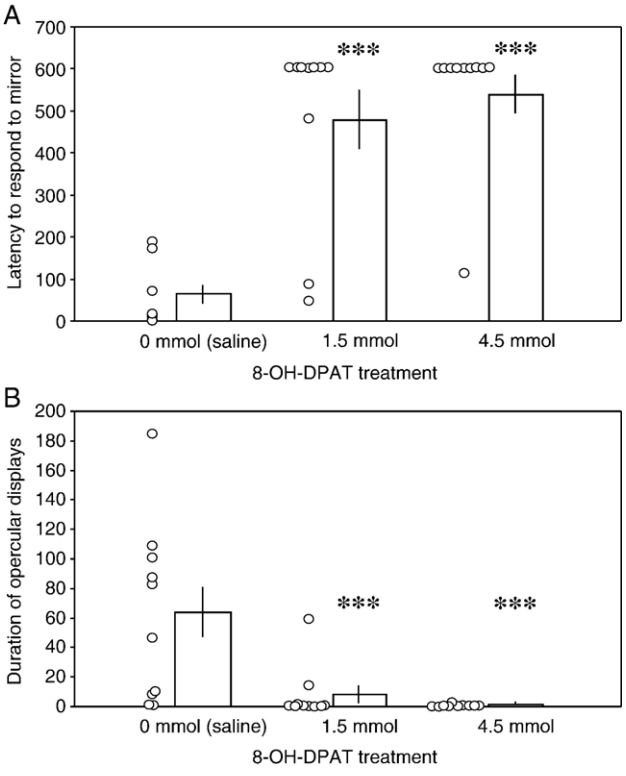

Fig. 2. Injection with the serotonin agonist 8-OH-DPAT significantly reduced aggressive behavior (in seconds) in male *B. splendens*; (A) the latency to respond to the mirror stimulus was increased ($F(2,27)=29.79$, $p<0.001$) and (B) the duration of aggressive displays toward the mirror was decreased ($F(2,27)=12.33$, $p<0.001$). Dunnett's post-hoc tests showed that these differences were highly significant for both the 1.5 mmol and the 4.5 mmol treatment groups relative to the control fish. Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text). ***$p<0.001$.

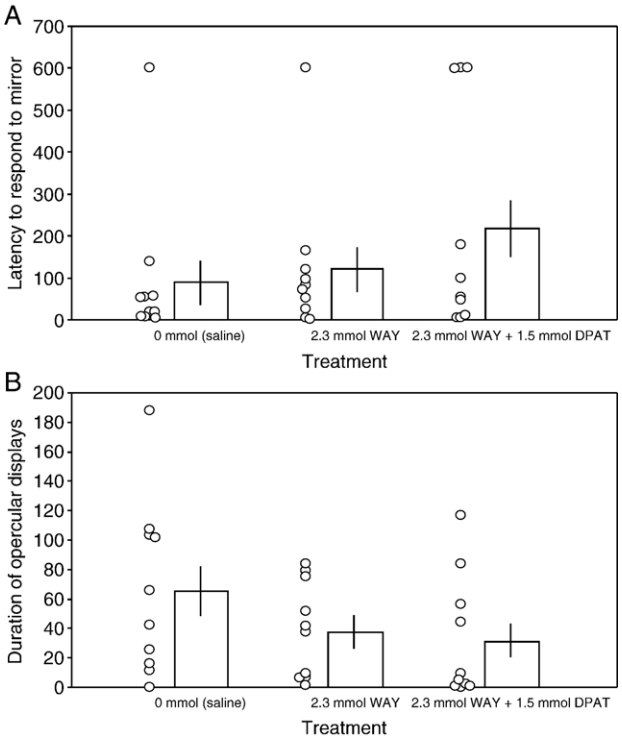

Fig. 3. The 5-HT_{1A} receptor antagonist WAY-100635 (WAY) had no effect on the latency to respond to the mirror stimulus (A; $F(2,27)=1.23$, $p=0.31$) or the duration of this response (B; $F(2,27)=1.26$, $p=0.29$) in male *B. splendens* (both in seconds). Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text).

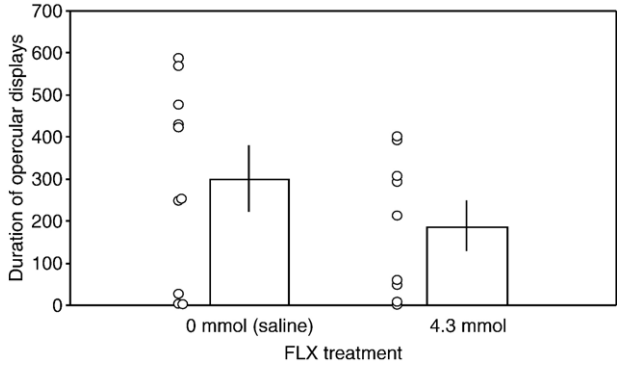

Fig. 4. The duration (in seconds) of agonistic displays performed by male *B. splendens* was unaffected by repeated injection with the selective serotonin reuptake inhibitor fluoxetine (FLX) ($t(17)=1.44$, $p=0.17$). Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text).

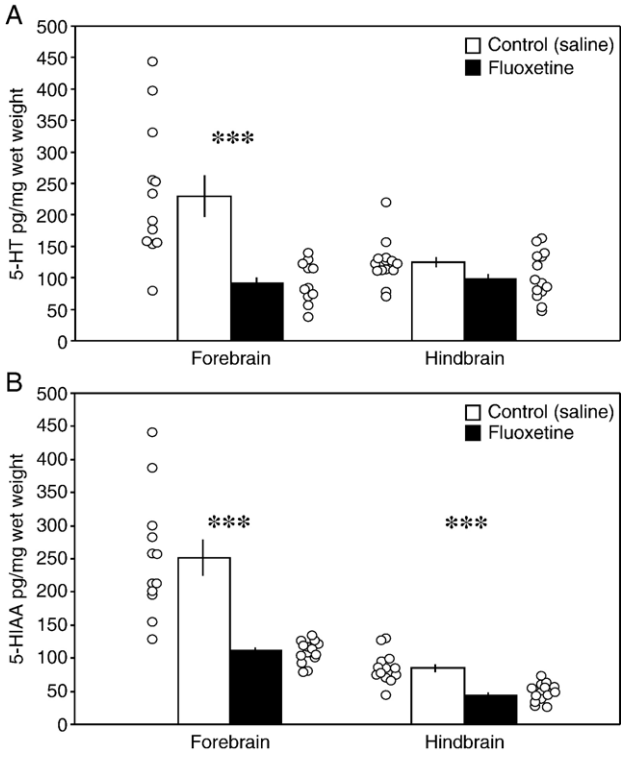

Fig. 5. (A) The selective serotonin reuptake inhibitor fluoxetine (FLX) caused a significant decline in 5-HT levels in male *B. splendens* forebrains ($t(24)=4.52$, $p<0.001$), but no change in the hindbrains. (B) FLX significantly reduced 5-HIAA levels in both forebrains ($t(26)=6.29$, $p<0.001$) and hindbrains ($t(26)=5.17$, $p<0.001$). Circles are individual data points and bars represent means±SE. ***$p<0.001$.

3.3. WAY-100635 (WAY)

We injected 10 *B. splendens* each with 0 or 2.3 mmol solutions of the 5-HT_{1A} receptor antagonist WAY, and an additional 10 fish with 2.3 mmol WAY followed 15 min later by 1.5 mmol 8-OH-DPAT. Neither the latency to attack ($F(2,27)=1.23$, $p=0.31$; Fig. 3A) nor the duration of the attack ($F(2,27)=1.26$, $p=0.29$; Fig. 3B) in the post-injection mirror test was significantly different among the three treatments.

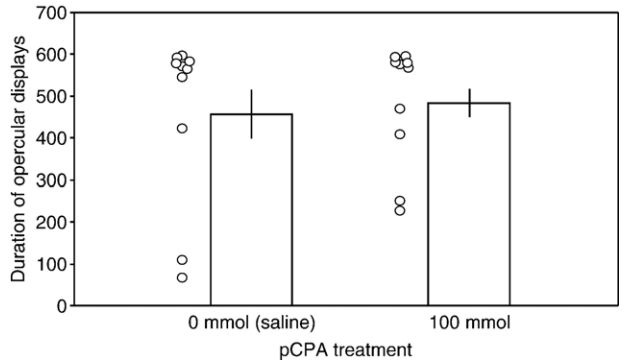

Fig. 6. The duration (in seconds) of agonistic displays performed by male *B. splendens* was unaffected by treatment with the serotonin synthesis inhibitor *p*-chlorophenylalanine (pCPA) ($t(18)=1.09$, $p=0.29$). Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text).

3.4. Fluoxetine (FLX)

We injected 9 *B. splendens* daily for 14 days with a 4.3 mmol solution of the selective serotonin reuptake inhibitor FLX; an additional 10 fish were similarly injected with equal volumes of teleost saline. We did not record latency to attack in this experiment. Fish injected with FLX showed decreased duration of displays compared to control fish, but this difference was not significant ($t(17)=1.44$, $p=0.17$; Fig. 4).

Another set of fish was injected daily for 14 days with a 4.3 mmol solution of FLX ($n=14$); a control group ($n=14$) was subjected to a similar injection regimen with teleost saline. After 14 days monoamine concentrations were measured in the forebrains and hindbrains. Fluoxetine caused a significant decline in 5-HT levels in the forebrains of male *B. splendens* ($t(24)=4.52$, $p<0.001$; Fig. 5A), but no change in the hindbrains. Furthermore, FLX significantly reduced 5-HIAA levels in both forebrains ($t(26)=6.29$, $p<0.001$) and hindbrains ($t(26)=5.17$, $p<0.001$; Fig. 5B). As a result, there was a highly significant reduction in serotonergic activity, as measured by the ratio of 5-HIAA to 5-HT, in the hindbrains of fish injected with FLX (control: 0.69, FLX: 0.47, $t(26)=5.76$, $p<0.001$), but no change in the forebrain (control: 1.19, FLX: 1.18, $t(24)=0.009$, $p=0.99$). The concentrations of DA and DOPAC, as well as

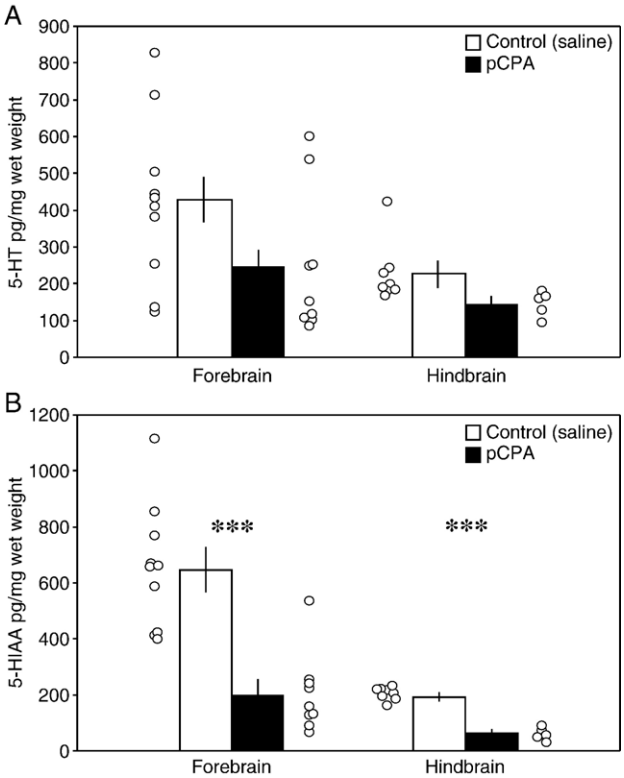

Fig. 7. (A) The serotonin synthesis inhibitor *p*-chlorophenylalanine (pCPA) caused nonsignificant decreases in 5-HT levels in forebrains ($t(17)=1.82$, $p=0.085$) and hindbrains ($t(11)=2.06$, $p=0.064$) of male *B. splendens*. (B) Levels of the metabolite 5-HIAA in forebrains ($t(17)=5.20$, $p<0.001$) and hindbrains ($t(11)=10.98$, $p<0.001$) were significantly reduced in the pCPA treatment group. Circles are individual data points and bars represent means±SE. ***$p<0.001$.

dopaminergic activity (ratio of DOPAC to DA), were all unaffected by FLX treatment ($p>0.24$ for all).

3.5. p-chlorophenylalanine (pCPA)

Ten fish each were injected with 0 or 100 mmol solutions of the serotonin synthesis inhibitor pCPA over 3 days. We found no significant behavioral effect of this manipulation. Duration of aggressive behavior in the mirror test was unaffected by pCPA treatment relative to control fish ($t(18)=1.09$, $p=0.29$; Fig. 6).

An additional nine fish received 3 days of daily 100 mmol injections of pCPA; another 10 fish served as controls. Monoamine analysis of these brains revealed that pCPA treatment caused nonsignificant declines in 5-HT levels in forebrains ($t(17)=1.82$, $p=0.085$) and hindbrains ($t(11)=2.06$, $p=0.064$) of male *B. splendens* (Fig. 7A), while 5-HIAA levels in forebrains ($t(17)=5.20$, $p<0.001$) and hindbrains ($t(11)=10.98$, $p<0.001$) were significantly reduced by the pCPA treatment (Fig. 7B). Treatment with *p*-chlorophenylalanine caused a modest decrease in the 5-HIAA:5-HT ratio in the forebrain (control: 1.86, pCPA: 1.53, $t(17)=2.10$, $p=0.051$) and significant decline in the hindbrain (control: 0.97, pCPA: 0.40, $t(11)=4.69$, $p<0.001$). DA and DOPAC levels and dopaminergic activity were unaffected by pCPA in either the forebrain or hindbrain ($p>0.11$ for all).

3.6. L-tryptophan (Trp)

To test the effects of a Trp-enhanced diet on aggressive behavior, we fed the control, 10X Trp and 100X Trp diets to 14, 15 and 14 fish, respectively. There were no significant effects of Trp concentration on *B. splendens* display behavior ($F(2,40)=1.51$, $p=0.23$), though fish fed for a week with the 10X Trp diet were slightly more aggressive in the mirror test than the 100X Trp fish ($p=0.16$, Fig. 8).

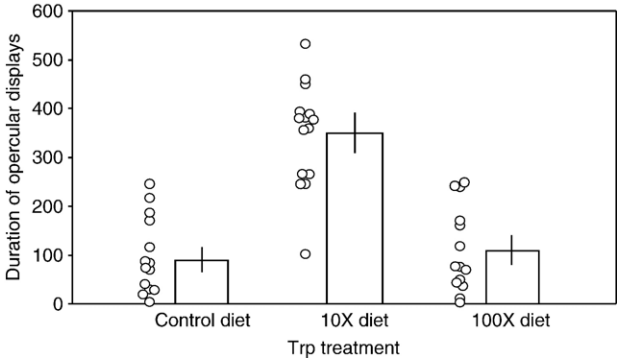

Fig. 8. Duration (in seconds) of agonistic displays performed by male *B. splendens* as a function of diet supplemented with the 5-HT precursor L-tryptophan (Trp). Fish fed the intermediate (10X) Trp diet had apparently higher levels of aggression, but most of this variation was explained by pre-treatment differences in behavior, and thus the relationship was not statistically significant once these differences were accounted for ($F(2,40)=1.51$, $p=0.23$). Data presented here (circles are individual data points, bars represent means±SE) are not corrected for male body size or pre-treatment behavior (see text).

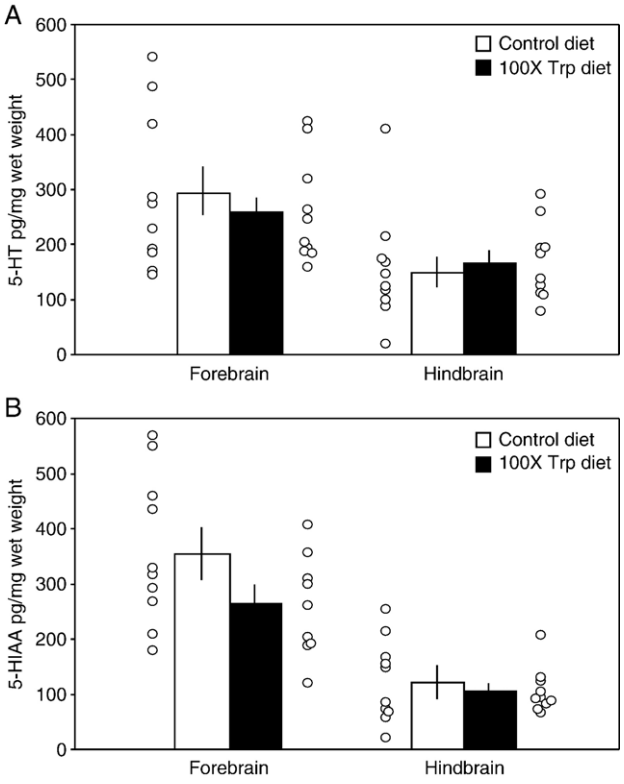

Fig. 9. The brains of male *B. splendens* fed a 100X Trp diet were analyzed for 5-HT (A) and 5-HIAA (B) levels. Only for 5-HIAA in the forebrain did these differences approach statistical significance ($t(18)=1.76$, $p=0.095$). Circles are individual data points and bars represent means±SE.

We fed another set of fish for one week on a control diet ($n=10$) or a 100X Trp diet ($n=10$), and then removed their brains for monoamine analysis. Dietary Trp caused a nonsignificant decline in forebrain levels of 5-HIAA ($t(18)=1.76$, $p=0.095$; Fig. 9A), but otherwise there were no changes in 5-HT or 5-HIAA levels ($p>0.51$ for all; Fig. 9A, B) as a result of 100X Trp consumption. The ratios of 5-HIAA to 5-HT showed nonsignificant declines in both forebrains (control: 1.31, Trp: 1.08, $t(18)=1.66$, $p=0.12$) and hindbrains (control: 0.82, Trp: 0.68, $t(18)=1.43$, $p=0.17$). Hindbrain levels of dopamine were marginally elevated in the Trp treatment ($F(1,17)=3.49$, $p=0.08$), but DOPAC and DOPAC:DA were generally unaffected by Trp supplementation.

4. Discussion

Serotonin has long been thought to play a key role in the inhibition of territorial male aggression. Many of the molecules implicated in the regulation of aggression, such as arginine vasotocin, arginine vasopressin or nitrous oxide, likely do so via serotonergic interactions (Delville et al., 1996; Ferris and Delville, 1994; Nelson and Chiavegatto, 2001; Semsar et al., 2004). In spite of the presumed primacy of serotonin, a direct causal relationship between increased serotonin and decreased aggression has yet to be made for any species. Only recently (Summers et al., 2005b) has it been demonstrated that pre-encounter serotonergic activity is decreased in more aggressive

males. In addition, the temporal dynamics of serotonergic activity and aggression are just beginning to be understood (Miczek et al., 2002; Summers and Winberg, 2006). Two emergent trends, particularly among fishes and lizards, are that the onset of aggressive behavior is associated with increased serotonergic activity, whereas prolonged elevated of serotonin metabolism results in inhibition of aggression and social subordination (Overli et al., 1999; Stoddard et al., 2003; Summers et al., 2005c; Summers and Winberg, 2006).

Our results in *B. splendens* are generally consistent with previous findings on the inhibition of territorial aggression by serotonin. Acute treatment with serotonin decreased aggressive behavior in a mirror image stimulus test in a roughly dose-dependent fashion. These results do not reveal, however, which serotonin receptor type mediated this effect. Injections with the 5-HT_{1A} receptor agonist 8-OH-DPAT were highly successful at decreasing aggression, as has been demonstrated previously in birds (Sperry et al., 2003) and rodents (de Boer et al., 1999; Ferris et al., 1999; Haug et al., 1990). It has also been shown that 8-OH-DPAT affects the hypothalamic–pituitary–interrenal axis in fishes (Hoglund et al., 2002; Winberg et al., 1997a), which may explain the reduction in aggressive behavior (DiBattista et al., 2005). The highest dose of 8-OH-DPAT reduced overall responsiveness of male *B. splendens*, but the intermediate dose did not (Sperry et al., 2003). As an agonist of both 5-HT_{1A} and 5-HT_7 receptors, 8-OH-DPAT could have affected behavior through either of these pathways. Antagonism of the 5-HT_{1A} receptor through the administration of WAY-100635 had no significant effect on aggression in *B. splendens*. Several rodent studies report that WAY only affects aggression if administered with a serotonin agonist or a serotonin reuptake inhibitor (Bell et al., 1999; Lopez-Mendoza et al., 1998; Sanchez, 1997), but when we gave WAY injections followed by 8-OH-DPAT injections we saw no change in male aggressive behavior. Additional receptor classes, such as 5-HT_{1B} and 5-HT_3, are also likely to be involved in the expression of aggression in *B. splendens* (Grimes and Melloni, 2005; Miczek et al., 1989; Ricci et al., 2005). Future studies should test additional receptor type antagonists to reveal the mechanism underlying this behavioral effect.

Chronic treatment with the selective serotonin reuptake inhibitor fluoxetine did not significantly decrease in territorial aggression in male *B. splendens*. This result was contrary to our prediction, which we based on a number of studies showing reduced aggression in FLX-treated animals across a range of taxa (Deckel, 1996; Delville et al., 1996; Dodman et al., 1996; Ho et al., 2001; Perreault et al., 2003; Sperry et al., 2003), though some studies have shown that chronic treatment with FLX and other anti-depressants can increase agonistic behavior (Mitchell and Redfern, 1992, 1997). It is possible that the dosage or duration of treatment we used, which were based on those used in bluehead wrasse *Thalassoma bifasciatum* (Perreault et al., 2003; Semsar et al., 2004), was insufficient to suppress aggression in *B. splendens*. Serotonin and 5-HIAA levels were generally reduced by FLX in the *B. splendens* brain, which is consistent with a number of rodent studies (Baldessarini et al., 1992; Frankfurt et al., 1994; Hall et al., 1995; Shishkina et al., 2006; Wong et al., 1995). Presumably this occurred due to reduced levels of 5-HT precursor 5-HTP, negative feedback via somatodendritic autoreceptors in the raphe nuclei, and decreased firing of the raphe due to this feedback (Wong et al., 1995). It is also possible that our injection regimen caused stress-induced alterations in the 5-HT_{1A} receptor system (Hoglund et al., 2002), though our experience suggests *B. splendens* is relatively robust against the stress of frequent handling. Future studies should address this question using different drug delivery techniques.

Aggressive behavior was similarly unaffected by the serotonin synthesis inhibitor pCPA, which had previously been shown to increase aggression in a similar mirror test in the firemouth cichlid *Cichlasoma meeki* (Adams et al., 1996). Treatment with pCPA also increases aggression in mice and domestic chicks (Buchanan et al., 1994; Chiavegatto et al., 2001). The lack of behavioral effects of pCPA in *B. splendens* could have been due to significant pools of 5-HT in the terminal regions of the brain, but this did not appear to be the case. We observed declines (albeit nonsignificant) in serotonin levels and significant decreases in the metabolism of 5-HT to 5-HIAA in both the forebrain and the hindbrain (Jones and Lucki, 2005; Kornum et al., 2006). The lack of any behavioral effect from this manipulation is unlikely to be due to insufficient 5-HT depletion, as Adams et al. (1996) found significant increases in mirror-induced aggression in cichlids only 24 h after the first pCPA injection. It may be easier to block aggression than to promote it, and during agonistic encounters 5-HT may actually be irrelevant (Summers and Winberg, 2006). Aggressive behavior may require the activation of hypothalamic arginine vasotocin (Santangelo and Bass, 2006; Semsar et al., 2004) and glutamate neurons. Thus, the increase in serotonergic activity by pCPA may be insufficient to cause the expression of mirror-induced aggression.

When we administered dietary l-tryptophan to *B. splendens*, we found generally no effect on aggressive behavior. There was a tendency for fish on the 10X Trp diet to be more aggressive than the 100X Trp fish, perhaps because at slightly elevated levels 5-HT could bind to 5-HT_3 receptors (Ricci et al., 2005) without stimulating a stress response that might reduce aggression (Summers et al., 2005b). In addition, there were no significant changes in 5-HT or 5-HIAA concentrations in *B. splendens* brains, or the ratio between the two. The former result is more surprising than the latter, as previous studies in fishes have shown that Trp supplementation suppresses aggressive behavior more robustly than it increases serotonergic activity (Lepage et al., 2002, 2003; Winberg et al., 2001). The lack of an effect of Trp could be due to saturation of the tryptophan hydroxylase enzyme, which has been shown to occur in rats at much lower doses of dietary Trp than we used here (Carlsson and Carlsson, 1988). If this were true, however, we should have observed elevated 5-HT levels. Future studies in *B. splendens* should examine brain levels of Trp to confirm assimilation of the amino acid from feed, as well as on feeding rate differences among treatment groups (Winberg et al., 2001).

Together, our results provide some of the first information regarding the serotonergic modulation of aggression in *B. splendens*, a species widely used in ethological studies for its

stereotyped agonistic displays (Clayton and Hinde, 1968; Clotfelter and Paolino, 2003; Clotfelter et al., 2006; Evans, 1985). Change in the availability or metabolism of 5-HT may be necessary but not sufficient to change aggression. The remaining element required to modify aggressive behavior, if the serotonergic activity is appropriate, must come from stimulation of the excitatory elements in the aggression neurocircuitry. Our results also highlight the potential utility of this species as a pharmacological or toxicological model system (Clotfelter and Rodriguez, 2006). Future studies using this system should focus upon, among other things, the efficacy of the opercular display as a sign stimulus to elevate serotonin metabolism, the localization of serotonergic activity in the brain, the role of pre-encounter serotonin levels in initiating and escalating aggressive interactions, and the role of glucocorticoids in modulating aggressive behavior.

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